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Review

Recent Progress in the Microbial Production of Pyruvic Acid

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Abstract: Pyruvic acid (pyruvate) is a cellular metabolite found at the biochemical junction of glycolysis and the tricarboxylic acid cycle. Pyruvate is used in food, cosmetics, pharmaceutical and agricultural applications. Microbial production of pyruvate from either yeast or bacteria relies on restricting the natural catabolism of pyruvate, while also limiting the accumulation of the numerous potential by-products. In this review we describe research to improve pyruvate formation which has targeted both strain development and process development. Strain development requires an understanding of carbohydrate metabolism and the many competing enzymes which use pyruvate as a substrate, and it often combines classical mutation/isolation approaches with modern metabolic engineering strategies. Process development requires an understanding of operational modes and their differing effects on microbial growth and product formation.

Keywords: auxotrophy; *Candida glabrata*; *Escherichia coli*; fed-batch; metabolic engineering; pyruvate; pyruvate dehydrogenase

1. Introduction

Pyruvic acid (pyruvate at neutral pH) is a three carbon oxo-monocarboxylic acid, also known as 2-oxopropanoic acid, 2-ketopropionic acid or acetylformic acid. Pyruvate is biochemically located at the end of glycolysis and entry into the tricarboxylic acid (TCA) cycle (Figure 1). Having both keto and carboxylic groups in its structure, pyruvate is a potential precursor for many chemicals, pharmaceuticals, food additives, and polymers. For example, pyruvate has been used in the biochemical synthesis of L-DOPA [1], *N*-acetyl-D-neuraminic acid [2], (R)-phenylacetylcarbinol [3], butanol using a three-enzyme cascade [4], and has been proposed as a starting material for the enzymatic synthesis of propionate [5]. A recent assessment of *Escherichia coli* as a cell factory concluded that pyruvate was one of the most useful metabolic precursors to a wide range of non-native commercial products [6]. Microbial pyruvate production has been the subject of previous reviews [7,8].

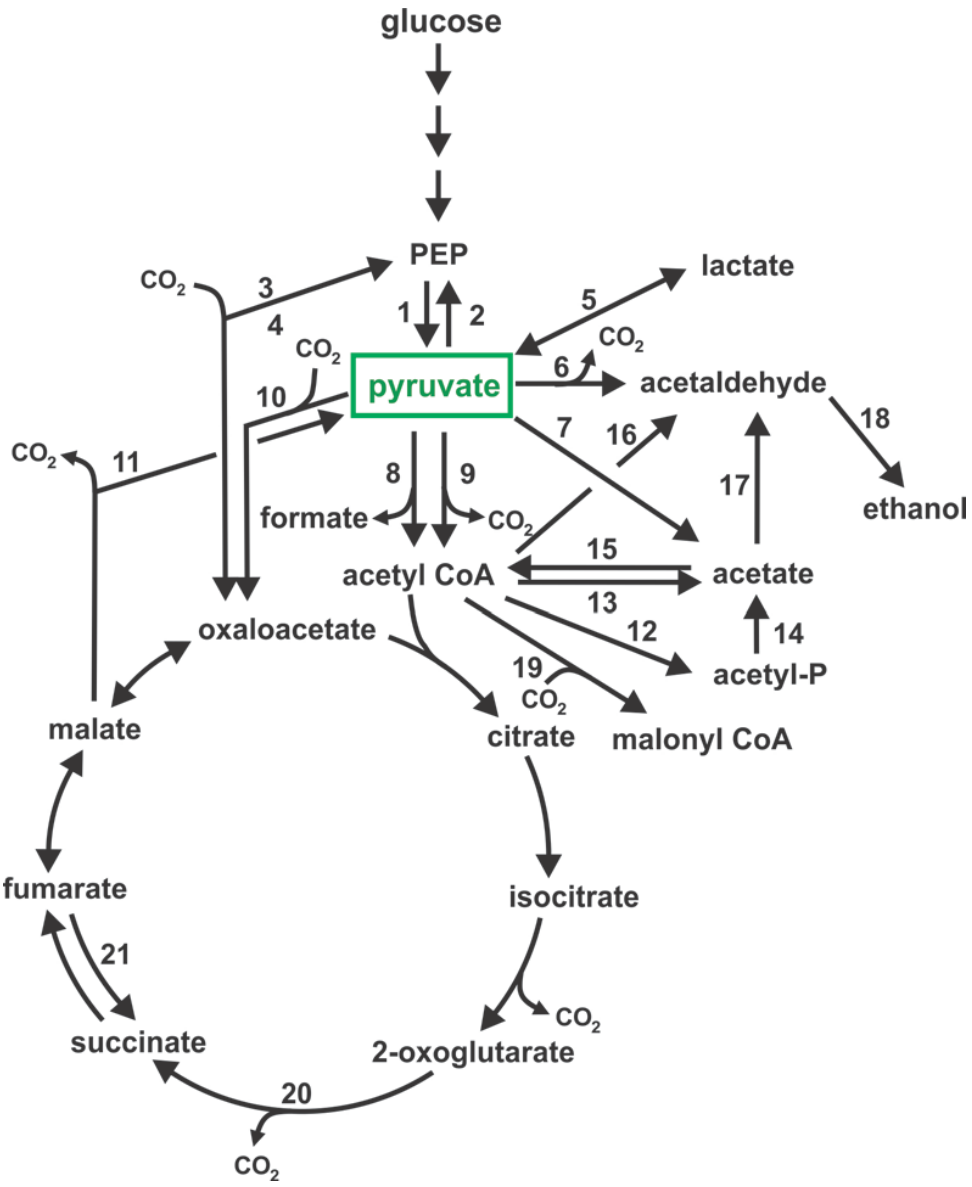

Figure 1. The key metabolic pathways of microorganisms involved in the formation and consumption of pyruvate. Enzyme cofactors (e.g., NAD and NADH) and compounds involved in energy transfer (e.g., ATP) are not shown. Not all organisms express each enzyme shown. The enzymes indicated by numbers are detailed in Table 1.

