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Review

A Review on the Production of C4 Platform Chemicals from Biochemical Conversion of Sugar Crop Processing Products and By-Products

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Abstract: The development and commercialization of sustainable chemicals from agricultural products and by-products is necessary for a circular economy built on renewable natural resources. Among the largest contributors to the final cost of a biomass conversion product is the cost of the initial biomass feedstock, representing a significant challenge in effective biomass utilization. Another major challenge is in identifying the correct products for development, which must be able to satisfy the need for both low-cost, drop-in fossil fuel replacements and novel, high-value fine chemicals (and/or commodity chemicals). Both challenges can be met by utilizing wastes or by-products from biomass processing, which have very limited starting cost, to yield platform chemicals. Specifically, sugar crop processing (e.g., sugarcane, sugar beet) is a mature industry that produces high volumes of by-products with significant potential for valorization. This review focuses specifically on the production of acetoin (3-hydroxybutanone), 2,3-butanediol, and C4 dicarboxylic (succinic, malic, and fumaric) acids with emphasis on biochemical conversion and targeted upgrading of sugar crop products/by-products. These C4 compounds are easily derived from fermentations and can be converted into many different final products, including food, fragrance, and cosmetic additives, as well as sustainable biofuels and other chemicals. State-of-the-art literature pertaining to optimization strategies for microbial conversion of sugar crop byproducts to C4 chemicals (e.g., bagasse, molasses) is reviewed, along with potential routes for upgrading and valorization. Directions and opportunities for future research and industrial biotechnology development are discussed.

Keywords: sugarcane; sugar beet; fermentation; biorefinery; platform chemicals; succinic acid; malic acid; fumaric acid; acetoin; 2,3-butanediol

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1. Introduction

Among the greatest challenges at present in chemical engineering research and development is the need to supplant fossil fuels and petrochemicals with renewable biomass-derived analogues. This issue is centered largely on the non-renewable nature of fossil resources and their deleterious effects on the environment, including climate change [1,2]. Because of the persistent need for carbonaceous fuels, chemicals and materials in the global economy, biomass is the only viable sustainable feedstock with the potential for carbon-neutral production [2–5]. In general, a major contributor to the final cost for a biomass-derived product is typically the cost of the initial biomass feedstock (particularly on a dry, ash-free basis) [6–9]. To overcome this economically driven obstacle, the effective utilization of biomass-derived wastes and by-products is critical. Using wastes is desirable because they are abundantly available at low cost, with the intrinsic benefit of affording new waste management opportunities [10,11].

A biomass sector with significant potential for greater by-product utilization is agricultural production of sugar crops, including sugarcane and sugar beets [12]. Global sugar (i.e., sucrose) production is nearly 200 million tons per year, of which 75–80% comes from

sugarcane and 20–25% comes from sugar beets [13,14]. For a given quantity of sugarcane, a larger mass of waste and by-products is generated than the total mass of final sugar produced. Meghana and Shastri report that for the production of 100 kg of sugar from 1000 kg of cane, a total of 300 kg bagasse and 40 kg of molasses are generated [13]. Lignocellulosic bagasse residues are typically consumed for energy to be used in sugar and ethanol plants, however, large surpluses often remain [15]. Sugarcane molasses is made up of a significant amount of fermentable sugars, containing roughly 30–35% sucrose and 10–25% glucose and fructose according to one estimate [16]. Roughly 30 kg of press mud (itself containing 5–10% sugar) per 100 kg of sugar (from 1000 kg cane) are also generated [13,16]. The processing of sugar beets for sucrose production also yields large quantities of lignocellulosic sugar beet pulp as a primary by-product. From 1000 kg of sugar beets (with comparable sucrose yields to sugarcane), approximately 70 kg of dried pulp (250 kg wet basis) are produced [17,18]. Sucrose production from sugar beets, like sugarcane, also generates sugar beet molasses containing approximately 50% sugar. Sugar beet molasses is typically used in fermentations for alcohol production and in animal feed or fertilizer [19]. Numerous studies have reported on successful inclusion of sugar crop processing products and by-products in biochemical conversions to various products [20–36]. Further reading on sugarcane and sugar beet industries, emphasizing waste valorization, is available in recent reviews from Meghana and Shastri [13] and Rajaeifar et al. [37].

In addition to effective feedstock selection, the implementation of a circular bio-based economy also relies on identifying and manufacturing appropriate platform chemicals [11,38–47]. Importantly, a key aspect of biomass platform chemical development is the need to target compounds for production that are easily synthesized or derived from biomass feedstocks. By leveraging the diversity of functionalities in biomass and the present capabilities of thermo- and biochemical conversion, significant potential exists for bio-based drop-in replacements for petrochemicals as well as new products beyond the scope of the current petrochemical industry [4,41]. This review aims to highlight opportunities to produce acetoin (3-hydroxybutanone), 2,3-butanediol (2,3-BDO), and C4 dicarboxylic acids (succinic acid, malic acid, fumaric acid) (structural representations in Figure 1). These are all promising C4 platform chemicals that can be generated through microbial biochemical conversion [39,48–56].

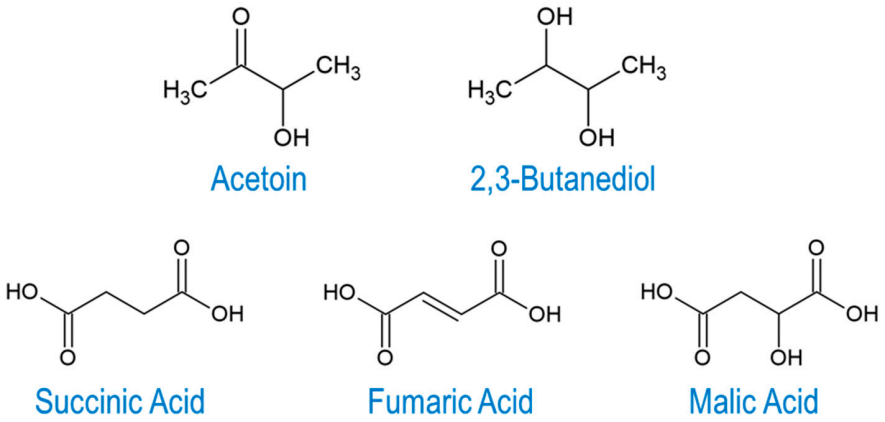

Figure 1. Structural representations of highlighted C4 platform chemicals.

Specific emphasis is given to highlighting recent work on the production of these chemicals from sugar crop processing products and by-products. Both acetoin and 2,3-BDO on their own have numerous applications, some of which are summarized in Table 1. Additionally, together, both acetoin and 2,3-BDO represent promising intermediates for downstream upgrading and diversification into a wide array of biofuels and other high-value products [50]. Succinic, fumaric, and malic acids have diverse applications in many industries, including pharmaceuticals, food and agriculture, and resins and polymers. Some of these existing applications are also summarized in Table 1. Like acetoin and 2,3-BDO, C4

carboxylic acids can be converted into a wide variety of final products, including biofuels and fuel additives, plasticizers, coolants, solvents, and other fine chemicals [38,57,58]. The overall goal of this review is to highlight the importance of careful feedstock selection coupled with targeted biochemical conversions in advancing the circular bio-based economy centered on the principles of green chemistry and engineering.

Table 1. Details on the existing applications of highlighted C4 platform chemicals.

Product	Applications	Market Price	Reference
Acetoin	Food additive, flavor/fragrance additive in tobacco products, cosmetics, soaps and detergents, chemical precursor, plant growth promoter and postharvest decay control	USD 30–50/kg	[48,58]
2,3-BDO	Chemical precursor for fuels and solvents, flavor and fragrance additives, pharmaceuticals, polymers and materials	USD 2–3/kg	[49–51]
Succinic Acid	Food additive, chemical precursor for fuels and solvents, pharmaceuticals, polymers and materials, precursor for 1,4-butanediol	USD 2–3/kg	[52–54]
Malic Acid	Primarily a food and flavor additive, further applications in pharmaceuticals, textiles and polymers/materials	USD 2/kg	[39,55]
Fumaric Acid	Primarily a food and flavor additive, further applications in polymers/resins and paper	USD 1.5/kg	[39,56]

2. Microbial Production of 2,3-BDO and Acetoin

Considering the cost to produce 2,3-BDO and acetoin from petrochemicals, there is considerable interest in renewable, inexpensive biomass conversion via microbial fermentation to high value chemicals and building blocks in an ecologically sustainable way. Since acetoin and 2,3-BDO are in the same pathway (Figure 2), several microorganisms naturally produce both acetoin and 2,3-BDO. However, some level of metabolic engineering may be required for accumulation of either acetoin or 2,3-BDO. For the sake of clarity in this review, we will discuss metabolic engineering for 2,3-BDO and acetoin production strategies separately.

