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Review

REVIEW – Biotechnology & Synthetic Biology

Lactic acid bacteria: from starter cultures to producers of chemicals

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One sentence summary: Lactic acid bacteria are among the most important group of industrial microorganisms, which besides being well established as starter cultures and probiotics, constitute promising biofactories for products for food and non-food sectors.

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ABSTRACT

Lactic acid bacteria constitute a diverse group of industrially significant, safe microorganisms that are primarily used as starter cultures and probiotics, and are also being developed as production systems in industrial biotechnology for biocatalysis and transformation of renewable feedstocks to commodity- and high-value chemicals, and health products. Development of strains, which was initially based mainly on natural approaches, is also achieved by metabolic engineering that has been facilitated by the availability of genome sequences and genetic tools for transformation of some of the bacterial strains. The aim of this paper is to provide a brief overview of the potential of lactic acid bacteria as biological catalysts for production of different organic compounds for food and non-food sectors based on their diversity, metabolic- and stress tolerance features, as well as the use of genetic/metabolic engineering tools for enhancing their capabilities.

Keywords: lactic acid bacteria; metabolic engineering; rerouting metabolism; biological catalysts; biobased chemicals

INTRODUCTION

Lactic acid bacteria (LAB) have been associated since time immemorial with fermentation of foods and their preservation, and today they are clearly the most important group of industrial microorganisms with a market in the range of multibillion dollars. LAB are used as starter cultures for fermentation of milk, vegetables, meat, fish and cereals, and also animal feed in the form of silage. The fermented dairy products are economically the most important with an estimated value of over 80 billion Euros (in 2011) (de Vos 2011). The well-known features of LAB that are utilized to formulate functional starter cultures are production of exopolysaccharides (EPS), organic acids, polyols, aromatic compounds, bacteriocins, among others, which are released into the food matrix giving improved characteristics in terms of texture, aroma, flavor, health effects and shelf life (Leroy and De Vuyst 2004). The application that has experienced growing global market is the use of LAB as probiotics—estimated at 20 billion Euros, a market that was predicted to grow 10% per year (de Vos 2011). Other important applications include their use as delivery vehicles for preventive and therapeutic drugs including proteins and DNA vaccines (Michon *et al.* 2016), and as biological catalysts for production of value added products for both food and non-food sectors from renewable feedstocks in a biobased economy. The efforts in the latter area have gathered

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momentum with the growing awareness of the environmental impact of the fossil based production in terms of greenhouse gas emissions, climate change, etc. Industrial biotechnology, considered to be a key enabling technology for transition from fossil to biobased economy, is dependent to a large part on the feasibility of development of efficient and economical microbial production systems and processes. So far only a few products are produced at industrial scale using a limited number of microorganisms, among them lactic acid produced by LAB has become an important product with a continuously growing demand.

The features favoring widespread industrial applications include the documented GRAS (Generally Regarded As Safe) status of the majority of LAB, their tolerance to various stress environments, their simple metabolism and ability to metabolize variety of carbon sources (Mazzoli *et al.* 2014). Furthermore, development of LAB as bio-factories benefits from the past and ongoing efforts inspired by their immense significance for foods and human health, which have led to increased understanding of genomics, metabolism and physiology, and tools for high throughput screening of microorganisms and development of improved strains (de Vos 2011; Derkx *et al.* 2014).

Both fundamental and applied aspects of LAB are extensively covered in the literature, reference is hereby provided to publications in recent years (Gaspar *et al.* 2013; Zhang and Cai 2014; Bosma, Forster and Nielsen 2017; Sauer *et al.* 2017; Wu, Huang and Zhou 2017). This paper provides a concise review of this important group of microorganisms, including their diversity, metabolism and the strain development options along with examples of their use as biological catalysts utilizing a metabolic pathway or a single enzyme for food and non-food uses.

LAB GENERAL FEATURES

LAB comprise a genetically and ecologically diverse group of non-motile, microaerophilic Gram-positive bacteria including several genera (*Enterococcus*, *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Oenococcus*, *Lactococcus*, *Streptococcus*, *Weissella*, etc. within the order *Lactobacillales*) belonging to the phylum Firmicutes, and anaerobic *Bifidobacterium* genus under the phylum Actinobacteria. The most commercially formulated starter cultures are represented by *Lactobacillus*, *Lactococcus*, *Streptococcus* species, while the most commonly reported probiotics belong to the genera *Lactobacillus* and *Bifidobacterium* (Corona-Hernandez *et al.* 2013), especially the former because of their known antimicrobial effects and other health benefits (Guandalini 2011).

LAB genomes are characterized by small size ranging from 1.23 Mb (*Lactobacillus sanfranciscensis*) to 4.91 Mb (*Lactobacillus parakefiri*). Since the early 21st century, genomic data from more than 200 LAB strains has been collected in different public databases (Douillard and de Vos 2014; Sun *et al.* 2015). Comparative genomic analysis has revealed vast diversity among the LAB, which is attributed to their varying interactions with the environmental niches involving gene loss and -gain through horizontal gene transfer (Wu, Huang and Zhou 2017). This diversity is reflected in the large phenotypic variability observed among the species and even among strains. Noteworthy is the phenomenon of reductive evolution of the genomes involving loss of several metabolic genes and related biosynthetic limitations, presence of pseudogenes, and also fewer higher-level genetic control systems as compared to many other microbes, which is attributed to their adaptation to nutrient rich niches (Schroeter and Klaenhammer 2009; Wu, Huang and Zhou 2017). The sequenced genomes have revealed the presence of several families of glycoside hydrolases in the CAZy database, many of which remain uncharacterized (Sun *et al.* 2015). Several LAB have retained transporter genes for enabling the microbes to acquire nutrients from their environment, and also genes that allow them to tolerate environmental stresses like temperature, pH, salts, etc. and inhibit pathogens (Zhang and Cai 2014). With respect to their safety features, genome analysis of different LAB has shown the absence of virulence-related and toxin encoding genes (Wu, Huang and Zhou 2017).