Pyruvate itself has also long been studied for a wide range of health benefits. For example, pyruvate protects against oxidative stress in human neuroblastoma cells [9,10] and rat cortical neurons [11], protects retinal cells against zinc toxicity [12], improves cerebral metabolism during hemorrhagic shock [13], and protects the brain from ischemia-reperfusion injury [14]. Pyruvate improves myocardial function and increases ejection fraction without increasing heart rate [15,16]. In one double blind study, supplementation with 6 g pyruvate per day for six weeks in conjunction with mild physical activity resulted in a significant decrease in body weight and fat mass [17], and a similar study involving 10 g calcium pyruvate daily for one month with supervised exercise increased very low-density lipoprotein (VLDL) cholesterol and triacylglycerol, and decreased HDL cholesterol [18]. Calcium or magnesium pyruvate is now accepted as a food supplement [19]. Interestingly, pyruvate appears to detoxify hydrogen peroxide in the environment and stimulate the growth of ammonia-oxidizing archaea [20].

Table 1. The key enzymes associated with the metabolism of pyruvate, including gene designations for *Escherichia coli*, *Corynebacterium glutamicum*, *Saccharomyces cerevisiae*, and *Candida glabrata*.

Figure 1 (ref)	Enzyme Accepted Name	EC [1] Number	Reaction	*E. coli* [2]	*C. glutamicum* [3]	*S. cerevisiae*	*C. glabrata*
1	pyruvate kinase	2.7.1.40	PEP + ADP → pyruvate + ATP	*pykA*, *pykF*	*pyk*	*PYK1*	CAGL0E05610g
						PYK2	CAGL0M12034g
2	PEP synthase	2.7.9.2	pyruvate + ATP + H_2O → PEP + AMP + Pi	*ppsA*	*cg0642*, *cg0644*	–	–
3	PEP carboxylase	4.1.1.31	PEP + HCO_3^- → oxaloacetate + Pi	*ppc*	*ppc*	–	–
4	PEP carboxykinase	4.1.1.49	PEP + CO_2 + ADP → oxaloacetate + ATP	*pck*	–	*PCK1*	CAGL0H06633g
		4.1.1.32	PEP + CO_2 + GDP → oxaloacetate + GTP	–	*pck*	–	–
5	D-lactate dehydrogenase	1.1.1.28	pyruvate + NADH + H^+ → D-lactate + NAD^+	*ldhA*	*dld*	–[4]	–[4]
	L-lactate dehydrogenase	1.1.1.27	pyruvate + NADH + H^+ → L-lactate + NAD^+	–	*ldh*	–[4]	–[4]
6	pyruvate decarboxylase	4.1.1.1	pyruvate → acetaldehyde + CO_2	–	–	*THI3*, *PDC1*,	CAGL0G02937g
						PDC5, *PDC6*	CAGL0L06842g
							CAGL0M07920g
7	pyruvate oxidase	1.2.5.1	pyruvate + ubiquinone → acetate + CO_2 + ubiquinol	*poxB*	*poxB*	–	–
8	pyruvate formate lyase	2.3.1.54	pyruvate + CoA → acetyl CoA + formate	*pflB*	–	–	–
9	pyruvate dehydrogenase complex:		pyruvate + CoA + NAD^+ → acetyl CoA + NADH + H^+ + CO_2				
	pyruvate dehydrogenase (E1)	1.2.4.1		*aceE*	*aceE*	*PDB1*	CAGL0K06831g
						PDA1	CAGL0L12078g
	dihydrolipoamide acetyltransferase (E2)	2.3.1.12		*aceF*	–	*PDA2*	CAGL0J10186g
	dihydrolipoamide dehydrogenase (E3)	1.8.1.4		*lpd*	*lpd*	*LPD1*, *IRC15*	CAGL0F01947g
10	pyruvate carboxylase	6.4.1.1	pyruvate + HCO_3^- + ATP → oxaloacetate + ADP	–	*pyc*	*PYC1*, *PYC2*	CAGL0F06941g
11	malate dehydrogenase (NAD^+, decarboxylating)	1.1.1.38	L-malate + NAD^+ → pyruvate + CO_2 + NADH	*maeA*	–	*MAE1*	CAGL0L02035g
	malate dehydrogenase ($NADP^+$, decarboxylating)	1.1.1.40	L-malate + $NADP^+$ → pyruvate + CO_2 + NADPH	*maeB*	–	–	–
12	phosphate acetyltransferase	2.3.1.8	acetyl CoA + Pi → acetyl-P + CoA	*pta*	*pta*	–	–
13	acetyl CoA hydrolase	3.1.2.1	acetyl CoA + H_2O → acetate + CoA	–	–	*ACH1*	CAGL0J04268g
14	acetate kinase	2.7.2.1	acetyl-P + ADP → acetate + ATP	*ackA*	*ackA*	–	–

