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REVIEW ARTICLE

Bioproduction of L- and D-lactic acids: advances and trends in microbial strain application and engineering

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ABSTRACT

Lactic acid is an important platform chemical used in the food, agriculture, cosmetic, pharmaceutical, and chemical industries. It serves as a building block for the production of polylactic acid (PLA), a biodegradable polymer, which can replace traditional petroleum-based plastics and help to reduce environmental pollution. Cost-effective production of optically pure L- and D-lactic acids is necessary to achieve a quality and thermostable PLA product. This paper evaluates research advances in the bioproduction of L- and D-lactic acids using microbial fermentation. Special emphasis is given to the development of metabolically engineered microbial strains and processes tailored to alternative and flexible feedstock concepts such as: lignocellulose, glycerol, C1-gases, and agricultural-food industry byproducts. Alternative fermentation concepts that can improve lactic acid production are discussed. The potential use of inducible gene expression systems for the development of biosensors to facilitate the screening and engineering of lactic acid-producing microorganisms is discussed.

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L-lactic acid; D-lactic acid; microbial production; metabolic engineering; analytical techniques; biosensor

Introduction

Lactic acid (2-hydroxypropanoic acid) is a chiral organic acid that can exist in two enantiomeric forms: L- or D-lactic acids. The two optical isomers are attributed to an asymmetric carbon adjacent to a hydroxyl and carboxyl group. These functional groups facilitate various chemical transformations such as: dehydration, decarboxylation, condensation, oxidation, hydrogenation, and esterification. Consequently, the lactic acid can be utilized as a platform chemical for the production of various chemicals including: acrylic, pyruvic and propionic acids, lactic and acrylic acid esters, acetaldehyde, pentane-2,3-dione, propylene glycol, propylene oxide and lactide, with the latter used for polymerization into biodegradable polylactic acid (PLA) [1,2]. Based on market data, properties, technical complexity of the synthesis pathway, and progress in production process development, the US Department of Energy listed lactic acid as one of the top 10 most important bio-based chemicals [3]. Recently, in the UK, lactic acid has been identified as a top high-value green chemical based on commercial viability, functionality, and sustainability [4]. The global lactic acid market was valued at USD 2.6 billion in 2018 and is expected to grow at a compound annual growth rate of 18.7% with a revenue forecast to be USD 8.7 billion in 2025 [5].

Lactic acid was first discovered in sour milk by the Swedish chemist C.W. Scheele in the 1780s [6]. Commercial production was established later in 1881, which allowed for widespread application of the lactic acid not only as a safe acidulant, flavor enhancer, or preservative in food and beverages [7], but also currently in: the pharmaceutical, personal care, and industrial sectors [5].

Most recently, due to its rising use in: packaging, 3D-printing filaments, agriculture, and textiles, the PLA has emerged as a dominant segment of industrial lactic acid application accounting for a 28.3% share of lactic acid market revenue in 2018 [5]. PLA can replace traditional plastics such as: polyvinyl chloride, low-density polyethylene, polypropylene, or polystyrene. The global PLA market is expected to reach an estimated value of USD 3.7 billion by 2024 [8]. However, to achieve high crystallinity in PLA, which enables an increase in the modulus and strength of elasticity and temperature resistance, optically pure lactic acid enantiomer, is

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Supplemental data for this article can be accessed here.

required [9]. Blending poly-(L-lactic acid) and poly-(D-lactic acid) at a specific ratio yields a highly regular stereocomplex of poly-lactic acid with improved crystallinity and thermal tolerances up to 230 °C [10]. Although demand for PLA is expected to increase in the near future, its annual production amount remains dwarfed by the billions of kilograms of commodity petroleum-based plastics produced globally. Moving forward, PLA and similar lactic acid derivatives will become more competitive material options if economically feasible production methods capable of producing optically pure L- or D-lactic acid are developed.

The lactic acid can be manufactured either by using chemical synthesis or microbial fermentation. Chemical synthesis uses petrochemical feedstocks that are less sustainable and results in racemic mixtures. Conversely, microbial fermentation involves fermenting renewable feedstock materials and, with appropriately selected microorganisms possessing either L- or D-lactate dehydrogenase activity, it enables production of optically pure L- or D-enantiomers [10,11]. Moreover, microbial fermentation is less energy demanding as it typically requires a lower temperature than chemical synthesis. The fermentation process has become the industry-standard method for lactic acid production [12] with this approach embraced by several major manufacturers including: Corbion, NatureWorks LLC, Galactic, and others [5].

Process-wise, the microbial fermentation of lactic acid involves the enzymatic breakdown of glucose- or another carbohydrate-rich material (upstream process), but requires product purification through downstream processes such as distillation to recover the optically pure lactic acid. Therefore, the cost of microbial fermentation processes can be seen as a balance between optimizing the upstream versus the downstream processes. For example, high purity sucrose from sugarcane enables the production of higher purity lactic acid product, which reduces the cost of downstream purification processes, but prohibitively increases the substrate's cost. Conversely, cheaper substrates such as agro-food byproducts reduce upstream processing cost, but their compositional complexity increases the separation and purification costs, which can represent 50% of the total production expenses [13].

This review explores recent advances in the production and analysis of L- and D-lactic acid presented here in five overarching sections. The first focuses on new developments in lactic acid production, reviewing different microorganisms and their ability to produce optically pure lactic acid from different substrates is defined. The second reviews advances in metabolically engineered microbial strains. The third discusses the use of alternative substrates for lactic acid fermentation. The fourth focuses on alternative fermentation concepts. In the last section, emphasis is placed on L- and D-lactic acid-inducible gene expression systems and their application for the development of genetically encoded biosensors as tools to facilitate microorganism screening and engineering for improved lactic acid production.

Microorganisms naturally producing lactic acid

This section reviews the latest research advances focusing on the wild-type microorganisms suitable for production of optically pure lactic acid. The microorganisms used for achieving the highest yield, productivity, and optical purity of either L- or D-lactic acid, along with modes of fermentation, are summarized in Table 1 and Supplementary Table S1, respectively. The non-modified lactic acid bacteria (LAB), some *Bacillales* species and filamentous fungi display the most prominent characteristics. Other microorganisms can naturally produce only small quantities of lactic acid, mainly as a minor fermentation byproduct. Nonetheless, many of these microorganisms have been extensively engineered aiming to develop strains exhibiting enhanced lactic acid biosynthesis and with advanced fermentation characteristics (discussed further in section "Metabolic engineering for improved lactic acid production").

Lactic acid production by lactic acid bacteria

Among bacteria, LAB, predominantly belonging to the *Lactobacillales* order, as well as species from: *Bacillus, Geobacillus*, *Sporolactobacillus*, and *Terrilactibacillus* genera (*Bacillales* order), naturally produce lactic acid as a major fermentation product.

LAB is typically non-respiring and non-sporulating bacteria with similar metabolic and physiological characteristics, primarily found in carbohydrate-rich environments, including: dairy products and decaying plants. They are mesophiles tolerating moderate temperatures between 5 and 45 °C and growing in the pH range of 3.5–10.0 [12]. Under oxygen limitation conditions, these bacteria direct carbon flux toward the lactic acid biosynthesis aiding the supply of adenosine triphosphate (ATP) and oxidized nicotinamide adenine dinucleotide (NAD^+) in the cell. Importantly, due to their natural occurrence in dairy and food products along with their role in sustaining a healthy microbiota in humans and

Table 1. L-Lactic acid production using different substrates in non-modified microorganisms.