LAB possess a rich ensemble of genetic elements like plasmids, conjugative transposons and bacteriophages (de Vos 2011). Megaplasmids with sizes in the range of 110–490 kb have been found in several species of LAB (Zhang and Cai 2014; Sun *et al.* 2015). The plasmids and conjugative transposons encode variety of functions like lactose and citrate metabolism, bacteriophage resistance, bacteriocin production, proteolysis, etc. (Schroeter and Klaenhammer 2009). Also, CRISPRs and associated *cas* genes are widespread in the genomes of a number of LAB (Barrangou *et al.* 2007; Sun *et al.* 2015), which provide adaptive immunity against potentially harmful invading foreign DNA, for example, from bacteriophages and plasmids present in the complex environments they inhabit (Schroeter and Klaenhammer 2009; Sun *et al.* 2015).

The LAB features of growth, nutritional requirements, metabolism and production of antimicrobial compounds are crucial for inhibiting the growth of pathogens and spoilage organisms and hence in the production of fermented foods (Wu, Huang and Zhou 2017). These bacteria used various strategies for countering environmental stresses in both industrial milieu and gastrointestinal tract, such as maintaining cell membrane functionality, regulating cellular metabolism, EPS production and expression of stress response proteins (Wu, Huang and Zhou 2012, 2014; Papadimitriou *et al.* 2016). Several LAB exhibit tolerance to stresses like low pH, high temperature, ethanol, high salt concentration, inhibitors generated on pretreatment of lignocellulosic biomass, etc. (Fiocco *et al.* 2007; Abdel-Rahman and Sonomoto 2016; Bosma, Forster and Nielsen 2017).

STRAIN DEVELOPMENT

Strain development of LAB has been a continuous process in the food industry to yield products with improved features such as texture, taste, reduction of additives, calorie content, modulating the compositions of acids produced and even eliminating a certain undesirable property such as antibiotic resistance. Due to strict food legislations and consumer acceptance, strain improvements have been limited to the use of natural strategies such as random mutagenesis, adaptive evolution, dominant selection and even natural transduction and conjugation systems (Derkx *et al.* 2014). Extensive characterization of the inherent genetic elements in LAB has led to the development of a number of gene cloning, expression and secretion systems, which are being applied for metabolic engineering of the bacteria for improving the production titers, modifying substrate utilization, making novel compounds, etc. in biotechnological applications (Gaspar *et al.* 2013; Bosma, Forster and Nielsen 2017). The genetic and metabolic studies were focused traditionally on *Lactococcus lactis* and *Lactobacillus plantarum* because of their importance as starter cultures and probiotics, respectively, and which still continue to be used because of the available genetic tools and high transformation efficiencies (de Vos 2011). Several other LAB strains have since been found to be accessible to genetic modification, however with efficiencies lower than the paradigm

strains of *L. lactis* and *Lb. plantarum* (de Vos 2011; Bosma, Forster and Nielsen 2017).

The currently available genetic tools developed for LAB are listed in Table 1. NICE (nisin controlled gene expression), based on the production of the antimicrobial peptide nisin has become a popular system for efficient, controlled gene expression in *L. lactis* for diverse applications like controlled lysis for accelerated cheese ripening, production of alanine, and other compunds (Hols *et al.* 1999; Mierau and Kleerebezem 2005). Later on, a prototype inducible gene expression system in *Lb. sakei*, nowadays also adapted to other *Lactobacillus* spp., was constructed based upon the sakacin A regulatory system (Axelsson, Lindstad and Naterstad 2003; Sørvig *et al.* 2003). Recently, potential of this so called pSIP system for efficient, tightly controlled expression of enzymes in *Lb. plantarum* WCSF1 was also shown, for eample, β-galactosidase production amounted to 55% of the total intracellular protein in the host cells (Halbmayr *et al.* 2008; Nguyen *et al.* 2015).

Besides the inducible gene expression systems, the development of specific gene regulatory elements in LAB, such as synthetic promoter libraries (Jensen and Hammer 1998; Solem and Jensen 2002; Rud *et al.* 2006), inducible promoters (e.g. P_{lacA}, $P_{lacSynth}$ and P_{xylA}), as well as an orthologous expression system for controlled gene expression in *L. plantarum* and reporter gene mCherry expression have been developed and characterized (Heiss *et al.* 2016). In the past decades, considerable effort has focused on the development of LAB genome modification in a markerless way. These include a system for generating chromosomal insertions based on conditional replication of Ori^+ $RepA^-$ vector pORI19 and a temperature-sensitive helper plasmid pVE6007 in *L. lactis* (Law *et al.* 1995), a Cre-*lox*-based system for multiple gene deletions in *Lb. plantarum* (Lambert, Bongers and Kleerebezem 2007), and a gene replacement system involving the use of *upp*- encoded uracil phosphoribosyltransferase (UPRTase) of *Lb. acidophilus* NCFM as a counter-selection marker (Goh *et al.* 2009).