Table 1. *Cont.*

Figure 1 (ref)	Enzyme Accepted Name	EC [1] Number	Reaction	*E. coli* [2]	*C. glutamicum* [3]	*S. cerevisiae*	*C. glabrata*
15	acetyl CoA synthetase	6.2.1.1	acetate + ATP + CoA → acetyl CoA + AMP + PPi	*acs*	–	*ACS1*	CAGL0B02717g
						ACS2	CAGL0L00649g
16	acetaldehyde dehydrogenase (acetylating)	1.2.1.10	acetyl CoA + NADH + H^+ → acetaldehyde + NAD^+ + CoA	*adhE*	–	–	–
17	acetaldehyde dehydrogenase (NAD)	1.2.1.3	acetate + NADH + H^+ → acetaldehyde + NAD^+ + H_2O	–	*xylC*	*ALD4-6*	CAGL0D06688g
						HFD1	CAGL0H05137g
							CAGL0J03212g
							CAGL0K03509g
	acetaldehyde dehydrogenase (NADP)	1.2.1.5	acetate + NADPH + H^+ → acetaldehyde + $NADP^+$ + H2O	–	–	*ALD2 ALD3*	CAGL0F07777g
18	alcohol dehydrogenase (NAD)	1.1.1.1	acetaldehyde + NADH + H^+ → ethanol + NAD^+	*adhE*	*adhA*	*ADH1-5*	CAGL0I07843g
	alcohol dehydrogenase (NADP)	1.1.1.2	acetaldehyde + NADPH + H^+ → ethanol + $NADP^+$	–	–	*ADH6-7*	CAGL0H06853g
19	acetyl CoA carboxylase	6.4.1.2	acetyl CoA + HCO_3^- + ATP → malonyl CoA + ADP + Pi	*accABCD*	*accABCD*	*HFA1, ACC1*	CAGL0L10780g
20	2-oxoglutarate dehydrogenase complex:		2-oxoglutarate + CoA + NAD^+ → succinyl CoA + NADH + H^+ + CO_2				
	2-oxoglutarate dehydrogenase (E1)	1.2.4.2		*sucA*	*odhA*	*KGD1*	CAGL0G08712g
	dihydrolipoamide succinyltransferase (E2)	2.3.1.61		*sucB*	*sucB*	*KGD2*	CAGL0E01287g
	dihydrolipoamide dehydrogenase (E3)	1.8.1.4		*lpd*	*lpd*	*LPD1, IRC15*	CAGL0F01947g
21	fumarate reductase	1.3.5.4	fumarate + quinone → succinate + quinol	*frdABCD*	–	–	–

(1) Enzyme Commission Number; (2). *E. coli* MG1655; (3). *C. glutamicum* ATCC 13032; (4). *S. cerevisiae* and *C. glabrata* have D-lactate dehydrogenase and L-lactate dehydrogenase (cytochromes) (EC 1.1.2.4 and EC 1.1.2.3).

2. Microbial Formation of Pyruvate

Because pyruvate is a central metabolite, small amounts of pyruvate have historically been reported in microorganisms under a variety of circumstances. The biochemical formation of pyruvate from glucose via glycolysis generally follows the stoichiometric equation:

$$\text{glucose} + 2\text{NAD} + 2\text{Pi} + 2\text{ADP} \rightarrow 2\text{pyruvate} + 2\text{NADH} + 2\text{ATP} \quad (1)$$

Equation (1) indicates that the maximum theoretical yield of pyruvate (as the ion) is 0.966 g/g glucose, and the equation becomes balanced if the microbial process is able to regenerate NAD and ADP needed to sustain the reaction. Of course, some of the carbon/energy source glucose must also be used to form cellular materials. Nevertheless, Equation (1) indicates that pyruvate theoretically could accumulate without other carbon by-products at a high yield. Also, Equation (1) suggests that the rate of pyruvate formation is affected by the rate of NAD and ADP formation. Thus, recurring themes in research have been reducing by-product formation (including cells themselves) and increasing the availability of NAD and ADP. The key enzymes involved in pyruvate formation and catabolism are listed in Table 1.

Significant progress was made when researchers linked pyruvate generation from glucose in certain fungi to the availability of thiamine, with the observation of about 3 g/L pyruvate in the absence of thiamine but no pyruvate in the presence of excess thiamine [21–25]. Similarly, pyruvate can be observed in lipoic acid auxotrophs [26]. Coupled with increased knowledge of the mechanisms for enzyme kinetics, researchers thus began to appreciate that pyruvate could accumulate in microbes having an impaired ability to decarboxylate pyruvate oxidatively (i.e., low pyruvate dehydrogenase activity), or which were auxotrophic for thiamine or lipoic acid. The observations made with these auxotrophs result from thiamine and lipoic acid each being essential cofactors for the activity of the pyruvate dehydrogenase multienzyme complex: thiamine binds to the E1 decarboxylase domain (coded by the *aceE* gene in *E. coli*) while lipoic acid facilitates acetyl transfer by attaching via an amide linkage to a single lysyl residue of the E2 transacetylase subunit (code by the *aceF* gene in *E. coli*).

Vitamin auxotrophy has therefore often been used to isolate pyruvate-accumulating microorganisms. For example, a thiamine-requiring *Acinetobacter* isolate was able to convert 20 g/L 1,2-propanediol into about 12 g/L pyruvate [27], *Schizophyllum commune* converted glucose into 19 g/L pyruvate in 5 days at a yield of 0.38 g/g [28], and *Debaryomyces coudertii* generated 9.7 g/L pyruvate in 48 h from pectin-containing citrus peel extract [29]. After screening 18 yeasts for pyruvate formation from glucose or glycerol, one thiamine-auxotrophic *Yarrowia lipolytica* generated over 61 g/L pyruvate from glycerol in 78 h at a yield of 0.71 g/g [30]. Thiamine or lipoic acid *Enterobacter* auxotrophs isolated after exposure to the mutagen *N*-nitrosoguanidine generated 4.7 g/L pyruvate from 20 g/L glucose after 72 h [26], and an *E. coli* lipoic acid auxotroph generated over 25 g/L pyruvate from 50 g/L glucose in 40 h in a controlled fermenter [31]. A study of 132 strains isolated *Trichosporon cutaneum* which generated nearly 35 g/L pyruvate from glucose at a yield of 0.43 g/g [32]. Another investigation of several genera of yeasts used oxythiamine, an analogue of thiamine, to select strains for pyruvate productivity [33]. These researchers isolated a strain of *Candida glabrata* (formerly *Torulopsis glabrata*) auxotrophic for thiamine, nicotinate, pyridoxine and biotin which generated 57 g/L pyruvate from glucose and 40 g/L peptone in 59 h at a yield of 0.57 g/g. Mutagenesis of a pyruvate-producing *C. glabrata* generated arginine and isoleucine/valine auxotrophs which accumulated about 60 g/L pyruvate from glucose in 43 h at a yield of 0.60 g/g [34]. Because of this strain's natural predisposition at accumulating pyruvate, *C. glabrata* remains the principal microbe used for pyruvate production [35]. More recently, a *Blastobotrys adeninivorans* isolate generated 43 g/L pyruvate from glucose in 192 h at a yield of 0.77 g/g [36].