Microorganism	Carbon source	Fermentation mode	L-Lactic acid				References
			Concentration (g/L)	Yield (g/g) consumed substrate	Productivity (g/L/h)	Optical purity (%)	
	Pure compound						
Lactococcus lactis BME5-18M	Glucose	Continuous feeding	210	0.97	2.2	ND	[14]
Lactococcus lactis IO-1	Xylose	Batch	20.62	1.00	ND	ND	[15]
Lactobacillus paracasei sp. *paracasei* CHB2121	Glucose	Batch	192	0.96	3.99	96.6	[16]
Enterococcus hirae BoM1-2	Glucose	Repeated fed-batch	39.6	1.00	39.9	99.7	[17]
Enterococcus faecium QU 50	Xylose	Batch	23.7	1.02	1.97	99.2	[18]
Enterococcus faecalis QU 11	Glycerol	Fed-batch, fermentation coupled with acetic acid reduction to ethanol	55.3	0.97	0.772	99.9	[19]
Bacillus coagulans arr4	Glucose	Exponential fed-batch	206.81	0.97	5.29	99.1	[20]
Bacillus coagulans H-2	Glucose	Long-term repeated fed-batch (20 batches)	203.3	0.93	6.43	99.6	[21]
Rhizopus oryzae LA-UN-1	Glucose	Fed-batch, one-step fermentation	158	0.79	5.45	ND	[22]
Rhizopus oryzae NRRL 395	Glucose	Fed-batch, rotating fibrous-bed bioreactor	126	0.89	2.52	ND	[23]
Rhizopus oryzae NRRL395	Glucose	Batch, static-bed bioreactor	75.28	0.5	1.05	ND	[24]
	Agro-food byproduct						
Lactobacillus rhamnosus ATCC 9595 (CECT288)	Apple pomace (glucose, fructose)	Batch, separated hydrolysis and fermentation (SHF)	32.5	0.88	5.41	ND	[25]
Lactobacillus paracasei LA104	Microalgae (*Hydrodictyon reticulum*)	Batch, SSF	37.1	0.46	1.03	95.7	[26]
Lactobacillus rhamnosus ATCC 7469	Brewer's spent grain hydrolysate (glucose, xylose)	Fed-batch, SHF	58.01	0.88	1.19	99.7	[27]
Enterococcus faecium K-1	Gelatinized starchy waste	Batch, 37 °C amylolytic microorganism	87.2	0.88	1.82	99.2	[28]
Enterococcus faecalis SI	Acid-impregnated steam explosion-treated plywood chips (glucose, xylose)	Batch, SHF	102.38	1.03	1.28	>99	[29]
Bacillus coagulans A162	Hemicellulosic hydrolysates (glucose, xylose, arabinose)	Batch, SHF	ND	0.83	2.4	ND	[30]
Bacillus coagulans	Coffee pulp hydrolysate (glucose, xylose, arabinose, sucrose)	Batch (pilot scale), SHF	ND	0.78	4.02	99.50	[31]
Bacillus coagulans	Crust bread waste (glucose, fructose, sucrose)	Continuous, SHF	50.3	0.8	10.11	99.7	[32]
Bacillus coagulans H-1	Corncob residue (glucose)	Fed-batch, SHF	79.1	0.76	0.94	ND	[33]
Bacillus coagulans DSM 2314	Walnut shell (glucose, xylose)	Batch, non-sterile conditions (microwave-assisted autohydrolysis)	ND	0.81	0.2	97.4	[34]
Geobacillus stearothermophilus DSM 494	Potato residues (starch)	Batch, 60 °C non-sterile conditions, amylolytic microorganism	59.6	0.81	ND	98.0	[35]
Rhizopus oryzae NLX-M-1	Corncobs waste (cellulose, hemicellulose, glucose, xylose)	Batch, SSF	60.3	0.60	1	ND	[36]
Rhizopus oryzae As 3.819	Tobacco waste (glucose, xylose)	Fed-batch, SHF	173.5	0.86	1.45	>99	[37]

ND: not determined; SSF: simultaneous saccharification and fermentation; SHF: separated hydrolysis fermentation.

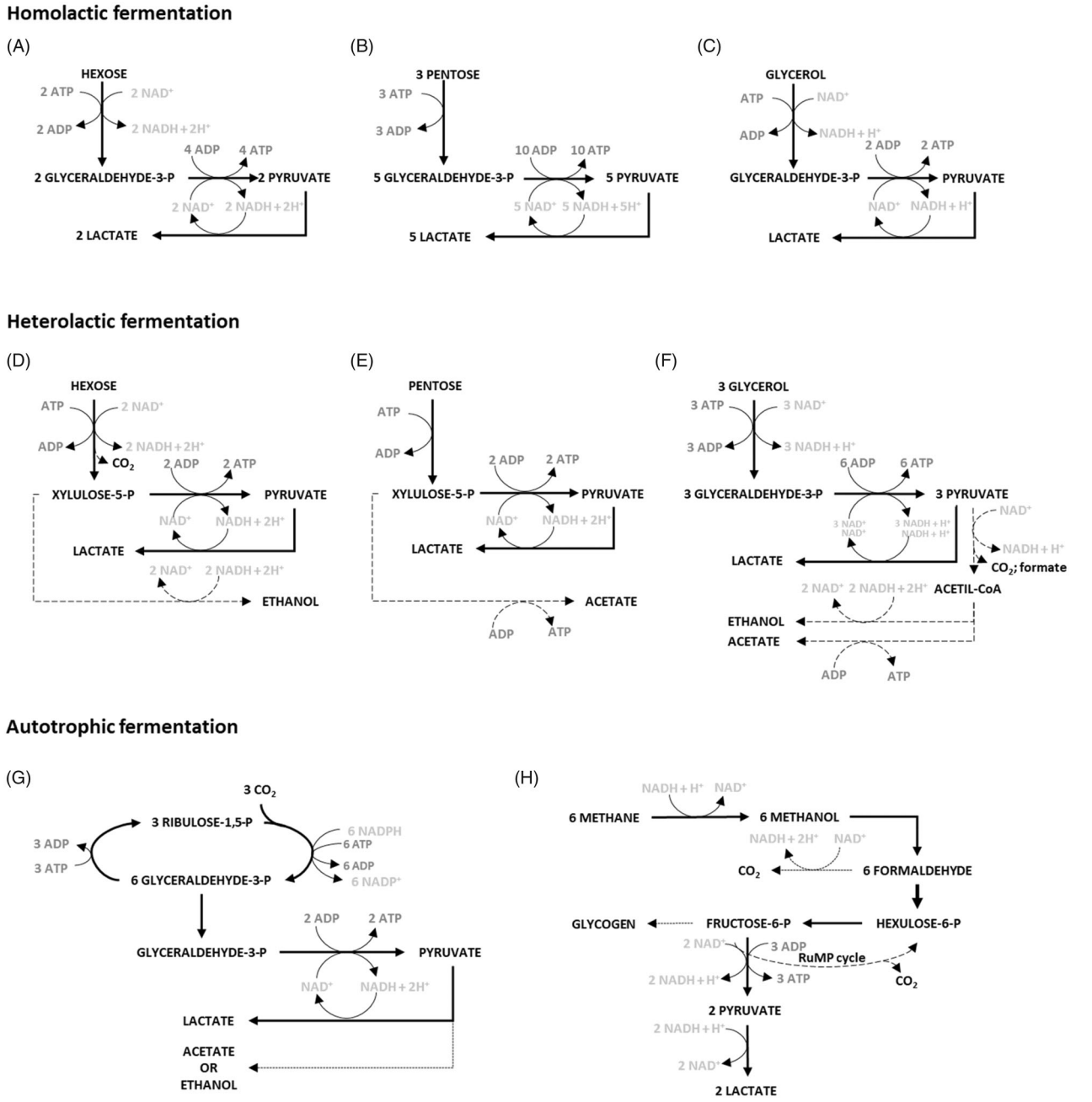

Figure 1. Metabolic pathway for lactic acid from different carbon sources. Homofermentation of hexoses (A), pentoses (B), and glycerol (C). Heterofermentation of hexoses (D), pentoses (E), and glycerol (F). Conversion of CO_2 (G) and CH_4 (H) to lactic acid in cyanobacteria and methanotrophs, respectively. The formation of reduced and oxidized NAD co-factors is indicated in light gray font; ATP consumption and synthesis are in dark gray; pathway of byproduct formation (e.g. ethanol) is indicated by dotted arrows. The diagram is based on previous research [38–40].

animals, these bacteria are generally recognized as safe for industrial use.

Implications of homo- and heterolactic metabolism

LAB can exhibit homo- and heterolactic metabolism, where the former produces lactic acid as the sole product of hexose (or pentose) fermentation, whereas the latter also generates byproducts such as: ethanol, acetate, and carbon dioxide (Figure 1). Species of: *Lactococcus, Streptococcus, Pediococcus*, *Vagococcus, Enterococcus* genera, and some *Lactobacillus* can perform homolactic fermentation under excess hexose and limited oxygen conditions. They ferment hexoses through the Embden–Meyerhof–Parnas (EMP) pathway, but only some of them can utilize pentoses [41]. The other LAB genera (*Leuconostoc*, *Oenococcus*, *Weissella*, and certain *Lactobacillus*) exhibit heterolactic fermentation.