More recent applications of LAB genome engineering involving single stranded oligonucleotide- mediated recombineering rather than double-stranded DNA, have been reported (van Pijkeren and Britton 2012). Some of the limitations of this approach are the need for high amount of oligonucleotide and the low recombineering efficiency. The advent of CRISPR technologies has now broadened the avenues for generating mutations, deletions, insertions, etc., and the LAB CRISPR-Cas systems provide great opportunities for improving starter culture functional characteristics and probiotic features (Barrangou and van Pijkeren 2016; Hidalgo-Cantabrana, O´Flaherty and Barrangou 2017) as well as for production of native or non-native metabolites. Targeted mutagenesis in *Lb. reuteri* chromosome through CRISPR-Cas single stranded DNA recombineering has been demonstrated with very high efficiencies (Oh and van Pijkeren 2014).

CENTRAL CARBON METABOLISM AND LACTIC ACID PRODUCTION

Besides the extensive application in the food industry, the main industrial application of LAB is in the fermentative production of lactic acid (a 3-carbon hydroxylacid), the primary product of carbohydrate metabolism. Lactic acid is among the most important industrial product to date, with a long history of use in food and nonfood sectors such as cosmetics, pharmaceuticals, chemicals and agriculture. But the demand for lactic acid during the past decades has been driven by the growing market for polylactic acid (PLA), a biodegradable and biocompatible thermoplastic polyester finding diverse applications in packaging materials, films, fibers, nonwoven fabrics, medical implants, drug delivery scaffolds, etc. (Castro-Aguirre *et al.* 2016). The global market size for lactic acid and PLA was estimated at USD 2.08 billion and 1.29 billion, respectively, in 2016, and is expected to grow in the coming years to meet the demand for packaging, personal care and textiles (http://www.grandviewresearch.com/industry-analysis/lactic-acid-and-poly-lactic-acid-market). Natureworks LLC, Corbion, Galactic, Archer Daniel and Pyramid Bioplastics are currently among the major producers of lactic acid and PLA. The possibility to make optically pure lactic acid required for PLA production has made microbial fermentation the most desired route for lactic acid production from sugars in contrast to the petrochemical route.

LAB metabolize sugars by fermentation under microaerophilic conditions and generate ATP by substrate level phosphorylation. Lactic acid is the main product from glucose in homofermentative LAB (*Streptococcus*, *Lactococcus*, *Enterococcus*, *Pediococcus*, and some *Lactobacillus* species) formed via the Embden–Meyerhof (EM) pathway that yields 2 mol pyruvate/mol glucose, which acts as an electron acceptor and is reduced to 2 mol lactic acid in a reaction catalyzed by lactate dehydrogenase (LDH) (Fig. 1A). Phosphofructokinase enzyme was shown to have an important role in the EM pathway flux, decreased or increased activity resulting in proportionally lowered or improved flux and lactate formation (Andersen *et al.* 2001). The homofermentative bacteria have been found to shift to mixed acid fermentation under conditions of carbon limitation, low growth rates and change in oxygen concentration. In the presence of oxygen, increase in NADH oxidase (Nox) activity leads to competition for the available NADH and hence shift in metabolism to yield a mixture of products (de Felipe, Starrenburg and Hugenholtz 1997). The heterofermentative strains (*Leuconostoc, Weissella* genera and certain *Lactobacillus* species) produce also ethanol/acetate and CO_2 (1 mol/mol sugar) besides lactate (1 mol) via the phosphoketolase (PK) pathway (Fig. 1B) (Bosma, Forster and Nielsen 2017).

Production of L-(+)-/D-(-)-lactic acid

The homofermentative bacteria, mainly *Lactobacillus* species, are used for industrial lactic acid production. Lactic acid production from a variety of raw materials by different LAB strains has been reviewed earlier (Mazzoli *et al.* 2014). The optical purity of the lactic acid produced depends on the type of LDH present, L- or D-LDH or both and also the presence of lactate racemases that interconvert the two isomers. *Lb. casei*, *Lb. paracasei* and *Lb. rhamnosus* produce L-lactic acid, *Lb. delbrueckii*, *Lb. coryniformis*, *Lb. jensenii* and *Lb. vitulinus* produce D-lactic acid, while *Lb. pentosus*, *Lb. plantarum*, *Lb. brevis*, *Lb. sake* and *Lb. acidophilus* produce DL-lactic acid (Mack 2004). L-Lactic acid is the main product available commercially; in recent years, stereocomplex PLA, a highly thermostable polymer composed of both L- and D-lactic acid monomers has led to interest even for the production of D-lactic acid (Okano *et al.* 2009a,b,c; 2010; 2017). Several studies have targeted increased production of optically pure lactic acid by different strains of LAB by disruption/deletion of the *ldh* gene encoding for the enzyme catalyzing the production of the unwanted isomer or by genome shuffling (Table 2). The application of genome shuffling to enhance glucose tolerance as well as L-lactic acid production by *Lb. rhamnosus* resulted in a