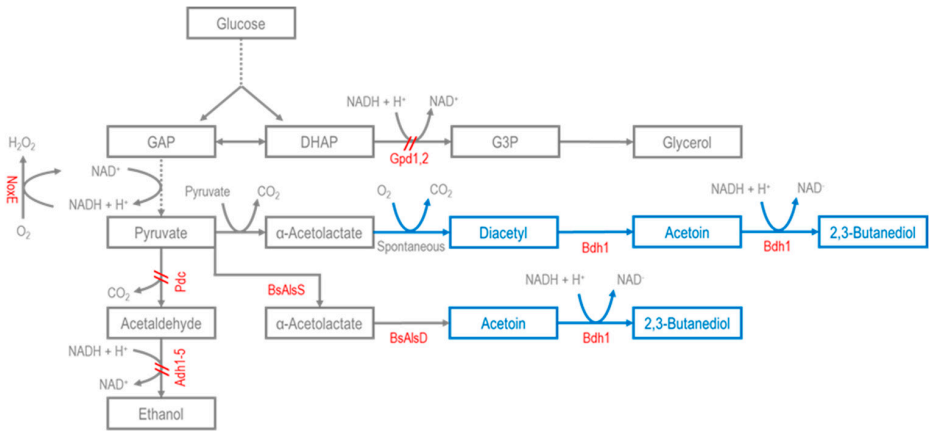

Figure 2. Example schematic of metabolic pathways in *Saccharomyces cerevisiae* for endogenous acetoin and 2,3-butanediol production and the heterologously expressed *Bacillus subtilis* acetoin/2,3-butanediol biosynthetic pathway with cofactor engineering utilizing NoxE from *Lactococcus lactis* (highlighting both acetoin and 2,3-butanediol). For metabolic engineering to produce acetoin, *BDH1* is typically deleted. Adapted from previously published work (with detailed abbreviation descriptions) from Hahn and associates, described in subsequent sections.

2.1. Microbial Production of 2,3-BDO

Some microorganisms naturally produce 2,3-BDO and have been reviewed by Ji et al. [59]. Using an organism that already produces 2,3-BDO such as *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Enterobacter aerogenes*, and *Serratia marcesans* would in theory be convenient and possibly bypass the need for any genetic engineering [60–63]. However, there are several reports of these microorganisms being further genetically optimized to improve 2,3-BDO production or chiral purity [60–62,64,65]. The caveat is that some of these organisms are considered pathogenic and may not be suitable for large scale industrial fermentation, despite high yields [59,66]. For example, one pathogenic bacterial strain reported to have the highest 2,3-BDO titer, is a *Klebsiella pneumoniae* isolate that produced 150 g/L 2,3-BDO in a medium optimized with corn steep liquor powder, in fed-batch fermentation [64]. The pathogenicity issue is such a concern that there have been some efforts to remove pathogenic properties from *K. pneumoniae* [67]. In addition, microorganisms produce various stereoisomers of 2,3-BDO, which may be irrelevant for biofuels but may pose problems for downstream catalysis or pharmaceutical/biological applications affected by stereochemistry [59].

Considerable effort has been devoted to metabolic engineering of microorganisms that are generally recognized as safe (GRAS) such as *Bacillus subtilis* and *Saccharomyces cerevisiae*, which are widely used and genetically tractable with conventional techniques [66,68]. Utilizing GRAS microorganisms is especially important in the development of any products meant for human consumption (e.g., food/fragrance additives, cosmetics) [69,70].

Optimized microbial biomass conversion can lower costs and improve profit margins for fermentation products by lowering the cost of carbon sources and directing metabolism toward the desired end-product with fewer side-products, thereby facilitating easier isolation or purification of the end-product. Fermentations can be optimized by reducing side products through gene deletions in side pathways and directing the carbon flux in the desired direction by heterologous expression of biosynthetic pathways. Metabolism pathways can also be further optimized by balancing the intracellular redox state by adjusting NAD^+ or NADH levels [71–74]. One method for increasing NAD^+ levels is to express a water-forming NADH oxidase from *Lactococcus lactis* [75]. When combined, multiple adjustments or adaptations facilitate improved productivity in terms of metabolite production, carbon source utilization, and time to completion. This review section will focus mostly on engineering strategies in *S. cerevisiae* to make highly optimized yeast cell factories for 2,3-BDO and acetoin production.

S. cerevisiae Production of 2,3-BDO

Given its GRAS status and genetic tractability, *S. cerevisiae* has been widely utilized in the fermentation industry for production of various biochemicals [68]. Several metabolic modifications have been reported in *S. cerevisiae* to improve 2,3-BDO production through various strategies. Under high glucose conditions, *S. cerevisiae* prefers to produce ethanol even under aerobic conditions and naturally produces a small amount of 2,3-BDO. This occurs in the mitochondria via α-acetolactate synthase (*ALS*) that synthesizes α-acetolactate. In this process, α-acetolactate undergoes spontaneous decarboxylation to diacetyl in the presence of oxygen and is subsequently converted to acetoin and 2,3-BDO in the yeast cytosol [76].

Strategies to increase 2,3-BDO production in yeast usually involve overexpressing biosynthetic pathway enzymes, reducing the formation of byproducts such as ethanol or glycerol, and balancing of the redox state in the yeast cell [68,76,77]. Glycerol production is blocked by deletion of glycerol-3-phosphate dehydrogenases, *GPD1* and *GPD2* [71,77,78]. Ethanol production can be eliminated by deletion of the alcohol dehydrogenases *ADH1*, *ADH4*, *ADH6*, and *ADH7*; however, accumulation of acetaldehyde and acetate can be problematic in some strains [68,79,80].

Another strategy is to delete the pyruvate decarboxylase genes *PDC5*, *PDC6*, and *PDC1* to block production of acetaldehyde, thereby leading to a buildup of pyruvate. However,

Pdc-deficient strains are known for their inability to utilize glucose as a carbon source and require additional adaptation measures as well as overexpression of the *MTH1*Δ*T* transcription regulator [81,82]. Normally, *MTH1* is involved in glucose sensing and hexose transporter expression. *MTH1*Δ*T* contains an internal deletion that increases the stability of the encoded protein resulting in lower intracellular glucose levels, thus removing glucose repression so that a Pdc-deficient strain can grow on glucose [81,82].

Collectively, these modifications result in increased carbon flux directed through pyruvate, which accumulates to higher levels that must be "directed" along toward the desired end-product. This is a critical step, where pyruvate can proceed toward the TCA cycle in the mitochondria, or in the case of metabolically engineered strains expressing either an endogenous cytosol-localized acetolactate synthase (AlsS) or a heterologously expressed *B. subtilis* AlsS, pyruvate carbon flux is "directed" toward 2,3-BDO production through formation of α-acetolactate, diacetyl, and reduction to acetoin and 2,3-BDO [82]. Pyruvate carbon flux is also pulled forward by overexpression of *B. subtilis* acetolactate decarboxylase (*BsALSD*) and NADH-dependent butanediol dehydrogenase (*BDH1*) (also known as acetoin reductase) to direct carbon flux to 2,3-BDO by ensuring efficient conversion of diacetyl and acetoin to 2,3-BDO [76,82].

The heterologous expression of the *B. subtilis* biosynthetic operon is a common strategy used in many studies [82]. Huang et al. demonstrated the value of a high-copy number approach using CRISPR technology to facilitate insertion of large DNA fragments into delta sequences in the *S. cerevisiae* genome [83]. The resulting strains with higher copy numbers of the 2,3-BDO biosynthesis genes correlated with higher 2,3-BDO production [83]. In this particular study, the final 2,3-BDO titer of 50 g/L during fed-batch fermentation was not as high as other reports with only partial deletion of competing pathways for ethanol and glycerol including Δ*ADH1*, Δ*PDC1*, Δ*PDC5*, and Δ*MTH1* deletions, which still allowed for substantial carbon flux diversion to ethanol and glycerol production. However, this method of integrating a high copy number (up to 25 copies) of 2,3-BDO biosynthetic genes into the genome provided an important proof of concept that copy number correlates with 2,3-BDO titer, and the engineered strain could be grown on nutrient rich fermentation medium without synthetic dropout medium to maintain the biosynthetic genes on a plasmid [83].

Although most metabolically engineered *S. cerevisiae* strains are haploid, another approach recently utilized was to produce a robust polyploid yeast strain with partial deletions in alcohol dehydrogenases (*ADH*) and pyruvate decarboxylases (*PDC*). Unlike other studies in haploid yeast utilizing fully Adh or Pdc strains with growth limitations, this method helped to decrease unwanted ethanol production without impairing glucose consumption or growth rate during fermentation [84]. The partial deletions also caused a redox imbalance that was alleviated by expression of an NADH oxidase from *Lactococcus lactis* [71,84]. These modifications resulted in a robust polyploid *S. cerevisiae* strain that produced 178 g/L 2,3-BDO using glucose as a carbon source, which may be the highest reported yield for *S. cerevisiae* to date [84]. Another important feature is that the same strain was also able to produce 132 g/L 2,3-BDO using hydrolyzed cassava starch as a fermentable glucose source, suggesting the possibility of high-yield fermentation with this strain on other alternative, inexpensive feedstocks [84].