Homolactic fermentation can theoretically yield 100% of lactic acid from glucose via the EMP pathway. In this fermentation mode, one hexose molecule is converted into two molecules of lactic acid and two ATP molecules are generated, whereas the NAD^+/NADH redox state remains neutral. Using either fed-batch or repeated fed-batch fermentation, homofermentative bacteria such as: *Lactobacillus lactis*, *Lactobacillus paracasei*, and *Enterococcus hirae* have been shown to achieve yields close to the maximum theoretical values with reported L-lactic acid yields of 0.97, 0.96, and 1.00 g/g consumed glucose, respectively [14,16,17] (Table 1 and Supplementary Table S1). Notably, maximum theoretical yields of D-lactic acid have been obtained by the continuous fermentation of *Lactobacillus delbrueckii* subsp. *lactis* QU41 [42].

Some, but not all, of LAB (e.g. *Enterococcus*, *Lactococcus*) can also utilize pentoses for homolactic fermentation. Similar to hexoses, the homolactic fermentation of pentoses can yield 100% of lactic acid. Here, three pentose molecules are converted into five molecules of lactic acid and seven ATP molecules are generated, whereas the NAD^+ redox balance remains neutral (Figure 1(B)). Complete conversion of xylose to L-lactic acid has been demonstrated using *Enterococcus faecium* QU 50 [18] and *Lactococcus lactis* IO-1 [15] (Table 1). However, the homolactic production of D enantiomer from pentoses has not been reported to date.

Heterolactic LAB, including: *Leuconostoc*, *Oenococcus*, *Weissella*, and certain *Lactobacillus*, can only utilize the pentose phosphate pathway due to the absence of fructose-1,6-bisphosphate aldolase. They convert only 50% of the carbon into lactic acid producing one molecule each of CO_2, ATP, and ethanol or acetic acid per molecule of glucose (Figure 1(D)). Here, the acetyl phosphate conversion into ethanol requires the oxidation of two NADH molecules resulting in a neutral redox state of heterolactic fermentation, whereas the formation of acetic acid allows the regeneration of one extra ATP and two NAD^+ molecules.

One of the key limitations in large-scale lactic acid production is the high cost of the carbon source used for fermentation. Utilization of raw materials, particularly agro-industrial byproducts, provides economically sustainable solutions and helps solve environmental problems. Utilization of materials rich in: starch, cellulose and other polysaccharides for lactic acid production using LAB has been widely demonstrated during the last three decades. The most interesting examples are summarized in Table 1 and Supplementary Table S1. Among various L-lactic acid producers, *Enterococcus* species have attracted particular attention. Due to high temperature tolerance (up to 55 °C), *E. faecalis* DUT1805, *E. faecium* QU 50, and *E. durans* BP130 were used in non-sterile continuous fermentation [43,44]. Whereas, strains such as *E. durans* BP130 and *E. faecium* K-1, possessing amylolytic activity suitable for starch hydrolysis, were simultaneously utilized for saccharification and fermentation [28], enabling production cost reduction.

Additional to hexoses and pentoses, glycerol can be used as a carbon source for both homo- and heterolactic fermentation with LAB *Enterococcus faecalis* [19,45] (Figure 1(C,F)). Two dissimilation pathways exist for bacterial glycerol metabolism: the dehydrogenation pathway (*dhaK* pathway; accompanied by NADH generation) and the phosphorylation pathway (*glpK* pathway; ATP consumption), both leading to a glycolysis intermediate, glyceraldehyde 3-phosphate [46]. Glycerol dissimilation has been most extensively studied in *E. faecalis* [19,47] and *E. coli* [48]. For *E. faecalis* W11, the glycerol metabolism is proposed via the *dhaK* pathway [45]. Given the higher reduced state of carbon in glycerol (4.67 degree of reduction per carbon) compared to glucose (4.00) and utilizing the *dhaK* pathway, two NADH molecules are generated for one pyruvate molecule formed, which is twofold higher compared to that generated during glucose dissimilation. Therefore, bacteria must adapt to the excess NADH or maintain the intracellular redox balance in some other way [46,47]. One possible strategy is coupling to the metabolism of another substrate with a lower reduction degree to restore the redox balance as demonstrated recently by employing the reduction of acetic acid to ethanol and achieving homolactic fermentation with a L-lactic acid yield of 0.97 g/g consumed glycerol [19] (Table 1).

It should be noted that only C1 gases such as carbon dioxide and methane have been applied as alternative carbon sources for lactic acid fermentation (discussed further in section "Metabolic engineering for improved lactic acid production"). On the contrary, other short-carbon-chain (C1–C4) compounds (e.g. relatively cheap short-chain fatty acids) have not yet been considered.

Despite the foregoing, for bacteria that have evolved to heteroferment and produce: formate, acetate, ethanol, carbon dioxide, or other byproducts along with the lactic acid, the advancement toward the homolactic fermentation mode remains a primary research challenge.

Bacterial lactate dehydrogenases

Independent of the fermentation mode, the carbon source or pathway (Figure 1), in the last step of lactic

acid biosynthesis, the pyruvate is converted to lactate through a reversible reaction catalyzed by a NAD-dependent lactate dehydrogenase (nLDH) regenerating the oxidized form of NAD from NADH formed at the earlier stage of the EMP pathway. The higher specificity constant (high k_{cat}/K_m value) and affinity (low K_m) for pyruvate than lactate and the excessive availability of NADH under oxygen-limited conditions enables the nLDH to maintain the reaction equilibrium shifted toward lactic acid synthesis. To enhance this, LAB often harbors several homologues of nLDHs [49]. Depending on whether the pyruvate is converted to the L- or D-lactate, nLDHs are divided into L-lactate dehydrogenase (L-nLDH; EC 1.1.1.27) and D-lactate dehydrogenase (D-nLDH; EC 1.1.1.28), respectively [50]. Either L-nLDH or D-nLDH enzymes encoding genes predominate in different LAB and some other bacteria genomes [50]. However, the coexistence of both LDH types results in the biosynthesis of both lactic acid enantiomers impeding to achieve the optically pure product and requiring genetic modification to eliminate undesirable enzymatic activity. Notably, the shift from the homofermentation of lactic acid to mixed-acid metabolism, exhibiting increased production of other metabolites, such as formate or CO_2, ethanol, and acetate, can occur due to reduced LDH activity [51].

Another type of LDH found in various bacteria is NAD-independent LDH (iLDH; EC 1.1.99.-). This enzyme, also called respiratory lactate dehydrogenase, catalyzes conversion of lactate to pyruvate by a flavin-dependent mechanism [50]. The iLDHs are mostly involved in lactic acid utilization [52] and there is little evidence that can be used in the lactic acid production catalyzing the reverse reaction from pyruvate to lactate. Nevertheless, gene expression control systems for iLDH have found some success in the design of biosensors [53].

Lactic acid production by Bacillales

Of the *Bacillales* order: *B. coagulans*, *B. subtilis*, *B. stearothermophilus*, *B. licheniformis*, and some other species possess a natural ability to produce L-lactic acid at high yields. For example, thermotolerant *B. coagulans* arr4 can achieve a very high yield (0.97 g/g) and productivity (5.29 g/L/h) of optically pure L-lactic acid (99.1%) from glucose using exponential fed-batch fermentation [20] (Table 1). Whereas, *Sporolactobacillus* spp. and *Terrilactibacillus laevilacticus* can be effective for D-lactic acid production [54] (Supplementary Table S1).

Notably, *Bacillus* strains and especially *B. coagulans* are the most studied and utilized for homofermentative L-lactic acid production [55]. If compared to LAB, *B. coagulans* exhibits low nutritional requirements and can grow on various carbon sources [56,57]. Moreover, due to its cellulolytic activity, it offers an advantage to combine the hydrolysis of complex carbohydrate substrates (saccharification) and fermentation into a simultaneous process. The thermophilic nature of this bacterium allows the fermentation to be carried out at a temperature range from 50 to 60 °C eliminating the need for sterilization and thus reducing the upstream process costs [56].