Table 1. Genetic toolbox for gene modifications in LAB

Tools		Purpose	Species studied	Characteristics	Reference
Expression System	Nisin system	Inducible gene expression	*L. lactis*, *Lb. helveticus*, *Lb. plantarum*	(a) Based on quorum-sensing regulatory mechanism (b) Under control of P*nisA* (c) Adapted for gene expression in lactobacilli and other Gram-positive bacteria (d) Food-grade markers available	Kuipers *et al.* 1995 Hols *et al.* 1999 Gaspar *et al.* 2011, Neves *et al.* 2006 Douillard *et al.* 2011
	Sakacin system	Inducible gene expression	*Lb. sakei*, *Lb. plantarum*, *Lb. casei*	(a) A variant of NICE system, but with tighter control and lower background expression (b) High production of target protein	Axelsson *et al.* 2003 Mathiesen *et al.* 2008 Nguyen *et al.* 2011
	Promoters development	Controllable gene expression	*L. lactis*, *Lb. plantarum*	(a) Constitutive gene expression (b) Provides a flexible genetic tool	Jensen and Hammer 1998 Heiss *et al.* 2016
	Reporter genes development	Measuring and tracking gene expression	*L. lactis*, *Lb. plantarum*, *Lb. reuteri*	(a) Established reported: *gusA*, GFP, CBRluc, mCherry (b) Aid studies on mechanisms of probiosis, localization and interaction with the host	Rud *et al.* 2006 Guo *et al.* 2013 Frese *et al.* 2011 Karimi *et al.* 2016
Chromosomal modification	pORI system	Gene deletion and insertion (markerless)	*L. lactis*	(a) Based on conditional replication of vector pORI19 (b) High stability of mutants and integration plasmid can be readily recovered (c) The stability of the insertional mutations after single-crossover HR requires maintenance of antibiotic selection (d) Same selection marker can not introduce multiple mutations	Law *et al.* 1995 Leenhouts *et al.* 1996
	pTRK system	Gene deletion and insertion (markerless)	*Lb. acidophilus*, *Lb. gasseri*, *Lb. casei*, *L. lactis*	(a) Independent of transformation efficiency (b) Allows growth of mutant strains at preferred growth temperatures and efficient recovery of stable integrants (c) Markerless gene replacement system that involves the use of *upp* as counter-selectable marker for positive system selection of double recombinants	Russell and Klaenhammer 2001 Goh *et al.* 2009 Song *et al.* 2014
	Cre-loxP system	Gene deletion and insertion	*L. lactis*, *Lb. plantarum*	(a) Based on double-crossover integration of nonreplicating vectors (b) Enables selectable marker removal upon marker selection of deletion variants (c) Introduction of multiple *loxP* sites recognizable by Cre might lead to genome instability	Campo *et al.* 2002 Lambert *et al.* 2007
	Single stranded re-combineering	Targeted mutations on chromosome	*Lb. reuteri*, *L. lactis*, *Lb. plantarum*, *Lb. gasseri*	(a) Mutations were incorporated in the chromosome of *L. reuteri* and *L. lactis* without selection at efficiency of 0.4–19% b) High ssDNA concentration, optimal electroporation and optimized oligonucleotide design were required	Van Pijkeren and Britton 2012
	CRISPR-Cas9	Targeted mutations on chromosome	*Lb. reuteri*, *S. thermophilus*, *Lb. gasseri*	(a) Counter-selection against wild-type cells after genome modification (b) Combined with single-stranded DNA recombineering for the generation of subtle mutations	Van Pijkeren and Britton 2014 Hidalgo-Cantabrana *et al.* 2017