2.2. Microbial Production of Acetoin

There are several examples of natural acetoin producers including *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Enterobacter cloacae*, *Serratia marcescens*, *Lactobacillus casei*, and *Paenibacillus polymyxa* [71,85]. There are also reports of optimizing these natural producers [86,87]. For example, in *Serratia marcescens* H32, even under fermentation conditions that favor acetoin production, a significant level of 2,3-BDO is also produced to regenerate NAD^+ [63]. Sun et al. reported a cofactor engineering strategy in *S. marcescens* that increased acetoin levels 33% to 75.2 g/L and reduced 2,3-BDO levels by 52% in acetoin-favoring fermentations by expressing the *nox* gene from *Lactobacillus brevis* encoding a water-forming NADH oxidase. This cofactor engineering strategy tipped the redox balance in favor of

acetoin production over 2,3-BDO by increasing NAD^+ levels 1.5-fold, thereby depleting NADH levels required by the NADH-dependent butanediol dehydrogenase Bdh1 [73]. However, despite its ability to produce acetoin, *S. marcescens* is an opportunistic pathogen and is a causative agent of nosocomial infections including outbreaks in neonatal intensive care units [65,88]. In addition, *S. marcescens* is resistant to multiple antibiotics, making this organism less than ideal for large-scale industrial fermentations [89].

In contrast to *S. marcescens*, a GRAS strain of *Corynebacterium glutamicum* was recently reported to produce 102.45 g/L acetoin with a yield of 0.419 g/g glucose at a rate of 1.86 g/L/h after extensive metabolic engineering. The modifications included integration of multiple copies of the *AlsSD* operon into the genome, the elimination of biosynthesis pathways for competing side products including lactate, acetate, and glycerin, for improved carbon flux toward acetoin production as well as disruption of 2,3-BDO synthesis [90].

S. cerevisiae Production of Acetoin

Since acetoin and 2,3-BDO are produced in the same pathway with acetoin being produced first, then converted to 2,3-BDO by butanediol dehydrogenase (Bdh1), the advances in metabolic engineering of either acetoin or 2,3-BDO production has helped to inform strategy for the other product as well since one is produced directly after the other [50,91]. This is also the case in *S. cerevisiae*, which naturally produces low levels of acetoin through pyruvate that is converted to α-acetolactate by acetolactate synthase in the mitochondria. Here, α-acetolactate undergoes spontaneous decarboxylation to diacetyl which is converted to acetoin by Bdh1. However, acetoin is not normally detected since it is readily converted to 2,3-BDO by Bdh [92]. Therefore, metabolic engineering strategies to produce and accumulate acetoin in yeast almost always include blocking this last conversion step to 2,3-BDO. This seems to be the biggest difference between acetoin and 2,3-BDO metabolic pathway engineering. Bae et al. reported a genetically engineered *S. cerevisiae* strain that produced 100.1 g/L acetoin during fed-batch fermentation [71]. Similar to strains engineered for 2,3-BDO, the starting yeast strain utilized deletion of all five alcohol dehydrogenases (*ADH1*, *ADH2*, *ADH3*, *ADH4*, and *ADH5*) to block ethanol production and deletion of two glycerol 3-phosphate dehydrogenases (*GPD1* and *GPD2*) to block glycerol production, thereby increasing pyruvate levels [79]. Carbon flux is then directed toward acetoin by expressing the biosynthetic genes acetolactate synthase (*BsAlsS*), which forms α-acetolactate from pyruvate and acetolactate decarboxylase (*BsAlsD*), which converts α-acetolactate to acetoin [71]. Acetoin accumulation was accomplished by deleting 2,3-BDO dehydrogenase *BDH1* to prevent conversion to 2,3-BDO and redox balance was improved by expressing the *Lactococcus lactis noxE* encoded, water-forming NADH oxidase to regenerate NAD^+ [71]. The resulting strain and acetoin titer might be further improved by employing a high copy number integration of the same biosynthetic genes *BsAlsS* and *BsAlsD* into the yeast genome to increase acetoin production and alleviate concerns about possible loss of the plasmid carrying the biosynthetic genes for acetoin production during fed-batch fermentation conditions, which do not maintain selective pressure for plasmid maintenance [83].

Further efforts to improve the metabolically engineered *S. cerevisiae* strain in the previous study were aimed at eliminating additional side products. For example, although deletion of *BDH1* blocked production of (R)-2,3-BDO, the strain still produced meso-2,3-BDO as a side product [93]. In order to improve acetoin yield, the group sought to identify and eliminate gene products involved in forming the meso-2,3-BDO side product as well as a new side product 2,3-dimethylglycerate resulting from the engineered acetoin pathway. To that end, two acetoin reductases, Ara1 and Ypr1, were identified and deleted after determining that Ara1 reduces (R)-acetoin and (S)-acetoin to form meso 2,3-BDO while Ypr1 acts on (S)-acetoin to form mostly meso-2,3-BDO [93]. The resulting strain had similar acetoin production in fed-batch fermentation 101.3 g/L, but purity improved to 96% [93]. This study also underscored one of the caveats of metabolic engineering with the realization that additional, unintended side products may be produced, in this case, requiring deletion

of Ora1 that was involved in the formation of 2,3-methylglycerate [93]. Adaptive laboratory evolution was also employed to improve tolerance to acetoin by gradually increasing acetoin levels over 19 subculture passages in YPD [93].

It could also be beneficial to determine whether acetoin production and yield could be further improved in this strain by implementing the strategy used by Huang et al., utilizing CRISPR technology to insert a high copy number of the biosynthetic operon encoding *BsAlsS* and *BsAlsD* into the genome that correlated with 3.9-fold higher 2,3-BDO production in a previous study [83].

2.3. Summary of Optimization Strategies for Acetoin and 2,3-Butanediol Production

For applications of fermentation technologies in any industrial setting, consideration must be given to strategies to improve the feasibility of microbial biomass conversion. In summary, the elimination of side products to redirect metabolic carbon flux is a major component of optimizing microbial conversion of biomass. The metabolic engineering of pathways may also generate new alternative side products [61,68,79,93]. The expression of biosynthetic pathways to direct carbon flux toward the desired end-product is at the central thrust of metabolic engineering, especially for 2,3-BDO or acetoin [76]. Furthermore, the copy number of plasmids or genomic integrations of biosynthetic pathways also play a role in 2,3-BDO or acetoin titers and yield [76,83,93].

Cellular cofactors levels in many metabolically optimized strains have been modified to balance intracellular redox levels of $NAD(P)^+$/NAD(P)H [72,75,77,94]. Adjusting cofactor levels in the cell helps to drive metabolic carbon flux since several enzyme reactions are cofactor-dependent [68,75,95]. Cofactor engineering strategies in yeast were recently reviewed [95]. Another facet to microbial strain development is adaptive laboratory evolution to improve overall growth rate and robustness. These methods can progress carbon source utilization or tolerance to inhibitors, metabolites, or product accumulation such as acetoin or 2,3-BDO [82,93]. Finally, the implementation of inexpensive, renewable feedstocks is central to making microbial production of high value biochemicals cost effective [24,75,96].

3. Microbial Production of C4 Dicarboxylic Acids

In addition to acetoin and 2,3-butanediol, another class of compounds that is readily derivable from biochemical conversions is C4 dicarboxylic acids; specifically, these are succinic (butanedioic acid), fumaric (trans-butenedioic acid), and malic (hydroxybutanedioic acid) acids. In order to make bio-based production of high value C4 dicarboxylic acids competitive and more economically feasible compared to fossil fuel-based production, it is imperative that microbial conversion of inexpensive biomass be optimized. Part of this process may involve metabolic engineering of strains, adaptation to inexpensive feedstocks, exporter engineering, and process development which includes optimization of aeration, pH, temperature, and isolation/purification steps. Microbial production of valuable, bio-based C4 dicarboxylic acids such as malic acid and succinic acid is performed primarily by filamentous fungi and anaerobic bacteria as well as yeast such as *S. cerevisiae.* In *S. cerevisiae*, the C4 dicarboxylic acids can be produced either in the cytosol by the reductive TCA branch or through modification of the mitochondrial TCA cycle [97]. Production strategies of C4 dicarboxylic acids in *S. cerevisiae* have been recently reviewed [97].

3.1. Exporter Engineering and Metabolic Engineering

One facet of engineering microbes to produce C4 dicarboxylic acids at a higher level involves ensuring efficient export to the cell surface through the process of exporter engineering. Keeping intracellular organic acid levels low circumvents feedback inhibition, toxic buildup, or utilization by another pathway within the cell. This strategy also facilitates easier isolation and purification from the fermentation medium. Multiple studies have implemented the heterologous expression of exporters or permeases belonging to different protein families in bacteria and fungi [75,98–100].

For instance, the *Schizosaccharomyces pombe* transporter Mae1, a member of the voltage-dependent slow-anion channel transporter (SLAC1) protein transporter family, has been found to transport succinic, malic and fumaric acids out of the cell when expressed in *Xenopus* oocytes. SLAC1 transporters contain two highly conserved phenylalanine residues in the transport channel involved in transport activity. Several Mae1, SLAC1 homologs, from fungal sources have been evaluated in *S. cerevisiae* through heterologous expression and were found to increase malate export. The SLAC transporter, Dct, from *Aspergillus carbonarius* (AcDct) increased malate secretion in yeast by 12-fold under neutral pH while the *S. pombe* Mae1 (SpMae1) expressed in yeast increased titers of succinic, malic, and fumaric acids by 3-, 8-, and 5-fold, respectively [98,99,101]. Since these SLAC transporters are independent of proton- or sodium-motive forces, this transport mechanism requires less energy than others, thereby enabling improved yields.