The repeated fed-batch fermentation enables not only to achieve yields close to the maximum theoretical values, but also ensures high concentrations of lactic acid, as reviewed recently for homolactic and other bacteria as considered previously [17]. Most recently, *B. coagulans* H-2 was used in a long-term repeated fed-batch strategy involving 20 batches, where L-lactic acid was produced to a final concentration of 192.7 g/L with the yield of up to 0.93 g/g and the productivity of 6.43 g/L/h [21] (Table 1). Although the same yield was obtained for *Bacillus* strains previously studied [58], the long-term repeated fed-batch approach allowed to achieve a twofold higher titer of L-lactic acid and proving to be superior to a single batch experiment or repeated batch fermentation.

Lactic acid production by filamentous fungi

Filamentous fungi form a structure known as hyphae and can secrete various enzymes for decomposing complex biopolymers from animal and plant biomass. During L-lactic acid production, *Rhizopus* species are widely utilized as they possess only the L-nLDH form [59–61]. However, when oxygen supply is the limiting factor in this species, the expression (and activity) of pyruvate decarboxylase and alcohol dehydrogenase is elevated, resulting in pyruvate conversion into ethanol (to the concentration of up to 5 g/L). Increasing oxygen content in the fermentation mixture through improved aeration can significantly reduce this byproduct formation. By immobilizing *R. oryzae* in rotating fibrous bed fermentation, the L-lactic acid yield has been improved to 90% and nearly 100% of theoretical value using glucose and corn starch as carbon sources [23]. On the other hand, the application of a static-bed fermentor has also proved beneficial for immobilized *R. oryzae* growth and lactic acid production [24]. This fermentor enables the elimination of substrate repression at high initial glucose concentrations in the batch culture, helps control the proper cell morphology, improves mass transfer, and supports continuous operation modes. Due to available amylolytic enzyme activity, *Rhizopus*

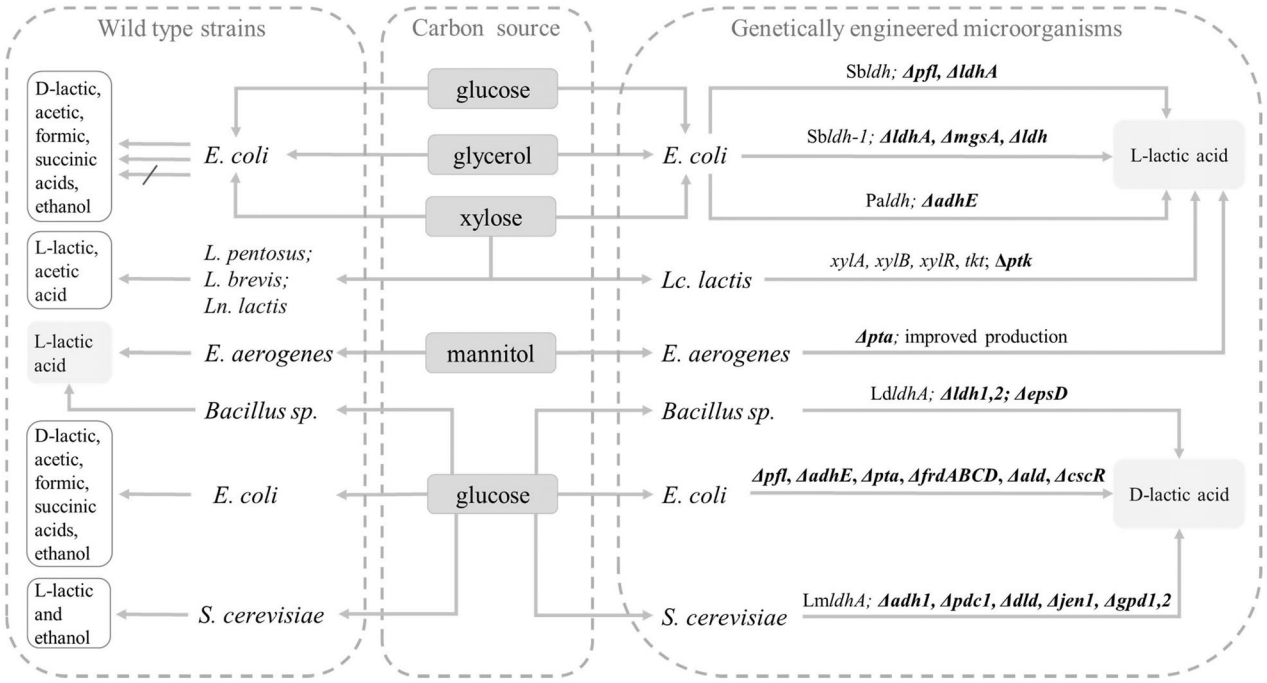

Figure 2. L- and D-lactic acid production in non-modified and metabolically engineered microorganisms using different carbon sources. Genes that have been introduced are in normal front: Sb*Ldh*, *Streptococcus bovis* L-lactate dehydrogenase; Pa*Ldh*, *Pediococcus acidilactici* L-lactate dehydrogenase; Ld*ldhA*, *Lactobacillus delbrueckii* D-lactate dehydrogenase, Lm*ldhA*, *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293 D-lactate dehydrogenase. Genes that have been deleted/mutated are in bold front: *pta*: phosphotransacetylase; *adhE*: alcohol/acetaldehyde dehydrogenase; *adh1*: alcohol dehydrogenase; *pdc1*: pyruvate decarboxylase; *dld*: D-lactate dehydrogenase; *jen1*: monocarboxylate transporter; *gpd1,2*: glycerol-3-phosphate dehydrogenase; *pfl*: pyruvate formate lyase; *frdABCD*: fumarate reductase operon; *ald*: aldehyde dehydrogenase acting on methylglyoxal conversion to L-lactic acid; *cscR*: sucrose utilization regulator; *mgsA*: methylglyoxal synthase; *tkt*: transketolase; *ptk*: phosphoketolase; *epsD*: exopolysaccharide biosynthesis gene; *xylA*: xylose isomerase; *xylB*: xylulokinase; *xylR*: xylose assimilation regulator. Additional information can be found in Supplementary Tables S2 and S3.

species can readily be employed to produce L-lactic acid from starch-rich products, other oligosaccharides, and lignocellulosic biomass achieving over 90% of theoretical yield [23,60,61]. One of the highest reported concentrations of L-lactic acid (173.5 g/L) was achieved using *R. oryzae* AS 3.819 from cellulose-rich tobacco waste (Table 1) [37].

To date, there are no reports of the direct production of lactic acid by other filamentous fungi. However, *Aspergillus niger* SL-09, has been used in a mixed-culture, with *Lactobacillus* sp. G-02 fermenting Jerusalem artichoke tubers to produce 120.5 g/L lactic acid after 36 h of fed-batch fermentation with a yield of 0.95 g/g and a productivity of 3.3 g/L/h) (Table 1). Since the Jerusalem artichoke is 70–90% (w/w) composed of inulin, the fungus provides the necessary enzymes inulinase (E.C. 3.2.1.7) and invertase (E.C. 3.2.1.80) to hydrolyze this polysaccharide into fructose or sucrose for the *Lactobacillus* sp. G-02 to convert them into lactic acid [62]. Such a fungus-bacterium mixed-culture enables the utilization of polymerized carbohydrates for the lactic acid fermentation.

Metabolic engineering for improved lactic acid production

In the last decade, significant efforts have been made to engineer other microorganisms, besides LAB, to produce optically pure L- and D-lactic acids (Figure 2), with the most interesting cases being listed in Supplementary Tables S2 and S3. Whereas LAB strains are an obvious choice, the engineering of other microorganisms opens an opportunity to access alternative carbon sources and exploit a flexible feedstock concept [63].

Organotrophic bacteria

LAB engineering has been primarily directed to facilitate the use of a biomass rich in pentose, starch, or other polysaccharides without the prior pretreatment or hydrolysis of feedstock reducing fermentation costs. As demonstrated previously, xylose assimilation genes from *L. pentosus* and *L. lactis* IO-1 can be introduced into other LAB bacteria enabling the utilization of xylose as a carbon source for L- and D-lactic acid

fermentation, achieving yields of 0.95 and 0.80 g/g, respectively [64,65]. For starch-rich substrates, bacteria were engineered by introducing the *AmyA* gene encoding α-amylase from *Streptococcus bovis* 148, enabling simultaneous saccharification and fermentation, resulting in high (0.85–0.90 g/g) lactic acid yields [66,67].