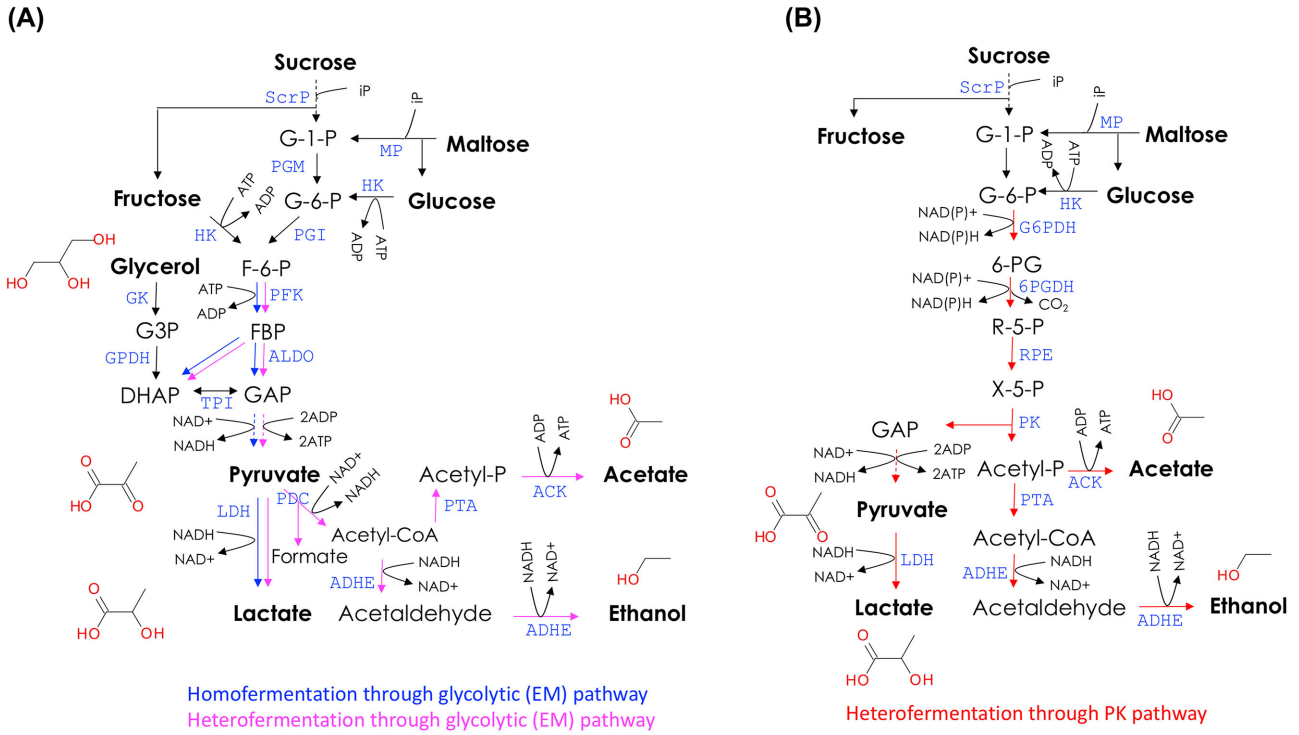


Figure 1. Overview of the central carbon metabolic pathways of glucose, maltose, sucrose, fructose, and glycerol in lactic acid bacteria. (A) Embden–Meyerhof (glycolytic) pathway showing the metabolism under standard fermentation conditions. Blue arrows indicate the different reactions of homolactic fermentation through glycolytic pathway, while purple arrows indicate the different reaction of heterolactic fermentation through the glycolytic pathway. (B) Phosphoketolase (PK) pathway showing the metabolism under standard fermentation conditions. Red arrows indicate the different reactions of heterolactic acid fermentation through the PK pathway. Substrates, major products, and important intermediates are marked in **bold**. Enzyme abbreviations (Blue capitals): ScrP: sucrose phosphorylase, HK: hexokinase, MP: maltose phosphorylase, PGM: phosphoglucomutase, PGI: phosphoglucose isomerase, PFK: phosphofructokinase, GK: glycerol kinase, GPDH: glycerol-3-phosphate dehydrogenase, ALDO: fructose bisphosphate aldolase, TPI: triose-phosphate isomerase, LDH: lactate dehydrogenase, PDC: pyruvate dehydrogenase complex, PTA: phosphotransacetylase, ACK: acetate kinase, ADHE: bifunctional aldehyde and alcohol dehydrogenase, G6PDH: glucose-6-P dehydrogenase, PGL: phosphoglucolactonase, 6PGDH: 6-P-gluconate dehydrogenase, RPE: ribulose epimerase, PK: phosphoketolase. Intermediates abbreviations: G-6-P: glucose-6-phosphate, G-1-P: glucose-1-phosphate, F-6-P: fructose-6-phosphate, FBP: fructose-1,6-biphosphate, GAP: glyceraldehyde-3-phosphate, DHAP: dihydroxyacetone phosphate, G3P: glycerol-3-phosphate, 6-PG: 6-phophogluconate, R-5-P: ribulose-5-phosphate, and X-5-P: xylulose-5-phosphate.

strain producing 184 g/L of L-lactic acid from 200 g/L glucose in batch fermentation (Yu *et al.* 2008). L-Lactic acid overproduction was also achieved in *L. lactis* strain subjected to UV-mutagenesis for enhancement of glucose and lactate tolerance; the mutant was found to possess reduced Nox activity and increased glucose uptake rate (Bai *et al.* 2004a). Inactivation of the *ldhD* gene has resulted in the formation of pure L-lactic acid in several LAB including *Lb. helveticus*, *Lb. paracasei* and *Pediococcus acidilactici* (Bhowmik and Steele 1994; Kylä-Nikkilä *et al.* 2000; Kuo *et al.* 2015; Yi *et al.* 2016), while selective production of D-LDH has been achieved by disruption of *ldhL* gene e.g. in *P. acidilactici* and *Lb. plantarum* (Yi *et al.* 2016; Okano *et al.* 2009a,b,c, 2017).

Substrate utilization

Lactic acid production at industrial scale uses primarily first generation feedstocks i.e. corn starch and cane sugar. Since LAB are able to metabolize a range of sugars as carbon sources (depending on the species/strains) they are suitable organisms for utilizing a wide range of biobased feedstocks including pretreated/hydrolysed lignocellulosic agricultural and forestry residues, algal biomass and municipal solid wastes (Abdel-Rahman, Tashiro and Sonomoto 2011a; Mazzoli *et al.* 2014; Bai *et al.* 2016; Overbeck, Steele and Broadbent 2016; Wang *et al.* 2017; Tarraran and Mazzoli 2018). Except for some bacteria that secrete amylases and have the ability to utilize starch directly (Reddy *et al.* 2008), LAB, in general, do not accept polysaccharides as substrates, thus a hydrolysis step normally precedes fermentation or can be performed simultaneously (SSF, simultaneous saccharification and fermentation) using externally added enzymes. Utilization of disaccharides including sucrose and lactose is common among LAB, while a few bacteria are able to metabolize also cellobiose and other cellodextrins (Gandini *et al.* 2017) or oligosaccharides (with up to 4–6 units) derived from hemicellulose hydrolysis (Ohara, Owaki and Sonomoto 2006; Lawley, Sims and Tannock 2013).