Additionally, transporter engineering is usually coupled with metabolic modifications. The creation of anapleurotic pathways is sometimes central to optimizing microbial production of downstream products. Several studies have reported efforts to enhance production of microbial pathways through the reductive and oxidative TCA pathways (Figure 3), which, combined with other strategies including exporter engineering, can significantly increase C4 dicarboxylic acid titer. Fumarate is between malate and succinate in the TCA cycle, and subsequently, emphasis is given to the microbial production of malic and succinic acids in the following sections.

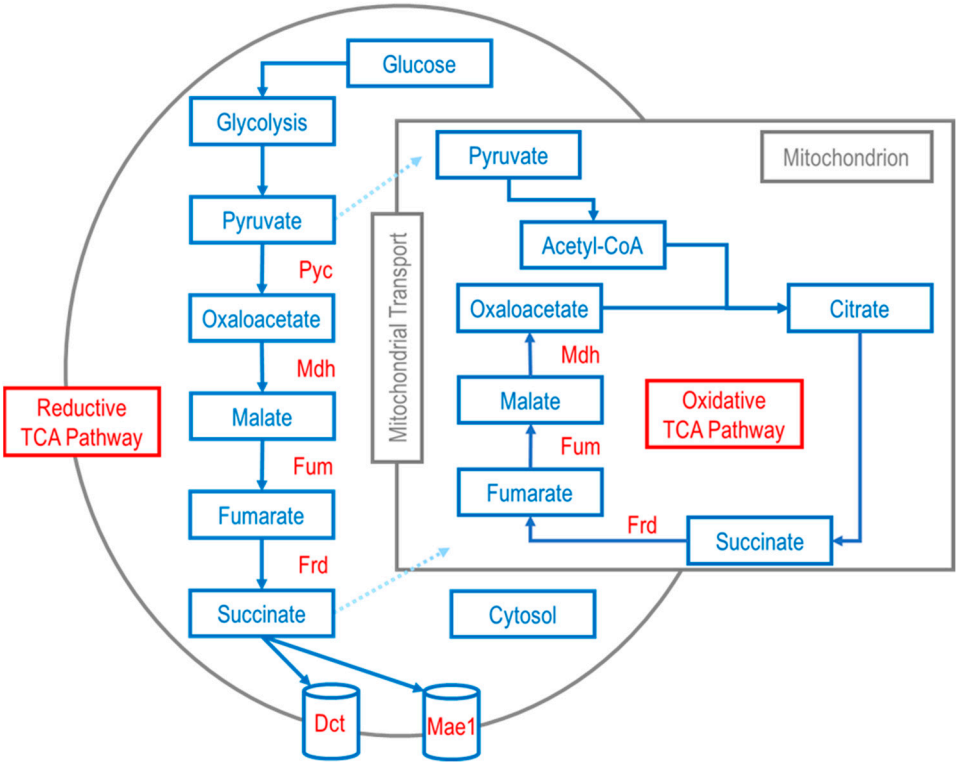

Figure 3. Example schematic of metabolic pathway(s) for C4 dicarboxylic acid production (succinate/succinic acid; fumarate/fumaric acid; malate/malic acid) in eukaryotes via the oxidative TCA pathway in the mitochondrion and the reductive TCA pathway in the cytosol. Enzymes of the depicted oxidative and reductive TCA pathways: pyruvate carboxylase (Pyc); malate dehydrogenase (Mdh); fumarase (Fum); fumarate reductase (Frd). Transport of C4 dicarboxylic acids out of the cytosol is aided by voltage-dependent slow-anion channel transporter (SLAC1) proteins transporters such as Mae1 and Dct. Adapted from previously published work [98,99,101].

3.2. Malic Acid

Aspergillus oryzae is a natural producer of malic acid and has been investigated for further optimization [102]. For instance, Liu et al. employed three strategic steps to dramatically improve L-malate titer in *A. oryzae*. First, they enhanced the reductive TCA (rTCA) pathway by overexpression of endogenous pyruvate carboxylase and malate dehydrogenase to drive carbon flux toward the rTCA pathway and improve malate titer. Next, an anapleurotic pathway to oxaloacetate was achieved by heterologous expression of *E. coli*-derived phosphoenol pyruvate caboxykinase and phosphoenol pyruvate carboxylase, which further increased malate titer. Third, to improve export from the cell and block malate transport back into the mitochondrial TCA cycle, they overexpressed the native C4-dicarboxylate transporter in *A. oryzae* and the *S. pombe* malate permease, Mae1. Additionally, the final strategy implemented was the identification of the potential rate limitation by 6-phosphofructokinase, which was overexpressed to further improve malate titer from 26.1 g/L in the parental strain to 93.2 g/L in flask culture and an impressive 165 g/L in fed-batch fermentation [103]. In another study, malic acid was produced by *A. oryzae* grown on lignocellulosic-derived acetate as an alternative carbon source, but much lower malic acid titers were achieved [104].

Aspergillus niger tolerates low pH and grows on a variety of renewable carbon sources, making this organism especially attractive for cost-effective fermentations. Recently, a Cre-loxP-based genetic system was developed in *A. niger* to construct "*A. niger* cell factories" that produce high levels of organic acids [105]. The deletion of *oahA* was found to block the oxalic acid biosynthesis pathway, allowing carbon flux from oxaloacetate to flow toward a higher production of malic acid instead. Furthermore, the expressions of pyruvate carboxylase (Pyc) and malate dehydrogenase (Mdh3) were used to enhance the rTCA cycle along with expression of a C4 dicarboxylic acid transporter c4t318 from *A. oryzae.* This strategy increased malic acid via the rTCA pathway to 120.38 g/L in flask fermentations and 201.24 g/L during fed batch fermentation [105].

3.3. Succinic Acid

Some natural producers of succinic acid include *Mannheimia succiniciproducens*, *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens*, and *Basfia succiniciproducens* [102,106]. For instance, a wild type strain of *A. succinogenes* was reported to produce 67.2 g/L succinic acid during batch anaerobic fermentation on glucose with a productivity of 0.8 g/L/h [107]. Another study used immobilized *A. succinogenes* cultures entrapped within alginate beads in a three-phase fluidized reactor to produce 31 g/L succinic acid with 35.6 g/L/h productivity [108]. Moreover, other strategies such as dual-phase fed batch fermentation have been reported for *C. glutamicum* during which the first phase involves cell growth to optimal OD followed by succinic acid production in the second phase [109]. Besides natural succinic acid producers, other studies have reported engineered bacterial strains that produce high levels of succinic acid including *E. coli* [110–112].

Further optimization of natural succinic acid producers has also led to improved succinic acid production. *Basfia succiniciproducens* was optimized to produce 20 g/L succinic acid by deleting *pflD* and *ldhA*, to eliminate formic acid and reduce lactic acid production resulting in increased carbon flux toward pyruvic acid and succinic acid [113,114]. Furthermore, 16s rRNA analysis indicates that *B. succiniciproducens* is very closely related to *M. succiniciproducens*.

The *M. succiniciproducens* strain MBEL55E was originally isolated from the rumen of a Korean cow and found to produce high levels of succinic acid [115]. In the capnophilic *M. succiniciproducens*, the formation of succinic acid involves carboxylation of phosphoenol pyruvate (PEP) by either PEP carboxykinase or PEP carboxylase to oxaloacetate during anaerobic respiration in the presence of CO_2 [116]. PEP carboxylation flux is decreased when CO_2 is replaced with N_2, but when H_2 is added to the fermentation in the presence of CO_2, succinic acid levels increased, likely due to additional reducing power [117]. Increased flux toward oxaloacetate flows into the reductive TCA cycle which proceeds

through malate, and fumarate as the terminal electron acceptor resulting in the formation of succinate. Furthermore, one patent reported fermentation with *M. succiniciproducens* using glycerol and sucrose as carbon sources to produce succinic acid with high productivity of 29.7 g/L/h [106,118]. Another study reported using a PALFK strain that was constructed with deletions in *ldhA*, *pta*, *ackA*, and *fruA* to produce 78.4 g/L homo-succinic acid with a productivity of 6.02 g/L/h [119].

Metabolic flux analysis based on updated genome metabolic information of pathways, metabolites, and gene deletions has been performed to optimize and balance cell growth rate with succinic acid production rate [120–122]. The *M. succiniciproducens* PALK strain was generated from the LK strain background containing a lactate dehydrogenase disruption Δ*ldhA*. Additional deletions in *pta* (phosphotransacetylase) and *ackA* acetate kinase were performed to dramatically reduce acetic acid and lactic acid byproduct formation, direct carbon flux toward succinic acid formation, and simplify recovery and purification efforts thereby lowering costs [122]. Even without the *pflB* deletion, no formic acid was formed, and pyruvic acid was the main byproduct. Pyruvic acid accumulation was ameliorated through implementation of chemically defined medium, which resulted in 66.14 g/L during fed-batch fermentation. Titer was further improved by pH control measures with magnesium hydroxide and ammonia, which improved succinic acid titer to 90.68 g/L underscoring the impact of optimized pH control [122].