Due to their natural ability to form and secrete significant amounts of organic acids as metabolic byproducts, *E. coli* and *C. glutamicum* are the most engineered bacteria for the production of various chemicals, including optically pure lactic acids [10,68]. Moreover, these bacteria are the preferred choice for their genetic tractability, availability of tools, ability to metabolize various carbon sources, and ability to grow rapidly in simple media under aerobic and anaerobic conditions. Several different approaches have been applied to *E. coli* with the view to modify its metabolism including: (1) eliminating or limiting byproducts [69]; (2) restricting production to a specific lactic acid enantiomer [70]; (3) enabling use of alternative carbon sources [71]; (4) inactivating the use of L-lactate as a nutrient, and; (5) removing the accumulation of toxic substances such as methylglyoxal [70]. One of the highest L-lactic acid concentrations (100 g/L) was achieved by deleting *pflB* and *adhE* genes required for formate and ethanol formation, respectively [69]. Whereas, to achieve the high level of D-lactic acid production (127.0 g/L), *E. coli* was engineered by disabling the *pflB* along with other genes responsible for succinate, acetate, ethanol, and L-lactate formation [72,73] (Supplementary Table S3).

Among other bacterial strains, thermophilic *Bacillus coagulans* has been successfully adapted for the industrial production of D-lactic acid. This strain is suitable for non-sterile fermentation, can utilize inexpensive carbon substrates and withstand harsh conditions such as: low pH, high temperature, and lack of aeration [74]. To enable exclusive D-lactic acid production, *Bacillus* strain was modified by deleting the native L-lactate dehydrogenase gene (*ldh*) and introducing the D-lactate dehydrogenase gene from *Lactobacillus delbrueckii*. Modified strain produced 140 g/L of D-lactic acid, with more than 0.9 g/g yield [74] (Supplementary Table S3).

In the future, the development of attractive industrial fermentation platforms is likely to depend on the successful engineering of strains suitable for efficient production of optically pure lactic acids from alternative sources.

Autotrophic bacteria

Methanotrophs and cyanobacteria have been engineered to enable lactic acid biosynthesis from methane (CH_4) and carbon dioxide (CO_2). Similar to the approach with *E. coli*, by introducing *Lactobacillus helveticus* and *Leuconostoc mesenteroides* L- and D-nLDH genes, methanotrophs *Methylomicrobium buryatense* and *Methylomonas* sp. DH-1 were modified, respectively, demonstrating the feasibility of optically pure lactic acid production from CH_4 [40,75,76]. However, in both cases, only relatively low concentrations (0.6 and 1.19 g/L) were achieved [40,76]. The production was primarily hindered by a high conversion of the assimilated methane to biomass (50–60%) and low methanotroph tolerance to the lactic acid [75,77]. Markedly, cyanobacteria such as *Synechocystis* sp. require even more complex genetic modifications [78,79]. In addition to incorporating LDH genes from the LAB, an improvement of the light-driven system is required to produce lactic acid from CO_2. Since cyanobacteria lack transporters to transport hydrophilic organic molecules across cell membranes, a lactic acid transporter is also required [79].

To increase L-lactic acid productivity, *Synechocystis* sp. PCC6803 has been further engineered by increasing L-nLDH of *L. lactis* affinity for NADPH, multiplication of *ldh* gene copy number, use of a self-replicating plasmid and elevating the metabolic flux toward lactic acid. The highest concentration of L-lactic acid obtained was 0.84 g/L after two-week fermentation [38]. The same strain was successfully modified for D-lactic acid production by overexpressing heterologous D-lactate dehydrogenase and endogenous malate dehydrogenase genes, and removing the acetate kinase gene [80]. The highest D-lactate production (26.6 g/L) and moderate productivity (0.186 g/L/h) were achieved after three days of photoautotrophic growth and three days of fermentation (Supplementary Table S3).

Yeasts

Various yeast species have been engineered for lactic acid production. Notable strains include: *Saccharomyces cerevisiae* [81], *Kluyveromyces lactis* [82], *Kluyveromyces marxianus* [83], *Zygosaccharomyces bailii* [84], *Pichia stipitis* [85], and *Candida* spp [86,87] (Supplementary Tables S2 and S3). In engineered yeast strains, the native lactate dehydrogenase genes are often substituted with a highly stereospecific D-lactate dehydrogenase gene from *Leuconostoc mesenteroides* or *Lactobacillus plantarum* [81,83]. Additionally, *PDC1* and *ADH1* genes encoding major pyruvate decarboxylase and alcohol dehydrogenase and *GPD1* and *GPD2* genes encoding glycerol-3-phosphate dehydrogenase are deleted to improve the carbon flux toward the lactic

acid. Other alterations include: disabling the monocarboxylate transporter gene, overexpression of the transcriptional activator and/or adaptive evolution depending on the yeast strain selection [81,83]. Despite these modifications, the maximum theoretical yield of D- and L-lactic acid has not been achieved, resulting in 0.60 g/g on glucose and 0.80 g/g on a glucose and xylose mixture, respectively [81,83].

In an recent study, *Pichia pastoris* has been engineered to produce D-lactic acid from methanol, yielding 0.22 g D-lactic acid per gram of substrate and a productivity of 0.0363 g/L/h [88]. Fermentation with this substrate has great potential if coupled with methanol synthesis from the synthesis gas (syngas), a mixture of hydrogen and carbon monoxide (CO) or renewable hydrogen derived from water electrolysis and CO [89].

Use of alternative substrates for lactic acid fermentation

Reducing the cost of the substrate material is an essential consideration for making lactic acid production more feasible. Unlike other production elements, the substrate cost cannot be intrinsically reduced by the process scale-up. The ideal substrate must be: inexpensive, readily available, and easily fermentable to a high product yield. Therefore, researching novel substrates plays an important role in improving lactic acid's production cost efficiency.

Plants such as corn, rice, or potatoes are undesirable as this imposes an additional burden on agricultural production. Therefore, a more sustainable option is to utilize non-food substrates rich in: carbohydrates, glycerol, lignocellulose, and other more complex biomass residues arising from: agriculture, marine, and the food industries [19,90–92]. Alternatively, lactic acid can also be produced from CH_4 and CO_2, as recently benchmarked [38,75].

Lignocellulose

Lignocellulose is formed of carbohydrate polymers: cellulose, hemicellulose, and lignin. Cellulose comprises glucose molecules and hemicellulose consists of xylose, galactose, arabinose, mannose, and other non-cellulosic sugars. Due to this complex polymeric structure of lignocellulose, feedstock pretreatment prior fermentation is required [93]. Typically, chemical methods (e.g. acid or alkali treatments) are employed. Application of alkaline treatment is an efficient way to treat lignocellulose because it enhances the enzymatic digestibility of fibers [94]. Optically pure D-lactic acid production was recently reported using *Lactobacillus delbrueckii subsp. bulgaricus* strain from the lignocellulose treated with a mild oxidative organosolv process [95] (Supplementary Table S1). In this study, despite the process optimization to reduce inhibitors' formation, only moderate yield and productivity (0.69 g/g and 0.86 g/L/h, respectively) were achieved. Chemical methods can restrict high lactic acid productivities due to inhibitor formation. Although, there are certain strains such as *Bacillus coagulans* DSM 2314 that have shown a high tolerance to inhibitors (5 g/L of furans and phenols) with high L-lactic acid productivity (2.4 g/L/h) (Table 1) [96]. Due to inhibitor formation and the environmental impact of chemical methods, alternative pretreatments such as enzymatic hydrolysis by: pullulanases, cellulases, or β-glucosidases can be used [93,96]. Moreover, "green solvents" such as ionic liquids have shown applicability for cellulose processing [97]. A recent study established that several LAB can tolerate residual ionic liquids and inhibitory compounds such as furfural and 5-hydroxymethylfurfural formed through this pretreatment. For instance, *Lactobacillus plantarum* SKL-22 can grow in 2% of an ionic liquid and is resistant to concentration up to 10 g/l of furfural or hydroxymethylfurfural, producing, however, both stereoisomers of lactic acid [98]. The challenge remains to have an all in one, the optimized digestion of lignocellulosic feedstock strains tolerant to inhibitory compounds with high optically pure lactic acid productivity. Thus, requiring more comprehensive studies of bacterial strain tolerance and productivity to expand the use of lignocellulosic substrates.