3. Medium Optimization

Because auxotrophy specifically and enzyme activity more generally are important to pyruvate accumulation in isolated strains, improvements in pyruvate production can be achieved by media optimization. For example, careful optimization of nitrogen sources and the key cofactors nicotinate and thiamine allowed the development of a fed-batch process using *C. glabrata* leading to nearly 68 g/L pyruvate from glucose in 63 h at a yield of 0.49 g/g [37]. A similar focused comparison of nitrogen nutrients for *C. glabrata* led to 57 g/L pyruvate from glucose in 55 h at a yield of 0.50 g/g [38], while a subsequent statistical optimization of vitamin concentration generated 69 g/L pyruvate from glucose in a 56 h batch process at a yield of 0.62 g/g [39]. Yeast extract is generally not suitable as a medium component because it is rich in thiamine [32]. These studies made clear the importance of vitamins, including those found in complex medium components, and the importance of aeration to pyruvate formation, necessary to ensure NAD availability. Recent genome-scale network analysis of *C. glabrata* confirms this yeast's propensity for glucose transport, its multivitamin auxotrophy and ability to transport organic acids, all attributes which contribute to pyruvate accumulation [40,41].

These early studies represent "classical" (e.g., pre-genomic) approaches to pyruvate formation, where production strains have been *isolated* for a particular target phenotype such as lipoic acid auxotrophy, and then the medium and environmental conditions optimized. Isolation/optimization approaches have continued to advance pyruvate formation. For example, researchers noted that urea is superior to ammonium chloride as the nitrogen source for *C. glabrata*, increasing final pyruvate concentration to about 86 g/L at a yield of 0.70 g/g [42]. The resulting increase in glucose consumption rate and pyruvate productivity was attributed to reduced futile cycling of ammonia ions and to elevated activities in enzymes which generate NADPH. Similarly, since the rate of pyruvate generation slows with an increased concentration of the base-neutralized product, researchers proposed increasing the NaCl-tolerance of the *C. glabrata* production strain [43]. Using continuous culture, a more salt-tolerant mutant was isolated which led to a 41% increase in the final pyruvate concentration compared to the parent strain to 94 g/L in 82 h. Supplementing a culture of *C. glabrata* with proline during growth also protects cells against a high osmotic pressure, and increased pyruvate production from 60 g/L to 74 g/L [44].

Being naturally tolerant to salt and osmotic pressure, halophilic microbes have recently gained attention for microbial production of organic acids [45–47]. One alkaliphilic, halophilic *Halomonas* generated 63 g/L pyruvate in 48 h under aerobic conditions using an unsterilized defined medium having high ionic strength [48]. Isolation/optimization over the recent three decades has proven quite successful in improving pyruvate titer, yield and productivity. In parallel with these classical approaches, the development of powerful genetic techniques over the last twenty years, as well as more sophisticated use of operational methodology, has facilitated the approach of "engineering" microbial metabolism toward the improvement of pyruvate production.

4. Metabolic Engineering of Pathways

Most broadly, metabolic engineering involves the use of genetic tools for the intentional optimization of pathways and regulatory circuitry in cells to affect the formation of an end product. Thus, the use of predictive algorithms and genetic tools to construct a strain *by design* is what distinguishes building a strain with the targeted characteristics to facilitate pyruvate formation from the simple isolation of pyruvate-accumulating strains. Like any product, improved pyruvate production primarily means increasing final concentration, yield from substrate and productivity. Since pyruvate is biochemically located at the end of glycolysis as a direct product from glucose or glycerol (Figure 1), efforts to accumulate pyruvate from these substrates ultimately involve restricting or eliminating the further metabolism of pyruvate, preventing by-product formation, and increasing the rate of glycolysis. Furthermore, any metabolic engineering approach for any product must account for other system constraints, such as the need to provide cells with sufficient NADPH and biochemical precursor molecules to satisfy biosynthetic demand.

An early metabolic engineering approach for pyruvate production focused on increasing the rate of glycolysis. Researchers have long understood that uncoupling oxidative phosphorylation by adding 2,4-dinitrophenol to the medium reduces the energy charge of a cell and increases the glucose consumption rate in *E. coli* [49]. Genetic tools have subsequently allowed the more direct uncoupling of respiration for *E. coli* by mutations in the *atp* operon, resulting in the doubling of glycolytic flux [50]. Essentially, cells compensate for reduced ATP generation from proton motive force by increasing the rate of ATP formation via glycolysis. Since pyruvate formation is so directly linked to glycolysis, researchers armed with modest gene manipulation tools proposed that introducing *atp* operon mutations would affect pyruvate formation. Thus, the introduction of an *atpA* mutation into a previously-isolated *E. coli* lipoic acid auxotroph resulted in a strain generating over 31 g/L pyruvate from glucose in 32 h at a yield of 0.64 g/g in batch culture [51,52]. Notably, as a result of the *atpA* mutation, the volumetric rate of pyruvate formation increased from about 0.8 g/L·h to over 1.2 g/L·h, and the biomass yield decreased from 0.26 g/g to 0.14 g/g. More sophisticated methods to control the intracellular ATP content continue to be developed. One example is constructing a copper-inducible F_0F_1-ATPase inhibitor, the *INH1* gene from *S. cerevisiae* [53]. When used with a pyruvate-overproducing strain of *C. glabrata*, this approach increased the volumetric productivity of pyruvate by 23% to 1.69 g/L·h.