Mixed LAB cultures have been used to maximize yield and productivity from mixtures of pentose and hexose sugars (Taniguchi *et al.* 2004; Cui, Li and Wan 2011); however, a few LAB strains, for example, *Lb. brevis*, *Lb. buchneri*, *Lb. plantarum* and *Enterococcus mundtii*, have been reported to consume C5 and C6 sugars simultaneously without carbon catabolite repression, (Guo *et al.* 2010; Kim, Block and Mills 2010; Abdel-Rahman *et al.* 2011b). Two different pathways are proposed for metabolism of pentoses, the PK pathway used by majority of pentose utilizing LAB yields 1 mol lactic acid/mol sugar, whereas the pentose phosphate (PP) pathway/glycolytic pathway provides a theoretical lactic acid yield of 1.67 mol/mol (Tanaka *et al.* 2002; Oshiro *et al.* 2009) (Fig. 2). Among wild type LAB, *E. mundtii* QU25 (Abdel-Rahman *et al.* 2011b), *Streptococcus* sp., *Lb. thermophilus* (Fukui *et al.* 1957) and *Lactobacillus* strain MONT4 (Barre 1978) were shown to display homofermentative metabolism of

Table 2. Examples of the metabolic engineering strategies used for production of optically pure lactic acid by different LAB species

Lactic acid isomer	LAB species	Engineering strategy	Comments	Reference
L-(+)-	*Lactobacillus helveticus* CNRZ32	*ldhD*-negative strains constructed by deletion of promoter region of the *ldhD* gene (GRL86) or by replacing the structural gene of *ldhD* with an additional copy of the structural gene of *ldhL* from the same species (GRL89)	Maximal productivity of pure L-lactic acid was 3.34 and 3.21 g/L.h for GRL86 and GRL89, respectively, in modified whey medium. Product yield of 76.2% for GRL86 and 91.6% for GRL89. At low pH when the growth and production were uncoupled, GRL89 produced 20% more lactic acid than GRL86	Kylä-Nikkilä *et al.* 2000
L-(+)-	*Lactobacillus lactis* BME5–18	UV mutagenesis to enhance tolerance to glucose and lactate repression	98.6 g/L L-lactic acid from 100 g/l glucose, volumetric productivity of 1.76 g/L.h	Bai *et al.* 2004a
L-(+)-	*Lactobacillus rhamnosus* ATCC11443	Genome shuffling by generating the starting population by UV and nitrosoguanidine mutagenesis, followed by recursive protoplast fusion	184 g/L of L-lactic acid produced at 99% enantiomeric purity from 200 g/l glucose in batch fermentation.	Yu *et al.* 2008
L-(+)-	*Lactobacillus paracasei* 7BL	Interruption of the *ldhD* gene	215 g/L of L-lactic acid produced during fed-batch fermentation on glucose. 99 g/L with 0.96 g/g yield and productivity of 2.25–3.23 g/L.h using non-detoxified wood hydrolysate. Productivity of 5.27 g/L.h using rice straw hydrolysate without detoxification	Kuo *et al.* 2015
L-(+)-	*Pediococcus acidilactici* TY112	*ldhD* gene disruption in the wild-type *P. acidilactici* DQ2 strain	77.66 g/L of L-lactic acid obtained at 25% (w/w) solids content of dry dilute acid pretreated and biodetoxified corn stover feedstock	Yi *et al.* 2016
D-(-)-	*P. acidilactici* ZP26	*ldhL* gene disruption in the wild-type *P. acidilactici* DQ2 strain	76.76 g/L of D-lactic acid obtained at 25% (w/w) solids content of dry dilute acid pretreated and biodetoxified corn stover feedstock	Yi *et al.* 2016
D-(-)-	*Lactobacillus plantarum* NCIMB8826	(i) L-LDH gene (*ldh*L1) deleted and a plasmid encoding *Streptococcus bovis* 148 α-amylase (*AmyA*) introduced (ii) Replacing the *ldh*L1 gene with an *amyA*-secreting expression cassette	73.2 g/L of lactic acid produced from raw corn starch with yield of 0.85 g/g of consumed sugar, and an optical purity of 99.6%.	Okano *et al.* 2009c
D-(-)-	*Lactobacillus plantarum* NCIMB8826	Disruption of L-LDH gene and adaptation of the cells to low pH condition during the early stage of simultaneous saccharification and fermentation	118–129.8 g/L of D-lactic acid obtained from brown rice at a yield of 0.93 g/g and optical purity of 99.6% and volumetric productivity of 2.18 g/L.h	Okano *et al.* 2017