Microalgae, certain strains of which are rich in carbohydrates (50–70% of biomass), are another promising feedstock for fermentative lactic acid production [26,90,99]. Since microalgae walls are composed of cellulose and hemicellulose, simple hydrolysis methods (acid or thermal treatment) can release the sugars [100]. Using *Chlorella vulgaris* ESP-31, treated with dilute sulfuric acid as a feedstock, a polyvinyl alcohol-immobilized *Lactobacillus plantarum* 23 strain, was allowed to achieve one of the highest lactic acid productivity (9.93 g/L/h) and yield (0.99 g/g) [90]. However, in this case, a racemic mixture was obtained. So far, using microalgae biomass, only moderate yields and productivities of optically pure lactic acid have been obtained [26,99] (Table 1 and Supplementary Table S1).

Carbon dioxide and methane

Recently, CO_2 and CH_4, two most abundant greenhouse gasses, which can also be derived by aerobic and anaerobic digestion of waste biomass, have found

application as carbon sources during lactic acid production [38,40,75–77,79,101,102]. The principal schematic diagrams for converting CO_2 and CH_4 to lactic acid are shown in Figure 1(G,H).

As discussed in section "Metabolic engineering for improved lactic acid production", L- and D-lactic acid biosynthesis from CO_2 by introducing LAB genes encoding either L-nLDH or D-nLDH was primarily engineered in *Synechocystis* sp. PCC 6803 [38,80]. Similarly to CO_2-fixing bacteria, the introduction of LDH genes into methanotrophs can allow to attract the CH_4 carbon toward the biosynthesis of lactic acid [75,76]. Particularly, group I methanotrophs (Gammaproteobacteria), in which methane assimilation is coupled with a highly efficient pyrophosphate (PPi)-dependent phosphofructotransferase-mediated EMP pathway [103], can be effectively used for lactic acid production using methane as a feedstock.

As the use of CO_2 and CH_4 for chemical and single cell protein bioproduction has recently gained an increased interest [104,105], the low lactic acid yield and productivity reported for C1 gas-fixing bacteria remains a primary limitation. For lactic acid production using C1 feedstock to become economically viable and competitive comparing to the use of food-based carbon sources, further research on the screening of strains with the highest growth rates and biomass concentrations, as well as metabolic engineering and synthetic biology efforts improving carbon flux are required.

Agro-food byproducts-derived substrates

Toward the development of a sustainable bioeconomy, considerable interest has been devoted to utilizing agro-food byproducts for lactic acid fermentation [106,107]. Agro-food byproducts, besides carbohydrates, may also contain lipids, proteins, vitamins, and minerals, all of which positively affects the fermentation process. Orange peel [92], bakery waste [32], brewers spent grain [27], corncobs waste [108], and other wastes were used as a feedstock for lactic acid production (Table 1 and Supplementary Table S1). Lignocellulose rich substrates have particular relevance in the agricultural areas of: Asia, South America, and Africa, where plants are predominantly cultivated, thus generating high quantities of low-value lignocellulosic biomass, typically considered as a waste. The development of microbial strains with the ability to utilize such alternative substrates has thus the potential to boost local bioeconomies.

Although certain strains allow L- or D-lactic acid yields of over 0.9 g/g on pure hexoses or/and pentoses, the fermentation using agro-food byproducts as a substrates yields range from 0.4 to 0.9 g/g with representative examples provided in Table 1 and Supplementary Table S1.

The above comparison highlights the complexities of pairing an appropriate microorganism to the right substrate. Some microorganisms exhibited higher theoretical yields on a control glucose substrate and performed worse with renewable waste substrates. From an industrial perspective, scale-up using waste materials as a substrate would likely require detailed knowledge of the waste's composition. It appears that indiscriminate use of agro-food byproducts with a given microbial family does not guarantee a consistent yield. The preliminary analysis of waste batch composition, establishing: glucose, xylose, starch, or other carbohydrates content is required. Such an approach will enable the use of alternative or engineered strains, which have the potential to produce excellent yields as it was shown with *B. coagulans* DSM1 (0.98 g/g) [74] and *E. coli* BW100 (0.97 g/g) [109] in D-(–)-lactic acid production.

A major remaining issue in the use of agro-food byproducts as a substrate is the relatively low yield of lactic acid compared to conventional substrates (Table 1 and Supplementary Table S1). Nevertheless, further process optimization followed by sustainable technologies with competitive economic performance is highly anticipated in the near future [106].

Nitrogen source

The use of inexpensive carbon sources can notably reduce fermentation expenditure. Further savings can be achieved by using a low-cost nitrogen source. Yeast extract and peptone, rich in amino acids, are traditionally used as nitrogen sources. However, it has been estimated that yeast extract may account for as much as 38% of the total cost of lactic acid production [110]. Agricultural residues or byproducts, including: corn steep liquor [110–112], malt rootlets and soybean meal [27], hydrolyzed rapeseed meal [113], cottonseed [114], and peanut meal [115], can replace expensive nitrogen sources enabling the achievement of L- or D-lactic acid greater than 0.9 g/g.

When choosing a nitrogen source, it is important to evaluate its composition for any inhibitory and toxic components, which may have negative effects on cell growth and fermentation. Moreover, supplementation with rare amino acids or vitamins, may be required for auxotrophic strains. For example, *Lactobacillus delbrueckii* ssp. exhibit high nutritional requirements

including B group vitamins and essential amino acids, which are lacking in corn steep liquor [110]. In addition, complex nitrogen sources require upstream processing involving enzymatic hydrolysis or the use of bacterial strains with sophisticated proteolytic systems [110,115]. Further techno-economic analysis is needed in order to ensure more sustainable lactic acid production.

Alternative fermentation concepts

Alkaliphilic and thermotolerant bacteria are often used for lactic acid production due to their tolerance to high salt and temperature levels, which reduce both contamination problems during processing and the amount of neutralizing agents required during lactic acid fermentation. Several such alkaliphilic strains including: *Halolactibacillus halophilus*, *Exiguobacterium* sp., *Enterococcus* sp., and *Bacillus* sp. have been used in the production of lactic acid [12,17].

Immobilized cell systems can be adapted for lactic acid production because of their advantages over traditional batch processes using free cells. They can offer: increased metabolic activity, cell concentrations, and cell stability, easier cell recovery, reduced chance of contamination, cell reusability, higher productivity, and lower production costs [116,117]. Immobilized cells are more resistant to environmental stresses such as: acidity, temperature, organic solvents, salts, inhibiting substrates and products, poisons, and self-destruction. Different natural (agar, carrageenan, alginate, or cellulose) and synthetic matrices (polyester, polyacrylamide, or polystyrene) have been utilized for immobilizing certain microbial species capable of producing lactic acid [117]. Cell insertion into alginate gels has been extensively studied, but the high cost and instability of the gels, which can be easily degraded by lactic acid, are major factors in preventing their practical application in lactic acid fermentation [116]. Despite this, a very recent study showed that immobilization of sterile poly(vinyl alcohol)/calcium alginate beads increased lactic acid productivity and concentration by 27% [118].

Several types of bioreactor have been used for lactic acid fermentation using immobilized cells [24,119]. For example, the application of a fibrous bed bioreactor enabled a 37.67% and 27.92% improvement in the D- and L-lactic acid comparing to the free-cell fermentation using *Sporolactobacillus inulinus* Y2-8 and *Lactococcus lactis* ATCC19435, respectively [119,120]. The immobilization of filamentous fungi has attracted a particular interest despite challenges in large-scale processes, as their morphology affects the mass transfer efficiency determining the rates of substrate uptake and metabolite production. For example, immobilization of *Rhizopus oryzae* increased L-(+)-lactic acid concentration by approximately 70% achieved in shorter times than free-cell fermentation [121].