Since increasing glycolytic flux usually has a limited impact on pyruvate yield, researchers have focused on other targets to direct more substrate to the product. Not surprisingly, an early target for knockout was the pyruvate dehydrogenase complex, which is the principal catabolic route for pyruvate (Figure 1). In contrast to earlier approaches which controlled pyruvate dehydrogenase by auxotrophy or by a naturally low enzyme activity, growth of a pyruvate dehydrogenase mutant usually necessitates introducing a secondary carbon source such as acetate or ethanol to provide cells with a source of acetyl CoA. A wide variety of *E. coli* mutants deficient in components of the pyruvate dehydrogenase complex (*aceE*, *aceF*, *lpd* genes) growing with an acetate supplement led to a high pyruvate yield from glucose [54]. The best performing strain *E. coli aceF ppc* generated 35 g/L pyruvate from glucose and acetate in 35 h at a yield of 0.78 g/g glucose [54]. The formation of acetate and lactate under certain conditions in *aceE* or *aceF* strains also implies that pyruvate oxidase (*poxB*), and, despite aerobic conditions, lactate dehydrogenase (*ldhA*) are important conduits for pyruvate metabolism and thus potential future targets for gene knockouts. The importance of pyruvate oxidase in *E. coli* lacking pyruvate dehydrogenase was shown through ^{13}C-flux analysis, which demonstrated that pyruvate oxidase as well as the Entner-Doudoroff and anaplerotic pathways are upregulated in the absence of a functional pyruvate dehydrogenase [55]. Another study using *E. coli aceEF pflB poxB pps ldhA* with a defined medium highlighted the relationship between acetate consumption, measurable CO_2 consumption and cell growth [56]. By careful control of both the acetate and glucose feeds (using online measurement, respectively, of CO_2 evolution rate and glucose concentration), these researchers achieved 62 g/L pyruvate in 30 h at a yield of 0.55 g/g. Integrating electrodialysis to separate pyruvate with a repetitive (fed)-batch fermentation process using this same *E. coli* strain reduced product inhibition and allowed an average yield of 0.82 g/g and productivity of 3.9 g/L·h over four cycles before a reduction in productivity was observed at 40 h [57]. An unstructured model incorporating pyruvate inhibition of growth and product formation was able to represent growth and pyruvate formation adequately, but did not account for the higher glucose consumption rate for pyruvate and maintenance than expected [58]. A neural network approach on the other hand was superior to predict the dynamics of substrate and product concentration changes during acetate feeding [59].

Although many researchers have exploited reduced activity in pyruvate dehydrogenase or have altogether eliminated one of the components of pyruvate dehydrogenase, metabolic engineering approaches can also use strategies which do not directly target this enzyme. In one study, *E. coli pflB poxB ackA ldhA adhE frdBC sucA atpFH* generated 52 g/L pyruvate from glucose as the sole carbon source in 43 h at a yield of 0.76 g/g [60]. This strategy combined a variety of features

including (1) preventing lactate (*ldhA* gene deletion), ethanol (*adhE*) and acetate (*poxB*, *ackA*) formation; (2) curtailing both oxidative (*sucA*) and reductive (*frdABC*) tricarboxylic acid cycle; and (3) increasing the rate of glycolysis by uncoupling respiration (*atpFH*). The process was operated under reduced oxygenation (5% of saturation) which would tend to increase the pool of NADH, an inhibitor of the dihydrolipamide dehydrogenase component of pyruvate dehydrogenase [61] and citrate synthase [62]. Thus, these operating conditions were critical for attaining a high yield of pyruvate (discussed in greater detail below). The study also confirmed the importance of pyruvate oxidase (*poxB*) in pyruvate accumulation. Obviously, the primary distinction between this approach and those involving a gene deletion in the pyruvate dehydrogenase complex is that maintaining some pyruvate dehydrogenase activity obviates an acetate requirement.

New and promising metabolic engineering approaches propose gene silencing rather than gene deletions. For example, silencing the *aceE* gene, particularly when combined with the silencing or deletions of other genes, resulted in 26 g/L pyruvate from glucose in 72 h [63]. A similar approach modified the promoters for the *accBC* genes coding acetyl CoA carboxylase as well as the *aceE* gene [64]. These promoters allowed the doxycycline-controlled expression of these two enzymes involved in pyruvate catabolism, leading to 26 g/L pyruvate in 73 h with a yield of 0.54 g/g [64]. Gene silencing seems particularly suited to targeting pyruvate dehydrogenase, as maintaining some residual activity in this enzyme allows glucose to be the sole carbon source. Gene silencing techniques are destined to become more widespread as methodologies are developed to tune finely the activity of targeted enzymes.