One study reported using elementary mode analysis with clustering to examine the *M. succiniciproducens* metabolic networks and predicted that overexpression of the *zwf* gene would increase succinic acid production [123]. The overexpression of *zwf* increased the NADPH levels that could be utilized by NADPH-dependent *Arabidopsis thaliana* malate dehydrogenase (Mdh) in the previously constructed LPK7 strain with deletions in *ldhA*, *pflB*, *pta*, and *ackA* that increase carbon flux to succinic acid [123]. The overexpression of both *zwf* and *mdh* revealed possible synergistic activity that resulted in improved succinic acid production.

In another study, high level succinate production was achieved by *M. succiniciproducens* heterologously expressing *Corynebacterium glutamicum* malate dehydrogenase (Mdh) with a higher specific activity for oxaloacetate reduction to malate and lower substrate inhibition than the endogenous MsMdh [124]. Fermentation with a high-inoculum, glycerol-glucose dual fed-batch fermentation yielded 134.25 g/L succinic acid and astonishing productivity of 21.1 g/L/h [125].

In addition, filamentous fungi such as *Aspergillus* sp. have been widely adapted and utilized for fermentation on various plant biomass feedstocks as microbial cell factories to produce organic acids [126]. The heterologous expression of *A succinogenes* phosphoenolpyruvate carboxykinase (*AsPEPCK*) and *E. coli* phosphoenolpyruvate carboxylase (EcPPC) in *A. carbonarius* has been used to direct enhanced carbon flux toward oxaloacetate and the reductive TCA pathway [127]. The later efforts of Yang et al. in *A. carbonarius* involved overexpression of the C4-dicarboxylate transporter Dct as well as heterologous expression of NADH-dependent fumarate reductase (Frd) from *Trypanasoma brucei* on a glucose oxidase-deficient parental strain background (Δ*gox*) [99,128]. The Δ*gox* genotype prevented conversion to gluconic acid, while Frd increased reduction in fumarate to succinate. The results showed a significant improvement in malic acid production and slight increase in succinic acid production with Dct overexpression, whereas the effect of Dct and Frd overexpression together significantly increased both malic acid and succinic acid levels (maximum reported titers of 32 g/L malic acid and 16 g/L succinic acid). Moreover, these results were obtained on wheat straw hydrolysate rich in both glucose and xylose, emphasizing the feasibility of organic acid production on renewable feedstocks [99].

More recently Yang et al. performed metabolic engineering in *Aspergillus niger* using ribonuceoprotein-based CRISPR-Cas9 technology to overcome low homologous recombination challenges. This approach enabled gene mutations in glucose oxidase (*gox*) and oxaloacetate hydrolase (*oah*) through non-homologous end joining and facilitated insertion of overexpression gene constructs for the *A. carbonarius* AcDct transporter and the

NADH-dependent fumarate reductase (Frd) into the genome [100,128]. The highest titer of succinic acid achieved by the resulting SAP-3 strain was (17 g/L) after three days at 35 °C. The resulting SAP-3 strain was also able to utilize sugar beet molasses and wheat straw hydrolysate as inexpensive carbon sources, resulting in succinic acid titers of 23 g/L and 9 g/L after 6 days.

3.4. Co-Production Strategies during Fermentation

Alternatively, some strains have been modified to co-produce two valuable chemicals during fermentation. For example, *Enterobacter cloacae* was engineered to produce both acetoin and succinic acid by deletion of *budC* to block 2,3-BDO production and deletion of *ldhA* to block lactic acid production thereby improving carbon flux toward acetoin and succinic acid [129]. Co-production of 2,3-BDO and succinic acid has also been reported for *E. cloacae* [130]. In *Propionibacterium acidipropionici*, propionic acid and succinic acid were co-produced by semi-continuous fermentation where propionic acid was removed by membrane separation and chromatography to negate end-product feedback inhibition [131]. Moreover, a co-fermentation method for *S. cerevisiae* and *A. succinogenes* was recently reported for the co-production of succinic acid and ethanol [26]. *S. cerevisiae* fermentation utilizing hydrolyzed lignocellulosic biomass produced ethanol and CO_2. The CO_2 was then assimilated by *A. succinogenes* to produce succinic acid [26]. Another group reported high-level production of ethanol and succinic acid on a similar substrate using a robust *S. cerevisiae* strain [132]. During the *S. cerevisiae* fermentation, the CO_2 produced during ethanol production was utilized to produce succinic acid via the reductive TCA pathway [133]. Another approach for co-production of 2,3-BDO and succinic acid by *Klebsiella pneumoniae* was to optimize pH and increase dissolved CO_2 levels in fermentation medium for improved succinic acid yield [134]. Optimized CO_2 levels have also been reported to improve succinic acid titers in *Actinobacillus succinogenes* fermentations [135]. Likewise, another *K. pneumoniae* strain, DSMZ2026, was reported to produce optimal levels of both 1,3-propanediol and 2,3-BDO during an anaerobic fermentation on glycerol when pH in the bioreactor was controlled and maintained at 7 [136].

3.5. Summary of Optimization Strategies for C4 Dicarboxylic Acids

Collectively, a diverse variety of strategies have been implemented to augment C4 dicarboxylic acid production, consideration of which may benefit and improve aspects of future studies. These include optimization of fermentation conditions including growth medium, growth rate, carbon source, pH control, O_2, CO_2, N_2, and H_2 levels [135]. In particular, the growth medium and method of carbon source feeding, whether in batch flask or fed-batch fermentations, can impact production. The sourcing of feedstock for carbon sources in the growth medium not only impacts growth and production of dicarboxylic acids, but also affects whether the microbial production is cost effective. Additionally, in some cases, bioreactor conditions such as pH determine whether the dicarboxylic acid or conjugate base is formed, as is the case with succinic acid or succinate. Additionally, specialized bioreactor conditions may also involve immobilization of cultures by adherence or entrapment [108]. Co-fermentation strategies that produce two high value chemicals in the same bioreactor with two different microorganisms can increase cost-effectiveness and overall productivity [129,130]. Another facet of engineering involves bioinformatic approaches that are informed by genomic data to conduct metabolic flux analysis, which considers pathways, genes, and metabolites and can further inform metabolic engineering approaches by predicting favorable genetic manipulations. [122,123].

Metabolic engineering strategies often involve the elimination of byproducts and unwanted anapleurotic pathways through targeted gene deletions. In addition, the heterologous expression of C4 dicarboxylic acid transporters can alleviate feedback inhibition and prevent utilization of accumulated end products by other pathways [75,98–100]. These strategies can also be paired with the creation of biosynthetic pathways in an engineered

organism to create cell factories that are specifically tailored to produce the desired end product [97,100,128].

4. Fermentation on Sugar Crop Processing Products and By-Products

By-products of sugar crop processing, specifically sugarcane and sugar beet molasses, are themselves high in sugar content. This makes the direct utilization of molasses as a substrate for fermentations promising for further biotechnological development. Although the major non-food by-product of sucrose production from sugarcane and sugar beet is lignocellulosic bagasse and sugar beet pulp, respectively, these products do not contain significant sugar content. Pretreatments are necessary to make available the holocellulosic components as a carbon source for microbial growth [137–139]. For this reason, emphasis is given in this section to studies utilizing molasses to produce C4 platform chemicals with GRAS microorganisms. Further reading is available in reviews (among many others) from Alokika et al. on sugarcane bagasse and Finkenstadt on complete sugar beet utilization [19,140].

4.1. Acetoin and 2,3-Butanediol from Molasses

The advances in metabolic, cofactor and adaptive engineering are certainly promising and continue to improve 2,3-BDO and acetoin production, yield, and purity. However, another facet to optimizing microbial conversion of sugars to valuable chemicals and building blocks is by utilizing inexpensive feedstock or biomass sources to improve cost effectiveness. Most metabolically engineered strains described previously herein were fermented on glucose, which can be an expensive substrate. However, there are numerous reports of using renewable sugar sources for fermentations to produce acetoin or 2,3-BDO [74,85,141–143].

In addition, fermentation of *Paenibacillus* species on several renewable carbon sources was recently reviewed [144]. In particular, the GRAS bacterium, *Paenibacillus polymyxa* was grown on crude glycerol waste and sugarcane molasses to produce optically pure levo-2,3-BDO at 19 g/L [145]. Although this yield is about half that for fermentation on glucose or hydrolyzed cellulose, the results underscore the potential for utilizing waste products as inexpensive feedstock. Similarly, Yang et al. have reported on the production of 2,3-BDO using non-pathogenic *Bacillus amyloliquefaciens* with biodiesel-derived glycerol and beet molasses as a co-substrate. In their initial work, these authors report that molasses supplementation increased fermentation productivity and conversion, with a titer of 83.3 g/L of 2,3-BDO and productivity of 0.87 g/L/h [146]. Subsequent optimization work by these authors gave a higher titer of 102.3 g/L and productivity of 1.16 g/L/h, suggesting that this microorganism has significant promise for further industrial development for 2,3-BDO production [147]. Maina et al. also report successful production of both acetoin and 2,3-BDO from a *Bacillus amyloliquefaciens* strain. In this work, the authors utilized very high polarity cane sugar and sugarcane molasses as substrates, giving titers of up to 28 g/L 2,3-BDO and 25.6 g/L acetoin, depending on operating conditions. These authors also indicate that manipulation of the oxygen transfer coefficient allows for diversion of bacterial metabolism towards either 2,3-BDO or acetoin production [148].