Alternatively, lactic acid can be co-produced with other compounds. Recently, a one-pot co-production of furfuryl alcohol and lactic acid using single *Bacillus coagulans* NL01 was reported. Fermentation with this strain allowed 264 mM furfuryl alcohol production along with 64.2 g/L L-(+)-lactic acid with 90.0% yield by a fed-batch strategy from furfural and glucose. Cells typically do not have a high tolerance for furfural. Therefore, the addition of glucose as co-substrate to regenerate the enzyme cofactor (NADH) is typically required. In the presence of glucose, cells can produce lactic acid via the EMP pathway [122]. The coupled biphasic system allows the simultaneous production of two different products and significantly reduces the production costs of glucose demand. Presumably, the separation process will not be expensive and complicated because the products are soluble in different phases.

Lactic acid can also be co-produced with hydrogen during capnophilic fermentation (requiring CO_2). Simultaneous production has been demonstrated using hyperthermophilic (>65 °C) bacteria *Thermotoga neapolitana* [123,124]. *T. neapolitana* strain DSMZ 4359^T TN-CMut was used to produce approximately 4.5 mol/mol H_2 and 8 mM lactic acid from 5 g/L lactose [124]. These exploratory studies show that the co-production of H_2 as a nonpolluting fuel and lactic acid as platform chemical has a future perspective.

Lactic acid-monitoring tools for microorganism screening and engineering

To complement efficient, cost-effective methods of producing lactic acid, it is necessary to use similarly efficient and inexpensive characterization and detection methods. Determination of the lactic acid content is important in order to detect acidity changes that affect the freshness, stability, and quality of the products. The quantitative determination of lactic acid can be achieved with various techniques. An overview, including advantages and disadvantages, of suggested analytical approaches is presented in Supplementary Table S4. Besides classical analytical techniques, considerable attention has recently been dedicated to the development of genetically encoded biosensors [125–127]. Unlike other characterization techniques, this type of biosensor can also be used *in vivo*. Moreover, they offer high sensitivity and specificity with a substantially

higher throughput than other analytical methods such as HPLC, electrophoresis, and titration.

Lactate-inducible gene expression systems: prospect for biosensors

Genetically encoded biosensors enable high throughput quantification by transducing the concentration of the target molecule into an easily measurable signal such as: fluorescence, luminescence, or absorbance [128]. This type of biosensor commonly consists of transcription factor (TF)-based inducible gene expression systems functionally connected to a reporter or an antibiotic resistance gene [129]. They allow real-time monitoring of product formation and measurement of the titer, that are: fast, inexpensive, specific, very sensitive, and suitable for high-throughput screening [128].

Several examples of lactic acid-inducible gene expression systems have been reported previously in several microorganisms and are summarized in this study (Figure 3), including: *Ec*LldR/P_{lldP} (*E. coli* MG1655) [130], *Cg*LldR/P_{lldP}, (*Corynebacterium glutamicum* ATCC 13032) [131], *Pa*LldR/P_{lldP} (*Pseudomonas aeruginosa* XMG) [132], *Bc*LutR/P_{lutA} (*Bacillus subtilis* 168) [133,134], *Dv*LurR/P_{por} (*Desulfovibrio vulgaris* Hildenborough) [135], and *Aw*LctA/P_{lctB} (*Acetobacterium woodii* DSM 1030) [136]. The systems are of two types. In the first type, found in *E. coli* (*Ec*LldR/P_{lldP}) and *Corynebacterium glutamicum* (*Cg*LldR/P_{lldP}) [130,131], the TF has a dual regulatory function acting as an activator (in the presence of L-lactate) and as a repressor (in the absence of L-lactate). In the second type, identified in *Pseudomonas aeruginosa* (*Pa*LldR/P_{lldP}), *Bacillus subtilis* (*Bc*LutR/P_{lutA}), *Acetobacterium woodii* (*Aw*LctA/P_{lctB}), and *Desulfovibrio vulgaris* (*Aw*LctA/P_{lctB}) [132–136], the TF acts as a repressor.

The most widely studied *E. coli* system *Ec*LldR/P_{lldP} (Figure 3(A)) controls, a *lldPRD* operon (formerly named *lct*), responsible for aerobic L-lactate metabolism. This operon encodes flavin adenine dinucleotide-dependent dehydrogenase (*lldD*), L-lactate-specific permease (*lldP*), and regulatory proteins (*lldR*) belonging to the *FadR* subfamily [130,137]. The expression of *lldD*, *lldP*, and *lldR* genes is increased by L-lactate but not by D-lactate under aerobic conditions [138]. *LldR* binds to both O1 (positions −105 to −89) and O2 (positions +22 to +38) operators, leading to DNA loop formation and transcription suppression when L-lactate is absent. If L-lactate is present, it binds to *LldR* and promotes conformational changes, which can disrupt the DNA loop and allow for the formation of an open transcription complex [130]. Moreover, the expression of *lldD*, *lldP*, and *lldR* genes increases under aerobic conditions and decreases under anaerobic conditions [130,139]. This switch is controlled by the two-component anoxic redox control system *ArcAB* [139]. To a large extent, the principal of gene expression regulation by *C. glutamicum* K051 ATCC 13032 system *Cg*LldR/P_{lldP} (Figure 3(B)) is similar to *Ec*LldR/P_{lldP}.

The *Pa*LldR/P_{lldP} from *P. aeruginosa* PAO1 controls the *lldPDE* operon containing both the L-lactate dehydrogenase (*lldD*) and D-lactate dehydrogenase (*lldE*) genes. Unsurprisingly, the gene expression here is activated in the presence of either L- or D-lactate [132]. The remaining three systems regulate the lactate utilization genes that are arranged in different and more complex operons. For example, *Bacillus subtilis* sp. *subtilis 168* has three genes *lutA*, *lutB*, and *lutC* responsible for the lactate utilization. However, the functions of these genes have not been fully investigated. Genes are thought to contain iron-sulfur clusters and the oxidation of lactate occurs via a cytochrome-like electron transfer chain [133]. Based on the existing data, the range of induction for systems from: *E. coli, C. glutamicum*, and *Pseudomonas denitrificans* can be calculated as 9.03-fold, 14.0-fold, and 6.0-fold, respectively [130,140,141].

An L-lactic acid biosensor has also been developed [142] using the *E. coli* inducible gene expression system [130]. This biosensor utilizes the green fluorescent protein (GFP) as a reporter, which responds to the P_{lldP} promoter, and contains the *lldR* TF gene under the control of a constitutive promoter to lower the baseline GFP expression. The biosensor has shown a 60-fold induction with high sensitivity (lowest detection limit ~0.05 mM). The developed sensor found application in monitoring the lactate content in mammalian cells [142]. A D-lactate biosensor has also recently been developed using the *Pseudomonas fluorescens* A506 lactic acid-inducible gene expression systems. A sixfold higher induction of 100 mM D-lactate than L-lactate was observed with a relatively broad (35–100 mM) dynamic range for D-lactic acid [141]. The examples discussed above show that lactic acid-inducible gene expression systems can be used to develop lactic acid biosensors.

Biosensors are established as effective high throughput screening methods with high sensitivity. They have found utility in biosensor-driven laboratory evolution, enzyme/pathway screening and engineering, as well as enabling the evaluation of a metabolic network bottleneck, among other multiple applications [127,143–145]. The process of optimizing lactic acid production is iterative, likely requiring a large number of permutations of microbe and substrate couplings to be assessed. The