Metabolic engineering approaches have also been applied to microbes other than *E. coli*. In yeast a key enzyme in pyruvate metabolism is typically pyruvate decarboxylase (*PDC* gene), which decarboxylates and reduces pyruvate to acetaldehyde, which is itself reduced to ethanol via an alcohol dehydrogenase. Disruption of *PDC* in *C. glabrata* resulted in significantly greater pyruvate accumulation and less ethanol formation: 82 g/L pyruvate and less than 5 g/L ethanol accumulated from glucose in 52 h at a yield of 0.55 g/g using a medium with 30 g/L peptone [65]. Similarly, disruption of the genes coding the two structural enzymes (*PDC1* and *PDC5*) in *Saccharomyces cerevisiae* increased pyruvate to 25 g/L with a yield of 0.30 g/g in a 96 h shake flask culture in a medium containing 20 g/L peptone, 10 g/L yeast extract and 2.5 g/L sodium acetate [66]. *PDC*-negative *S. cerevisiae* unfortunately require acetate or ethanol for growth, and also cannot tolerate a high concentration of glucose. In one study, a *PDC*-negative strain able to grow on glucose without acetate or ethanol was isolated in a chemostat using progressively lower acetate concentration in the feed, resulting in a strain accumulating 135 g/L pyruvate in a repeated batch process from glucose in 100 h at a yield of 0.54 g/g [67]. In a similar study, a *PDC*-negative strain able to grow on glucose without acetate or ethanol was isolated after 1000 generations of sequential batch cultures incrementally exposed to reduced ethanol and increased glucose concentrations [68]. These approaches thus combine targeted metabolic engineering with more classical mutation isolation methodology.

Increasing interest in the use of lignocellulosic hydrolysates as an inexpensive source of carbohydrates has created an interest in cells which are able to metabolize glucose and xylose simultaneously. Disrupting *PDC1* and *GPD1* (coding for glycerol-3P dehydrogenase) and introducing a xylose transporter and several metabolic genes in *Kluyveromyces marxianus* led to 29 g/L pyruvate in 36 h from a sugar mixture, although glucose was still the favored substrate [69]. Recently, a study generated pyruvate from alginate using the bacterium *Sphingomonas* having an *ldh* knockout [70]. The strain generated nearly 3 g/L pyruvate in about 3 days, and the study demonstrated the importance of oxygen availability.

Corynebacterium glutamicum is widely used industrial bacterium for the production of several amino acids such as L-lysine and L-glutamate. In one study focused on the production of L-valine, formed in four enzymatic steps from pyruvate, strains having an *aceE* gene deletion were observed also to accumulate over 2 g/L pyruvate [71]. A subsequent study examined the additional deletion of pyruvate oxidase (*pqo*, or *poxB* gene) which improved L-valine production and also introduced an

acetate growth requirement, suggesting that in *C. glutamicum* the combination of pyruvate oxidase, acetate kinase and phosphotransacetylase can bypass pyruvate dehydrogenase to allow acetyl CoA generation [72]. Further knockouts in the genes for lactate dehydrogenase (*ldhA*), two transaminases (*alaT* and *avtA*), and the replacement of native acetohydroxyacid synthase with an attenuated variant (C-T *ilvN*) led to 46 g/L pyruvate from glucose and acetate in 105 h at a yield of 0.48 g/g [73]. It should be noted that *C. glutamicum* and various yeasts including *S. cerevisiae* and *C. glabrata* have an active pyruvate carboxylase whereas *E. coli* do not.

5. Cofactor Engineering

Cofactor engineering focuses on altering the availability of NADH/NAD, and it is another means to improve pyruvic acid production. Since the conversion of glucose into pyruvate generates 2 mol NADH per mol glucose (Equation (1)), and NADH is an inhibitor of the dihydrolipamide dehydrogenase component of pyruvate dehydrogenase [61], the redox state has a direct impact on the pyruvate production rate and yield. Elevated NADH is one means of suppressing pyruvate catabolism in strains having pyruvate dehydrogenase activity. On the other hand, enhancing the oxidation of NADH to elevate NAD should increase the rate of glycolysis. Researchers have thus examined contrasting cofactor/metabolic engineering approaches to optimize pyruvate formation, depending on the genetic background of the strain. In one case, overexpression of water-forming NADH oxidase from *Streptococcus pneumoniae* (*nox* gene) in *E. coli* lead to a 70% increase in glucose uptake rate compared to a wild-type background [74]. However, in *E. coli aceEF pflB poxB pps ldhA* under acetate-limited conditions, no improvement in the rate of glycolysis was observed as a consequence of overexpressing *nox* [75]. Only additional knockouts in *atpFH* and *arcA* caused NADH oxidase to have a 12% increase in glucose uptake, and a modest 8% increase in the specific rate of pyruvate formation [75]. Similarly, overexpression of water-forming NADH oxidase (*nox*) or the endogenous mitochondrial alternative oxidase (*AOX1*) in *C. glabrata* increased the pyruvate yield and productivity by 15%–30% to 0.79 g/g and 1.63 g/L·h, respectively [76]. Expression of water-forming NADH oxidase from *Lactococcus lactis* (*noxE* gene) and *E. coli* transhydrogenase (*udhA*) in an evolved *PDC*-knockout *S. cerevisiae* improved pyruvate production to 75 g/L in 120 h with a yield of 0.63 g/g [68]. Another approach enhanced pyruvate formation by introducing acetaldehyde into the medium, which was then reduced to ethanol in a *C. glabrata* strain with high alcohol dehydrogenase activity. The resulting increases in pyruvate productivity, concentration and yield were attributed to elevated NAD concentration in the acetaldehyde-reducing strain [43]. The goal of each of these studies was to decrease NADH concentration. In contrast, for a strain having pyruvate dehydrogenase activity, the generation of more NADH was accomplished by expressing formate dehydrogenase from *Mycobacterium vaccae* (*fdh*) in *E. coli pta* [77]. In a medium with glucose, formate and peptone, using formate dehydrogenase increased pyruvate production from 6.8 to 9.0 g/L in 24 h with a yield of 0.48 g/g glucose.