Sweet sorghum syrup and sugar beet juice have also been used as sugar-rich carbon sources for *Bacillus subtilis* fermentations to produce acetoin with titers ranging from 30–60 g/L, depending on the percentage of sweet sorghum or sugar beet juice used as the carbon source as well as additional nitrogen in the form of corn steep liquor [24]. Work from Xiao et al. on the utilization of *Bacillus subtilis* with sugarcane molasses and soybean meal hydrolysate reports titers of roughly 35–40 g/L acetoin in batch fermentations [149]. More recent work from Dai et al. reports a titer of 61.2 g/L of acetoin from a marine *Bacillus subtilis* strain using sugarcane molasses, compared to a higher yield of 76.0 g/L when using glucose in batch fermentation [150]. In addition to molasses, sweet sorghum syrup, and sugar beet juice, glycerol has also been utilized as an inexpensive carbon

source to produce 2,3-BDO. For example, Ripoll et al. have studied glycerol for 2,3-BDO production using the organism *Raoutella terrigena* [151–153].

4.2. C4 Dicarboxylic Acids from Molasses

The most commonly reported C4 dicarboxylic acid found in the published literature is succinic acid, which is frequently produced in studies of *Actinobacillus succinogenes* [54,154–157]. Several of these works have explored the utilization of *A. succinogenes* with sugarcane molasses. Cao et al. analyzed the biosynthesis of succinic acid from both sugarcane molasses and a model sugar mixture, reporting the highest production of 64 g/L from 150 kDa ultrafiltration membrane-pretreated molasses. Untreated molasses produced 55 g/L succinic acid, and a glucose control resulted in 42 g/L. The observed increase in succinic acid production on molasses was attributed by the authors to the presence of essential vitamins/nutrients present in molasses, which are absent in model sugar solutions. The authors also report that ratios of glucose, fructose and sucrose in molasses had little effect on succinic acid production [158]. A study from Wang et al. similarly reports the highest production of succinic acid from *A succinogenes* utilizing molasses as a carbon source, in comparison to experiments using glucose, fructose, sucrose, and a sugar mixture. These authors further report concentrations of 84 g/L succinic acid (with 93% yield) in a fed-batch microbial electrolysis cell bioreactor using polyacrylamide-pretreated molasses [159]. Shen et al. report a maximum production of 65 g/L of succinic acid with 86% yield from molasses using *A. succinogenes* and yeast extract as a nitrogen source in a fed-batch reactor. However, these authors also show a comparable production of 61 g/L succinic acid when using corn-steep liquor and peanut meal as significantly lower-cost nitrogen supplements [160]. Klasson et al. have also studied succinic acid production from sweet sorghum syrup. In this work, the authors report 27 g/L of succinic acid produced from genetically engineered *E. coli* strain AFP184 when using hydrolyzed syrup as a carbon source (in comparison to 60 g/L succinic acid from pure glucose solutions) [161].

Two other potentially high-value C4 dicarboxylic acids in the TCA cycle, in addition to succinic acid, are fumaric acid and malic acid. Although these have not received the same level of attention in published work as succinic acid, recent studies have explored their production using molasses as a carbon source. The most commonly employed microorganism for malic acid production is *Aureobasidium pullulans* (an opportunistic human pathogen), where polymalic acid is the primary product, and is subsequently hydrolyzed to yield the malic acid monomer [162,163]. In one study of polymalic and malic acid production from sugarcane molasses from *A. pullalans*, Feng et al. report a highest final polymalic acid titer of 82 g/L from fed-batch fermentation. This corresponds to 94 g/L malic acid after hydrolysis, with reported malic acid yields of 62% and productivity of 0.67 g/L/h [164]. Wei et al. have also studied the production of polymalic acid from sugarcane by-products, although they do not provide any results on malic acid. These authors report titers of 50–120 g/L for polymalic acid with productivity of 0.41–0.66 g/L/h, concluding that *A. pullulans* can utilize either sugarcane juice or diluted sugarcane molasses without any pretreatment of nutrient supplementation [165]. The production of fumaric acid among recently published studies primarily relies on fungi of the *Rhizopus* genus, particularly *R. oryzae* and *R. arrhizus* (both opportunistic human pathogens) [166]. Papadaki et al. report highest fumaric acid production from *Aspergillus oryzae* (GRAS) of 40 g/L on very high polarity cane sugar as a carbon source; however, inhibitors present in molasses resulted in lower fumaric acid production in this study [167]. Although fumaric and malic acids are comparatively less researched, relative to succinic acid, opportunity exists for future work on the development of these acids from suitable organisms (like *A. oryzae*) that may be ideal for scale up in an industrial biotechnology setting.

4.3. C4 Platform Chemicals from Lignocellulosic By-Products

The most abundant wastes/by-products from sugar crop processing are lignocellulosic residues (viz., sugarcane bagasse and sugar beet pulp). Sugarcane bagasse especially has

received considerable attention in published studies for many years for a wide variety of applications [15,140,168–170]. Unlike molasses, which contains significant fermentable sugar content, lignocellulosic sugar crop by-products require pretreatment prior to any biochemical conversion processing [171]. Typical pretreatment processing for bagasse relies on strategies to delignify the feedstock, followed by hydrolysis of holocellulosic components to yield fermentable sugars [140,172]. Broadly similar approaches are also applied in the pretreatment of sugar beet pulp to yield fermentable sugars from hydrolyzed holocellulosic residues [173].

A summary of some recently published work on the production of C4 platform chemicals from lignocellulosic by-products of sugar crop processing is given in Table 2. These examples illustrate the feasibility of biochemical conversion as a tool for processing these wastes into value-added final products. An attractive strategy for efficient integration of lignocellulosic by-product and molasses processing is the co-fermentation of molasses with bagasse hydrolysates. This approach has been illustrated in recent experimental work from Zetty-Arenas et al. and from Chacon et al. for the production of biobutanol [27,174]. An example block flow diagram from Zetty-Arenas et al. is given in Figure 4. Although in this published study the authors emphasize acetone-butanol-ethanol fermentation, alternative biochemical conversion schemes could be explored to yield different products (e.g., 2,3 BDO or succinic acid) [175].

Table 2. Details on reported applications of sugar crop lignocellulosic byproducts utilized in the production of highlighted C4 platform chemicals.

Product	Feedstock	Brief Summary	Reference
2,3-BDO	Sugarcane bagasse	2,3-BDO is produced from mutant *E. ludwigii* with xylose-rich hydrolysate from sugarcane bagasse as a feedstock. Fed-batch fermentation resulted in accumulation of 68 g/L with yield of 38% and productivity of 0.9 g/L/h, with acetic acid by-product. Separation with an optimized aqueous two-phase system resulted in recovery of 97%. Techno-economic analysis using ASPEN Plus software is also presented for estimation of capital and operating costs.	[176]
2,3-BDO	Sugarcane bagasse	Metabolic/pathway engineering of *E. aerogenes* with gene deletions is explored for the improvement of xylose consumption and 2,3-BDO yield. Sugarcane bagasse hydrolysate was utilized as a feedstock for 2,3-BDO production. A carbon yield of 70% is reported after 36 h, decreasing to 40% at 72 h. By-products include succinate, acetate, ethanol, and acetoin.	[177]
Succinic Acid	Sugarcane bagasse	Sugarcane bagasse is pretreated with three different methods (hot water, ethanol, sodium hydroxide) and subsequently utilized in fermentations with *A. succinogenes* for succinic acid production. Sodium hydroxide pretreatment is reported as the most successful. The authors report maximum yield of 41 g/L with productivity of 0.3 g/L/h and present an assessment of energy and water consumption of the developed process.	[178]

Table 2. *Cont.*

Product	Feedstock	Brief Summary	Reference
Succinic Acid	Sugarcane bagasse	The co-utilization of glucose and xylose from sugarcane bagasse hydrolysates is explored for the production of succinic acid from *Y. lipolytica*. Mixed glucose-xylose carbon source resulted in a titer of 28 g/L with 55% yield and 0.36 g/L/h productivity; bagasse hydrolysates resulted in a higher titer of 33 g/L and higher yield of 58% with 0.33 g/L/h productivity.	[179]
Succinic Acid	Sugar beet pulp	Sugar beet pulp is used in an integrated bio-refinery study for the extraction/production of antioxidants and pectins along with fermentation of hydrolysates for succinic acid from *A. succinogenes*. Fed-batch pilot-scale (50 L) fermentation resulted in production of 30 g/L succinic acid, with 90% yield and 0.75 g/L/h productivity (similar to 5 L lab-scale fermentation). Estimated succinic acid production cost (at 40 kton capacity) is reported to be USD 2.4/kg.	[173]
Succinic Acid	Sweet sorghum bagasse	Phosphoric acid-pretreated sweet sorghum bagasse hydrolysate is used in the production of succinic acid from *A. succinogenes*. The authors report final concentration of 17.8 g/L with 61% yield, comparable to yield from pure glucose as sole carbon source. The authors utilize a 3.5 L, CO_2-sparged bioreactor and suggest the feasibility of this process as a sustainable route for carbon sequestration.	[180]
Polymalic Acid	Sugarcane bagasse	Bagasse hydrolysates are utilized in the production of β-poly(L-malic acid) from *A. pullulans*. The authors conclude that the mixture of acid and enzyme hydrolysates is ultimately not recommended as a fermentation substrate, due to effects from sugar ratios and concentration. Further processing is suggested to optimize suitable quantities/concentrations of sugars and acid for effective bagasse hydrolysate utilization.	[181]
Fumaric Acid	Energy cane bagasse	Hydrolysates from pre-treated energy cane bagasse were utilized in the production of fumaric acid with *R. oryzae*. Powdered activated carbon was applied to hydrolysates to remove potential fermentation inhibitors. Optimized conditions resulted in the production of 34 g/L fumaric acid with 43% yield and 0.2 g/L/h productivity, comparable to fermentation using pure glucose and xylose media.	[182]