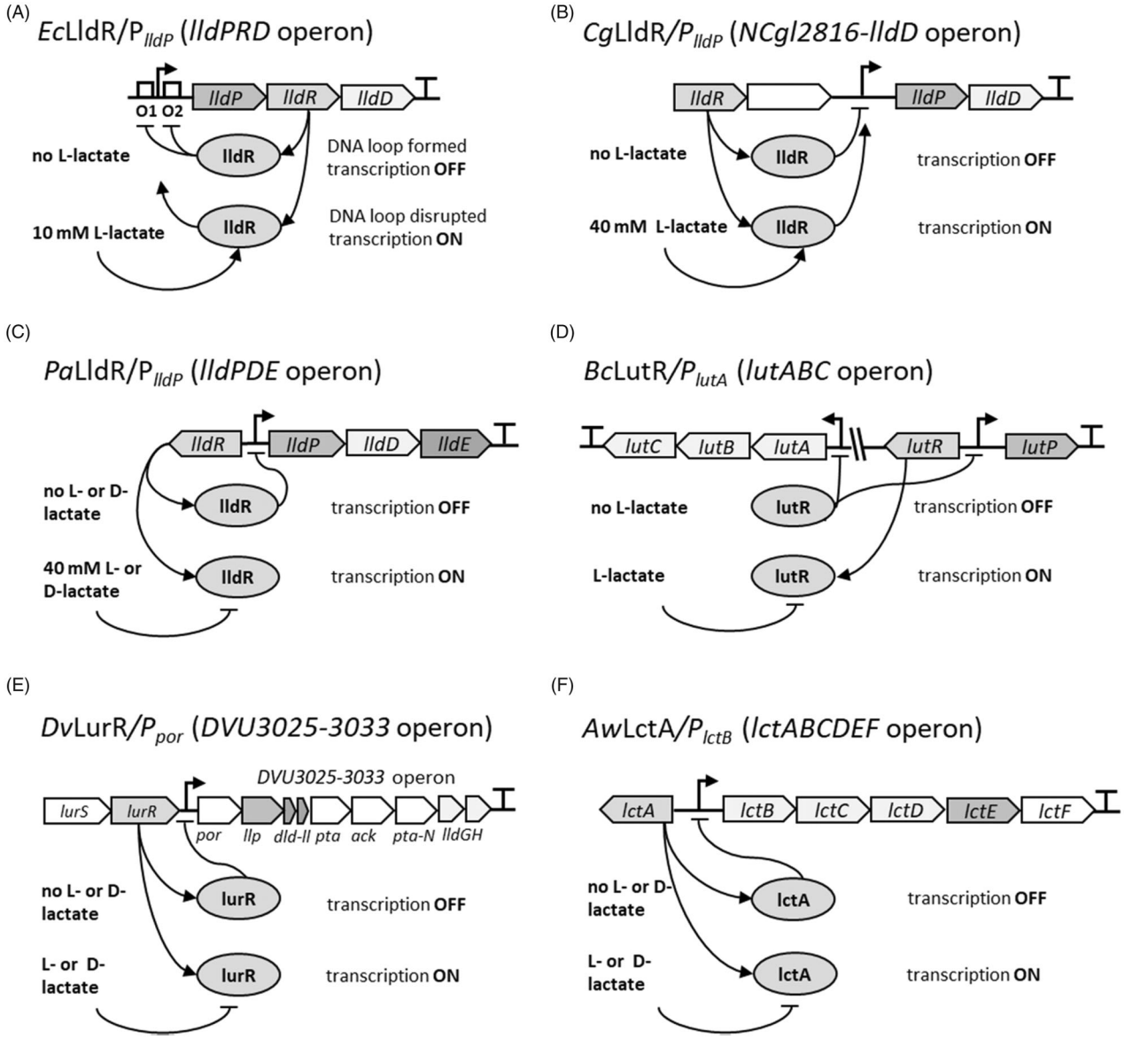

Figure 3. D- and L-lactic acid inducible gene expression systems. (A) Gene clusters in *Escherichia coli* MG1655 encoding the enzymes required for L-lactic acid catabolism. The inducible gene expression system is referred to as *Ec*LldR/P_{lldP}, where LldR is a transcriptional regulator (locus tag b3604) [130]. (B) Gene clusters in *Corynebacterium glutamicum K051* ATCC 13032 encoding the enzymes required for L-lactic acid catabolism. The inducible system is referred to as *Cg*LldR/P_{lldP}, where LldR is a transcriptional regulator (locus tag WA5_2814) [131]. (C) Gene clusters in *Pseudomonas aeruginosa* XMG encoding the enzymes required for L- and D-lactic acid catabolism. The inducible system is referred to as *Pa*LldR/P_{lldP}, where LldR is a transcriptional regulator [132]. (D) The gene clusters in *Bacillus subtilis* sp. *subtilis* 168 encoding the enzymes required for L-lactic acid catabolism. The inducible system is referred to as *Bc*LutR/P_{lutA}, where LutR is a transcriptional regulator (locus tag BSU_34180) [133, 134]. (E) Gene clusters in *Desulfovibrio vulgaris* Hildenborough encoding the enzymes required for L- and D-lactic acid catabolism. The inducible system is referred to as *Dv*LurR/P_{por}, where LurR is a transcriptional regulator [135]. (F) Gene clusters in *Acetobacterium woodii* DSM 1030 encoding the enzymes required for L- and D-lactic acid catabolism. The inducible system is referred to as *Aw*LctA/P_{lctB}, where LctA is a transcriptional regulator (locus tag AWO_RS04405) [136]. *LldP*, *lutP*, *lctE*: L-lactate permease gene; *lldR*, *lutR*, *lurR*, *lctA*: transcriptional regulator; *lldD*, *lldGH*: L-lactate dehydrogenase gene; *lldE*, *dld-ll*: D-lactate dehydrogenase gene; *lutABC* operon (*lutA*: iron-sulfur oxidase subunit used in L-lactate utilization; *lutB*: component of an iron-sulfur oxidase linked to L-lactate utilization; *lutC*: component of an iron-sulfur oxidase for L-lactate utilization; *por*: pyruvate ferredoxin oxidoreductase gene; *llp*: lactate permease gene; *pta*: phosphotransacetylase gene; *ack*: acetate kinase gene; *lctB*: electron transfer flavoprotein subunit *beta*; *lctC*: electron transfer flavoprotein subunit *alpha*; *lctD*: FAD-binding oxidoreductase (putative lactate dehydrogenase) gene; *lctF*: nickel-dependent lactate racemase gene).

use of biosensors would be well-placed in this context, given its suitability for the rapid turnaround of results. The presented cases highlight opportunities for broader use of lactic acid biosensors, both in production/optimization studies and the food and beverage industry at large. In the future, optimizing biosensor integration into fluidic chips, or hybridization with other sensing technologies will enable simultaneous detection/characterization of multiple chemicals.

Conclusions

Optimizing lactic acid production is an iterative process, requiring relatively rigorous studies that compares the performance of a single microbe strain across numerous substrates and vice versa. Similarly, determining the most cost-effective production strategy will best be represented as a cost–benefit analysis. Studies that consider the whole life cycle of the production process including: the cost of raw materials, separation, and CO_2 footprints, or even the cost of cultivating specialist engineered microbes strains as a function of improved yield, will undoubtedly be informative in focusing research efforts.

This review highlights recent successes and significant improvement in the following areas of lactic acid production: (1) production of highly optically pure lactic acid using non-modified and genetically modified microbial strains; (2) achieving high yields of L- and D-lactic acids close to 100% theoretical value using pure carbon sources (glucose and xylose) and more complex materials rich in di- and polysaccharides (starch and sucrose); (3) increasing titers and productivity to over 100 g/L and 10 g/L/h), respectively; (4) improving strain tolerance to carbon source (glucose) and product (lactic acid); (5) advance in engineering microorganisms such as: *E. coli*, *L. plantarum, L. lactis, C. glutamicum, Bacillus sp.*, and *S. cerevisiae* for lactic acid production; (6) identification and development thermophilic strains and their application in non-sterile fermentation processes; (7) development of immobilized cell systems; (8) application of mixed-microorganisms cultures for complex substrate fermentation. Nevertheless, several critical challenges remain. The main ones include: strain growth and inhibition of lactic acid biosynthesis associated with utilizing waste materials, tolerance to physical and chemical stress, and utilizing cheap renewable substrates and waste materials. Moreover, limited information is available on D-lactic acid-producing strains, especially the fermentation of pentoses and low-cost agro-industrial wastes with complex composition. In addition, to enable the cost-effective large-scale L- or D-lactic acid production, further research on the adaptation of alternative fermentation strategies or repeated fed-batch fermentation is required. This needs to be supported by the techno-economic analysis helping to assess the feasibility and sustainability of fermentation processes.

Technologies that can accelerate screening and further development of new microbial strains tolerant to: inhibitors, low pH, elevated temperatures or other stresses, and possessing the ability to utilize cheap, simple (CO_2, CH_4), complex (polymers), or mixed carbon sources that will provide powerful solutions for remaining challenges of the lactic acid biotechnology. No doubt the development of genetically encoded biosensors suitable for lactic acid monitoring *in vivo* will be very beneficial for application in a wide range of screening strategies directed to identify the best lactic acid producers.

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