6. Process Engineering

The formation of any microbial product relies not only on the best strain characteristics (genotype), but also on an optimal operating process. Generally, the optimal conditions for growth are different from those for product formation, and one strategy to accomplish both high growth/productivity and high yield often can be best performed by two distinct process stages. For example, low oxygenation surprisingly favors glucose uptake in *C. glabrata*, while high oxygenation favors pyruvate yield, so that a two-stage high-to-low oxygenation process improves overall performance over any single oxygenation process [78,79]. This compromise comes at the cost of the highest yield, which occurs at the highest oxygenation, demonstrating the difficulty in maximizing both yield and productivity. Because the conversion of glucose to pyruvate generates NADH as noted (Equation (1)), high oxygenation is often required in pyruvate formation. In addition to contributing to a high NAD concentration to drive glycolysis, oxygenation can prevent the formation of reduced by-products in strains lacking knockouts. For example, lactate formation by *E. coli* and pyruvate formate lyase activity can both

be discouraged by high oxygenation during pyruvate generation [77]. In stark contrast, for strains containing a functional pyruvate dehydrogenase, pyruvate generation is enhanced by low oxygenation which encourages intracellular NADH accumulation [80]. pH and other environmental conditions also impact pyruvate formation. Pyruvate production by an *E. coli* lipoic acid auxotroph was maximal at a pH of 6 [31], while *E. coli* with a pyruvate dehydrogenase deletion showed greatest pyruvate generation at 32 °C and a pH of 7 [54]. Not surprisingly, the optimal conditions depend on the genetic background of the strain and the associated strategy used to accumulate pyruvate.

Processes often use nutrient-limited conditions to control growth rate and direct carbon to the desired product. In other words, a limiting nutrient is supplied at a rate below what the microbes can maximally metabolize, resulting in growth being controlled by that nutrient. Glycolytic rate and pyruvate productivity by *E. coli poxB aceEF pps pflB ldhA* were compared at steady-state using glucose, acetate, nitrogen, or phosphorus limitation [75]. The highest yield (0.70 g/g) and specific productivity (1.11 g/g·h) were attained under acetate-limited conditions, allowing the design of an exponential acetate-limited fed-batch process to maintain a constant growth rate of 0.15 h^{-1}. Using an *E. coli* strain with additional *atpFH* and *arcA* knockouts, the process achieved 90 g/L pyruvate in 44 h at a yield of 0.68 g/g glucose [75]. A similar acetate-limited fed-batch process using glycerol as the primary carbon source attained about 40 g/L in less than 40 h with a yield of 0.62 g/g glycerol [81]. The yield from glycerol was greatly improved to 0.95 g/g by the addition of a small amount of glucose into the feed, presumably because the glucose could be catabolized for the generation of NADPH and biomass precursors "above" the entry point of glycerol into glycolysis [81].

7. Other Biological Methods

Pyruvate can be formed through biotransformation of a biochemical using non-growing cells. Advantages of biotransformation over fermentation include using a medium which is typically free of salts and other impurities, resulting in a more easily purified product, and the prospect of using the biocatalysts for several cycles. Of course, the cell "biocatalysts" must be generated and harvested previously in a separate process, so the advantage of improved productivity only exists if several cycles are accomplished. The obvious choices for starting material are compounds which are more reduced than pyruvate, thus allowing the generation of NADH in the cells, which provides the cells with some maintenance energy to sustain the process. For example, *Acinetobacter* sp. at a concentration of 6 g/L (dry cells) converted 0.5 M L-lactate into pyruvate in 12 h [82]. Permeabilized yeast cells of either *Hansenula polymorpha* or *Pichia pastoris* expressing glycolate oxidase and catalase were able to oxidize 0.5 M L-lactate to pyruvate for 12 cycles with 98% conversion of the lactate [83]. An L-lactate concentration above 0.5 M showed substrate inhibition of a whole-cell biocatalyst based on recombinant *P. pastoris*, and oxygen, a substrate for glycolate oxidase, was important for the conversion [84]. These conversions based on catalase/glycolate oxidase were operated at 5 °C to prolong enzyme activities. *Pseudomonas stutzeri* can serve as a biocatalyst for the conversion of D/L-lactate into pyruvate, generating about 23 g/L pyruvate at a yield of 0.89 g/g [85]. Another study with *P. stutzeri* generated 48 g/L pyruvate at a yield of 0.98 g/g after 29 h at a pH of 8 and a temperature of 30 °C [86]. Similarly, 50 g/L D/L-alanine was converted to 14.6 g/L pyruvate in 30 h by previously-grown *E. coli* having deletions in native pyruvate and alanine transporters and overexpressing membrane-bound L-amino acid deaminase [87].

Most often glucose or glycerol is used as the carbon source for pyruvate production. However, because lignin is a large and untapped feedstock, there is growing interest in using the components of lignin for the production of commodity chemicals. Recently, researchers have demonstrated that manipulation of the aromatic catabolic pathway, replacing the *ortho*-cleavage pathway with a *meta*-cleavage pathway, in *Pseudomonas putida* significantly increases pyruvate production from *p*-coumarate and benzoate in an *aceEF* knockout strain [88].