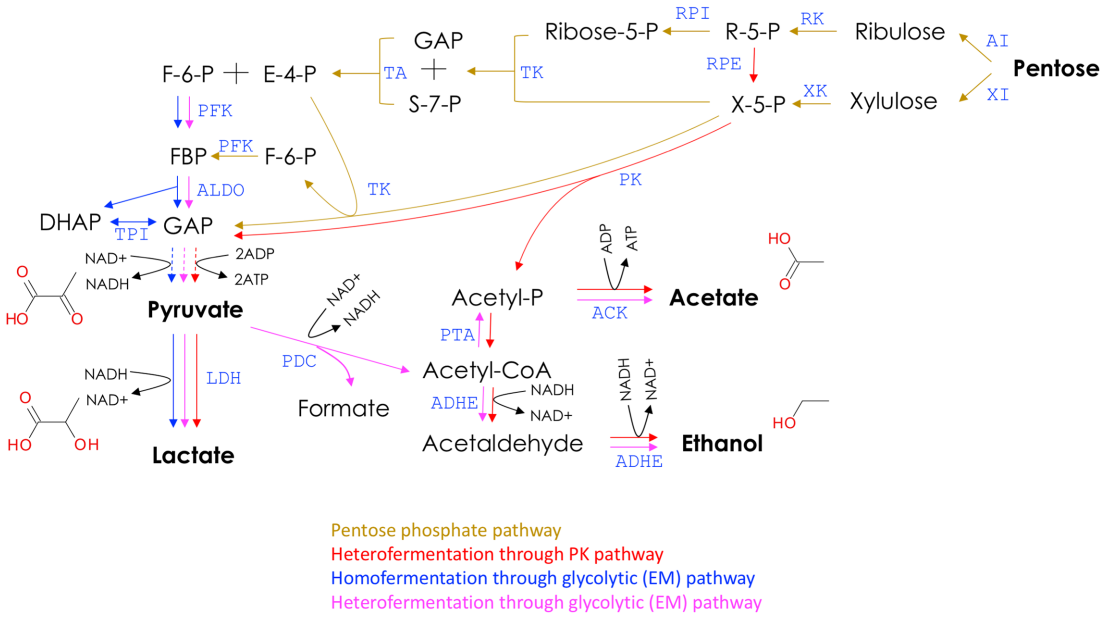


Figure 2. Overview of the central carbon metabolic pathways of pentoses in lactic acid bacteria under standard fermentative conditions. Golden arrows indicate the different steps of pentose phosphate (PP) pathway, Red arrows indicate the different reactions of heterolactic acid fermentation through the phosphoketolase (PK) pathway, Blue arrows indicate the different reactions of homolactic fermentation through glycolytic pathway, and Purple arrows indicate the different reactions of heterolactic fermentation through the EM pathway. Substrates, major products and important intermediates are marked in **bold**. Enzyme abbreviations (Blue capitals): PFK: phosphofructokinase, ALDO: fructose bisphosphate aldolase, TPI: triose-phosphate isomerase, LDH: lactate dehydrogenase, PDC: pyruvate dehydrogenase complex, PTA: phosphotransacetylase, ACK: acetate kinase, ADHE: bifunctional aldehyde and alcohol dehydrogenase, RPE: ribulose epimerase, XI: xylose isomerase, AI: arabinose isomerase, XK: xylulose kinase, RK: ribulose kinase, RPI: ribose-5-phosphate isomerase, TK: transketolase, TA: transaldolase, PK: phosphoketolase. Intermediates abbreviations: F-6-P: fructose-6-phosphate, FBP: fructose-1,6-biphosphate, GAP: glyceraldehyde-3-phosphate, DHAP: dihydroxyacetone phosphate, E-4-P: erythrose-4-phosphate, S-7-P: Sedoheptulose-7-phosphate, R-5-P: ribulose-5-phosphate, and X-5-P: xylulose-5-phosphate.

pentose sugars. An interesting observation made in case of *Lactococcus lactis* IO-1 was the effect of increase in xylose concentration from <5 g/L to >50 g/L on shift in xylulose 5-phosphate metabolism from the PK pathway to PP/glycolytic pathway, and of pyruvate metabolism from cleavage to acetyl-CoA and formic acid to the reduction to L-lactate by LDH (Tanaka *et al.* 2002). Depleting the PK gene and overexpressing a heterologous transketolase gene in *Lb. plantarum* also shifted the carbon metabolism from PK to PP pathway with arabinose and glucose as substrates to give high molar yields of lactic acid (Okano *et al.* 2009a,b). In another study, two copies of *xylAB* operon of *Lb. pentosus* have been introduced in the genome of *Lb. plantarum*, which converted a mixture of 25 g/L xylose and 75 g/L glucose without carbon catabolite repression effects, to produce D-lactic acid with a yield of 0.78 g/g of consumed sugar (Yoshida *et al.* 2011).

Alternative strategies are being investigated to develop systems wherein the lignocellulosic material can be used directly as the feedstock and consequently lower the dependence on pretreatment and enzymatic hydrolysis and the associated costs. These strategies include the use of consortia of cellulolytic microorganism and LAB, or engineering cellulolytic activities in LAB (Tarraran and Mazzoli 2018). A stable consortium of *Trichoderma reesei*, a well-known cellulolytic fungus and *Lb. pentosus* was used for consolidated bioprocessing of pretreated beech wood slurry with a lactic acid yield of 85.2% of the theoretical maximum (Shahab *et al.* 2018). For engineering LAB, the cellulolytic enzymes from aerobic fungi and bacteria as well as cellulosome complexes present in anaerobic microorganisms have been used. Although several studies have reported the expression of a single enzyme often using inducible promoters (Tarraran and Mazzoli 2018), increasing number of reports are emerging on developing strains expressing more than one polysaccharide degrading enzymes, which allow the recombinant strains to grow on the feedstocks being treated (Nguyen *et al.* 2016; Gandini *et al.* 2017). Since expression of multiple enzymes puts a burden on individual cells, the genes for the different enzymes have been introduced in different cells to maximize their ability to grow and produce each enzyme. Recombinant *Lb. plantarum* strains developed by expression of *Thermobifida fusca* cellulase Cel6A and xylanase Xyn11A-encoding genes have shown a synergistic effect on the release of soluble sugars from hypochlorite-pretreated wheat straw when used as a two-strain consortium secreting the enzymes (Morais *et al.* 2013). In another study, *Lb. plantarum* consortium secreting the enzymes was much more efficient than the one with anchored enzymes (Morais *et al.* 2014). On the other hand, the consortium containing an additional strain expressing a scaffoldin on the cell surface for capturing the secreted dockerin-containing cellulase and xylanase to make up a functional cellulosome had only slightly reduced activity than the secreting consortium but provided higher stability of the enzymes (Morais *et al.* 2014). The same research group has further developed adaptor scaffoldins with divergent cohesins for selectively binding enzymes containing different dockerins—an approach that can potentially allow the display of several enzymes by the cell consortia (Stern *et al.* 2018).