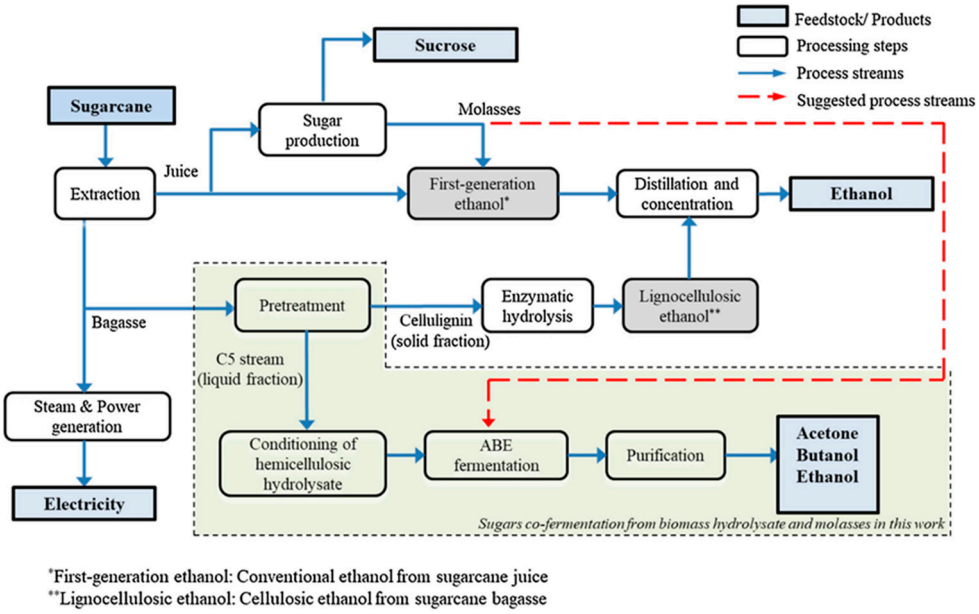

Figure 4. Proposed sugarcane biorefinery process(es) to produce sucrose, power, and fuels/solvents. Reproduced with permission from Zetty-Arenas A, et al. [174], Copyright 2021, Elsevier.

5. Opportunities for Downstream Upgrading

A critical value-added component of C4 platform chemical development is the amenability to downstream upgrading [39]. This has potential for creating a greater market share for sustainable, biochemically-derived intermediates due to the diversity in options for final product development. Following the production of targeted compounds through fermentation and extraction/purification (by means of distillation, membranes, solvent extraction, ion exchange, salting-out and/or sugaring-out, for example [51,183]), C4 compounds can be chemically/catalytically upgraded to a variety of chemicals and high-value material precursors. This review section highlights recent literature on routes to produce these final products from the C4 platform chemicals identified herein.

5.1. Upgrading Acetoin and 2,3-Butanediol

In comparison with 2,3 BDO, significantly less attention has been given to upgrading opportunities for acetoin as a platform chemical in its own right. Acetoin, along with the related C4 diketone butane-2,3-dione (diacetyl), have high value for direct use in flood, flavor and fragrance applications [48,184,185]. One upgrading route for acetoin is through its oxidative dehydrogenation to produce diacetyl. Huchede et al. analyzed this pathway, reporting 85% selectivity and near-complete conversion in air at 465 °C [186]. Another upgrading route for acetoin is through aldol condensation reactions with biomass-derived aldehydes (e.g., furfural, 5-hydroxymethylfurfural) to generate oxygenated fuel precursors. These compounds can then undergo hydrodeoxygenation for the production of sustainable hydrocarbon fuels, as reported in work from Zhu et al. [187]. Acetoin (and diacetyl) has also been used in the catalytic production of 2,3-BDO via vapor-phase hydrodeoxygenation. Duan et al. report high yields of greater than 90% 2,3-BDO from both acetoin and diacetyl, with best results coming from Ni and Cu catalysts at the low temperature of 150 °C [188]. Once 2,3-BDO has been generated, there are a myriad of further upgrading applications for the production of sustainable fuels and chemicals [49–51].

A significant number of studies and reviews have been published in recent years highlighting the opportunities available for microbial 2,3-BDO production and subsequent upgrading strategies. In a recent review from Maina et al., 2,3-BDO is highlighted as a platform chemical precursor for solvents, fuels, paints and coatings, polymers, fertilizers, pharmaceuticals, cosmetics, and food/flavor additives. The 2,3-BDO derivatives typically reported as food and flavor additives are acetoin and diacetyl, which are described previously.

Perhaps the most intensively studied applications of 2,3-BDO are for the production of bio-based fuels, solvents, and polymers [50,51]. Some targeted derivatives for these classes of chemicals are methyl ethyl ketone (MEK), dioxolanes, olefins (especially butenes), and 1,3-butadiene. These compounds are the result of upgrading through the dehydration of 2,3-BDO. In a recent techno-economic analysis study, Maina et al. illustrate the feasibility of a process for MEK production from glycerol-derived 2,3-BDO. The authors report direct aqueous conversion of 2,3-BDO to MEK with subsequent pressure swing distillation to be capable of producing bio-based MEK that is cost-competitive with petroleum-derived MEK [189]. The coproduction of MEK and dioxolanes from 2,3-BDO in catalytic upgrading process has also been reported in recent studies from Bai et al. and Harvey et al. [190,191]. Specific dioxolanes include 2-ethyl-2,4,5-trimethyl-1,3 dioxolane (TMED) and 2-isopropyl-4,5-dimethyl-1,3-dioxolane (IDMD). TMED and IDMD, like MEK, are attractive for their potential as bio-based oxygenated additives in gasoline and diesel fuels [192,193]. A hybrid pathway for sustainable aviation fuel from 2,3-BDO has been studied by Adhikari et al. In this work, the authors report catalytic conversion of 2,3-BDO to olefins (primarily butenes), followed by oligomerization to higher hydrocarbons. Notably, the authors also report that catalyst stability is not affected when co-feeding up to 40% water and 10% acetoin [194]. Finally, several studies have explored butadiene production from 2,3-BDO for sustainable polymer development [195]. Rare earth phosphate catalysts have specifically been highlighted in recent works for their effectiveness in 2,3-BDO dehydrate to butadiene [196]. A summary of several upgrading routes for 2,3-BDO to sustainable final products is given in Figure 5.

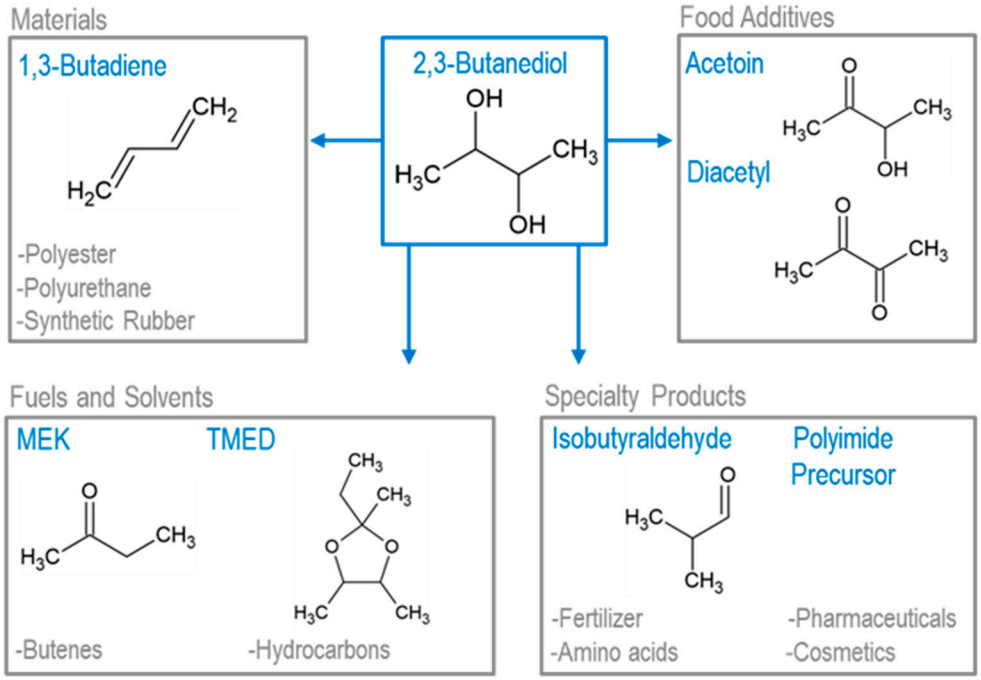

Figure 5. Schematic of potential upgrading possibilities for conversion of 2,3-butanediol (with acetoin highlighted as a food additive) to high-value products.