8. Downstream Processes

Several general separations methods may be considered for the recovery of pyruvate from fermentation broth. A traditional method for many organic acids is to precipitate a poorly soluble calcium salt followed by acidification with H_2SO_4 to generate gypsum and free acid, a method disfavored because of the use of large volumes of acid, the generation of a $CaSO_4$ waste stream and the poor selectivity of the process. One alternative to precipitation is liquid-liquid extraction. To be practical, extraction should selectively transfer the acid product from a fermentation broth to an immiscible solvent, and then regenerate the product-free solvent through back extraction. A useful modification of extraction is reactive extraction, whereby an additional chemical (an "extractant") is introduced into the solvent-aqueous extraction system [89]. The extractant is selected for its ability to undergo a reversible complexation with the acid product. Typical extractants for organic acids include phosphoryl-containing extractants such as tributyl phosphate (TBP) or trioctyl phosphine oxide (TOPO) and aliphatic amines such as tri-*n*-octylamine (TOA). The chemical properties of the functional groups present on both the extractant and the diluent (i.e., the solvent) affect the extraction equilibria, and therefore impact the selectivity of the specific desired extraction. Extractant and diluent are regenerated by introducing a pH shift, a temperature shift or by changing the composition of the diluent. Aliphatic amines combined with a suitable diluent are very commonly chosen for carboxylic acids because this combination provides high distribution coefficients at pH values near or below the pK_A of the target carboxylic acid [90].

Numerous studies have compiled extraction equilibria for pyruvate in extractant/diluent mixtures. For the tertiary amine Alamine 336 as extractant, cyclic alcohols provide a high extraction efficiency as diluents [91] and among aliphatic alcohols 1-dodecanol/Alamine yielded the largest separation factors [92]. TOA diluted in decanol/kerosene resulted in 98% pyruvate recovery, with TOA/decanol being a superior system. [93]. An examination of the kinetics and mass transfer of the tributylamine/butyl acetate system demonstrated that the reaction was second order in the diffusion film and independent of hydrodynamic conditions [94]. Equilibrium and kinetics studies for the TOA/1-octanol system revealed that the reaction is first order [95]. Recent studies have also considered mixing phosphate extractants with amine extractants in decanol, resulting in >98% pyruvate recovery [96]. Extraction equilibria for TBP with several diluents suggest that *n*-heptane and toluene provide the greatest extraction [97]. Most systems show that the pyruvate and extractant react with a 1:1 molar ratio. Care must be taken for selecting a system which limits co-extraction of other organic acids and also water. Comparatively little research has been completed in the selective recovery of pyruvate from a solution containing the multiple acids or directly from a fermentation broth. One study examined TOA with various diluents focused on the separation of lactate and pyruvate, taking advantage of the pK_A differences between lactate (3.86) and pyruvate (2.49) to accomplish a pH swing extraction. Using pH swing extraction with trimethylamine as a back extractant, these researchers were able to obtain 97% pyruvic acid purity after removal of the amine with simple distillation [98]. One recent comprehensive study using the TOA/1-octanol system showed significant competition for the amine between lactic acid, acetic acid and pyruvic acid, but pyruvic acid could be recovered selectively in a process which removes acetic acid first, followed by separation of lactic acid and pyruvic acid [99]. The process is most readily accomplished when the lactate concentration is no greater than 20% of the pyruvate concentration and the initial TOA concentration is at least equivalent to the pyruvic acid concentration [99].

A similar recovery method is repulsive extraction, also known as salt-assisted solvent extraction, and this method is applicable to a wide range of fermentation-derived biochemicals [100,101]. The approach involves selectively reducing the solubility of the desired product. Acetone is the most effective extractant at reducing the solubility of pyruvate from the fermentation broth, and integrating acetone extraction with microfiltration, evaporation (to concentrate the mixture) and crystallization led to 97% pyruvate purity and 75% recovery [102].

Another general type of recovery process possible for organic acids is electrodialysis, wherein current is applied to alternating anion exchange and cation-exchange membranes. This method has the advantage of requiring no organic solvent. Electrodialysis has also been successfully applied to pyruvate recovery [103]. Ideally cells are removed prior to the electrodialysis step using ultrafiltration. Electrodialysis can be operated either in a mono-polar mode to generate sodium pyruvate or in a bipolar mode to generate pyruvic acid and a hydroxide, the latter which can potentially be recycled back to the fermenter for pH control. Thus, the fermentation process can be fully integrated with the electrodialysis [57]. Some hurdles remain to make electrodialysis fully compatible with fermentation, including membrane fouling and co-separation of other medium components.

One additional method to recover pyruvic acid is using adsorption, and a primary amine, weakly basic sorbent can be used to uptake pyruvic acid near the pK_A of pyruvic acid (2.49) [104]. Generally, ion exchange has a low capacity compared to extraction or electrodialysis [57].

9. Conclusions

Pyruvate lies at the center of metabolism, at a key junction where carbon partitions between the energy-generating tricarboxylic acid cycle, anaplerotic reactions to replenish this cycle and several biochemical branches leading to building block components such as amino acids and fatty acids. There has been a long history of producing pyruvate by both bacteria and yeast. Original production strains were based on vitamin auxotrophy, as several of the steps from pyruvate involve enzymes which use vitamins as cofactors, while more recent approaches have developed engineered strains, often starting from the high-producing auxotrophs. Approaches which combine evolution or selection of desirable characteristics with targeted engineering of strains will likely dominate future improvements.

Although a great detail of research has used glucose as the carbon source, surprisingly limited research has involved the conversion of glycerol or pentoses derived from lignocellulosic biomass (xylose, arabinose). Of particular interest would be processes which are able to generate pyruvate from mixed substrates. There also continue to be opportunities to isolate or develop strains which are tolerant not only to high osmolarity, but also to low pH, higher temperature and to lignocellulosic hydrolysates or multiple stresses, factors which could reduce production costs. We foresee increasing interest in the integration of continuous or semi-continuous fermentation processes, with their inherent higher productivity, with downstream pyruvate recovery operations. Improving existing microbial processes to produce pyruvate will necessitate an understanding of how cells respond to imposed operational conditions.

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