5.2. Upgrading C4 Dicarboxylic Acids

Like acetoin relative to 2,3-BDO, malic acid and fumaric acid are comparatively less studied in the published literature relative to succinic acid. Malic acid and fumaric acid both primarily have direct applications as food additives [39,163,166]. For fumaric acid, recent work from Lima et al. has explored upgrading to dimethyl fumarate for pharmaceutical applications [197]. Alternative upgrading routes for fumaric acid include scalable production of butyrolactones and enzymatic hydration to malic acid [198,199].

Succinic acid has been recognized as one of the biomass-derived platform chemicals with significant promise for future development [39,52,54,57,183,200–202]. This is largely attributed to its potential ability to supplant applications for petrochemically-derived maleic anhydride, provided that a cost-competitive status can be reached [57,183,202]. In addition to direct applications, the primary fate of upgrading pathways for succinic acid is through its reduction to 1,4-butanediol [39,183,201,202], whose derivatives include tetrahydrofuran, polybutylene terephthalate, polyurethanes, polybutylene succinate and γ-butyrolactone [39]. Other products derived from succinic acid upgrading include pyrrolidines and succinic acid esters, as detailed in work from Silva and Bogel-Lukasik and Aguzin et al. [202,203].

The catalytic conversion of succinic acid to 1,4-butanediol was recently studied by Vardon et al. [204]. In this work, the authors utilize Ru-Sn on activated carbon support for the aqueous phase reduction in succinic acid. Operating at a temperature of 170 °C and hydrogen pressure of 124 bar (at 200 sccm flow rate), a near-complete conversion of succinic acid was achieved, producing roughly 70% 1,4-butanediol and 15% tetrahydrofuran, with γ-butyrolactone and butanol also generated as products. Similar results are reported in work from Kang et al., who also show complete conversion of succinic acid, yielding approximately 70% 1,4-butanediol and lesser quantities of tetrahydrofuran and γ-butyrolactone. Reactor conditions in this experimental work were temperature of 200 °C and hydrogen pressure of 80 bar. The authors conclude that bimetallic Re-Ru catalyst on mesoporous carbon can serve as a stable and reusable catalyst for succinic acid hydrogenation to 1,4-butanediol [205]. Complete conversion of succinic acid with ~80% yields of 1,4-butanediol (also at 80 bar hydrogen pressure and 200 °C) using hydroxyapatite-supported Cu-Pd catalysts has been reported in more recent work from Le and Nishimura [206]. An overall summary of some succinic acid upgrading routes, including and beyond 1,4-butanediol, is given in Figure 6.

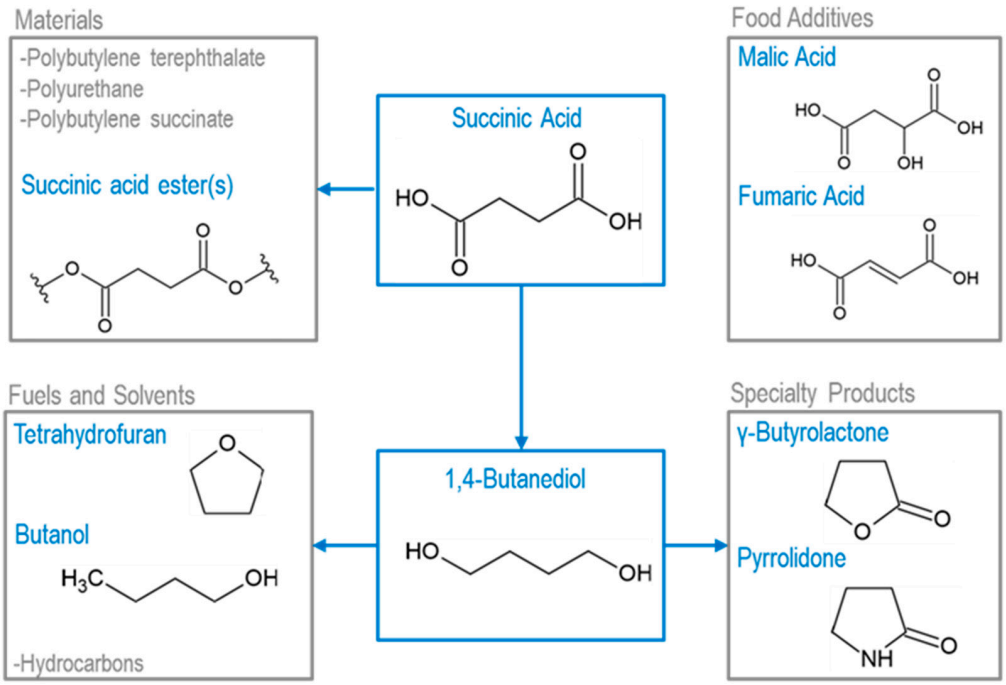

Figure 6. Schematic of potential upgrading possibilities for conversion of succinic acid (with malic and fumaric acids highlighted as a food additive) to high-value products.

6. Summary and Outlook

Biorefinery systems are one of the most prevalent emerging tools for combating climate change while driving industrial development. Although not without their own environmental challenges, biorefineries utilizing fossil-free feedstocks do not introduce

new carbon into the atmosphere. Continued improvements and implementation of lifecycle analyses serve to verify these sustainability gains from green engineering [207,208]. The role of "exnovation" also cannot be overlooked in the industrial transition to renewables [209].

Presented very simply, two critical choices in biorefinery development are (1) the identification of suitable starting feedstock(s) and (2) identifying suitable desired product(s). The utilization of agricultural wastes is desirable because it allows for (more) complete biomass conversion from a given crop, wastes and by-products are readily available in large quantities, and perhaps most importantly, they are available at low cost [210]. This review specifically highlights the agricultural sugar industry, an attractive candidate for on-going sustainable development [12,13]. Utilizing agricultural wastes also affords benefits for ecosystem and social services (e.g., greenhouse gas reduction, air quality, waste management, job creation, food security) which are often-overlooked factors in technoeconomic analyses and business model value streams [211].

With respect to product selection from the conversion of biomass feedstocks, oxygenated commodity chemicals play an important role. In a recent review, Krishna et al. indicate that advantages of biomass-derived oxygenates as targeted products are (1) higher final value, allowing for profitability at smaller scales; (2) lower deoxygenation requirements (relative to hydrocarbons), allowing for higher mass yields; and (3) the conservation of inherent functionalities present in biomolecules [212]. Although the review from Krishna et al. specifically focuses on biomass-derived heterocycles, these three primary points are still broadly true for the C4 platform chemicals emphasized herein. Both acetoin and 2,3-BDO, along with C4 dicarboxylic acids, are naturally produced by many microorganisms. Exploiting microorganisms through industrial biotechnology, for which many obstacles to large-scale commercialization are still acutely present, has several important advantages over existing (petro)chemical industry—especially in terms of sustainability [213–215].

Finally, the highlighted C4 compounds have existing price ranges (viz., ~USD1–3/kg, Table 1) that are attractive for development as platform chemicals to sustain profitable commercial exploitation [39]. The ultimate challenge is achieving these prices from by-product/waste biomass-fed biorefineries. Traditional, rigorous technoeconomic analysis (that incorporate ecosystem and social services) and newer tools like process network synthesis methodology can support the identification of optimum biorefineries based on gross profit and societal benefit [57,216,217].

7. Conclusions

Sugar production from crops like sugarcane and sugar beets generates significant quantities of wastes and by-products. The management and valorization of these by-products (e.g., sugar-rich molasses, lignocellulosic bagasse) is an ongoing challenge facing the sugar industry. One attractive approach for waste management and product diversification is the development of biorefineries utilizing sugar crop processing products/by-products to produce C4 platform chemicals. This review specifically highlights the production of acetoin (3-hydroxybutanone), and 2,3-butanediol, which are in the same pathway, and malic acid (hydroxybutanedioic acid), fumaric acid (trans-butenedioic acid), and succinic acid (butanedioic acid), which are parts of the TCA cycle. These compounds are naturally produced by a wide variety of microorganisms (including GRAS fungi and bacteria) with opportunities for greater utilization of inexpensive feedstocks (agricultural wastes and by-products). These compounds also have suitable market prices in the USD1–3 range for development within the platform chemical framework (as described by Gerardy et al. [39]), to sustain profitable commercial exploitation. C4 platform chemicals can be readily upgraded to diverse classes of chemicals, including: food and flavor additives; solvents, fuels, and fuel additives; polymers and materials (and their precursors); and fine specialty chemicals. Future work can seek to develop these potential biorefineries at both laboratory and pilot scale, coupled with prudent lifecycle assessments and technoeconomic analyses to identify criteria for maximum profitability and sustainability.

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