Besides the sugar containing feedstocks, glycerol generated in large amounts as a by-product from biodiesel, ethanol and soap manufacture has made it a renewable raw material of choice for a variety of chemicals. Glycerol is used as a carbon source for growth by some LAB, in which it enters the EM pathway at dihydroxyacetone phosphate (Fig. 1A). Although glycerol was predicted as a likely carbon source for *Lb. plantarum* according to the flux balance analysis model based on its genome

sequence, the organism showed only limited growth on it. Adaptive evolution under growth conditions using oxygen as the electron acceptor resulted in a *Lb. plantarum* strain converting glycerol completely to lactic acid, the evolved strain revealing promoter mutations that relieved the catabolite repression of the glycerol operon (Teusink *et al.* 2009).

Overcoming lactic acid process limitations

LAB acquire their nutrition from the environment and metabolize the substrate at a very high rate giving high yields and productivity of lactic acid at low cell biomass concentrations, which is interesting from an industrial perspective as much of the carbon source is used for product formation. However, the need for complex nutritional media for growth increases the production cost. Hence, besides using surplus/residual biomass as the feedstock inexpensive nutritional options, for example, cheese whey, soybean meal hydrolysate, corn steep liquor, vinasse, residual protein hydrolysates from poultry processing and other wastes have been of interest for LAB processes (Kwon *et al.* 2000; Kim et al. 2006; Vázquez and Murado 2008; Salgado *et al.* 2009; Lazzi *et al.* 2013). Lactic acid fermentation is subject to substrate inhibition, which has been overcome by performing fermentations in fed-batch mode with resultant high product yield and concentration (Bai *et al.* 2004b; Ding and Tan 2006).

Lactic acid fermentations are invariably controlled at pH5.0–5.5. Traditionally, the pH control has been achieved using calcium hydroxide but lactic acid recovery by acidification of the calcium salt results in the generation of large amounts of gypsum, which becomes an environmental burden. pH control can also be achieved using NH_4OH or NaOH but requires alternative separation techniques such as electrodialysis or chromatography for the recovery of pure lactic acid, which are cleaner but more expensive to run. The most desirable option is to run the fermentations at low pH, i.e. close to pH 3.8, the pK value of lactic acid, which besides lowering the chemical cost, would allow the product to be recovered in the acid form by, for example, extraction.

Different non-specific approaches such as genome shuffling, adaptive evolution, and error-prone whole genome amplification have been used to obtain strains that are able to grow at substantially lower pH than the wild type strains (Patnaik *et al.* 2002; Zhang *et al.* 2012; Ye, Zhao and Wu 2013). Studies with acid resistant *L. lactis* mutants showed that the organism possesses several means for survival at low pH, one of which results in multiple stress resistance (Rallu *et al.* 2000). In particular, levels of intracellular phosphate and guanine nucleotides determined the extent of the stress response. Amino acid regulation inside the cells is considered to be one of the main mechanisms applied by LAB to counter acid stress. Acid resistant *Lb. casei* strain obtained by adaptive evolution was shown to have higher intracellular pH, NH_4^+ concentration and lower inner membrane permeability besides higher amounts of arginine and aspartate levels under acid stress (Zhang *et al.* 2012). Ammonia production and consequent intracellular pH control was attributed to arginine metabolism via arginine deiminase pathway that is active at low pH; even aspartate is likely to be converted to arginine. Amino acid decarboxylation pathway, identified in different LAB like *Lb. buchneri* and *Lb. brevis*, is proposed to provide resistance by way of releasing CO_2 that results in consumption of protons, hence counteracting cytosol acidification and generating a proton motive force (Wolken *et al.* 2006). Engineering of such a pathway (histidine decarboxylation) in *L. lactis* was shown to result in enhanced tolerance to acid stress (Trip, Mulder and Lolkema 2012). Overexpressing glutathione biosynthetic genes from *Escherichia coli* into *L. lactis* also showed improved resistance to acid stress, which was ascribed to increased intracellular pH and/or stabilization of glyceraldehyde-3-phosphate dehydrogenase activity (Zhang *et al.* 2007). On the other hand, overexpression of endogenous genes in trehalose catabolic pathway, *trePP* and *pgmB* encoding trehalose 6-phosphate phosphorylase and β-phosphoglucomutase, respectively, together with *Propionibacterium freudenrichii otsB* gene encoding trehalose 6-phosphate phosphatase in *L. lactis* showed improved tolerance to acid as well as to cold and heat shock (Carvalho *et al.* 2011).

REROUTING CARBON METABOLISM TO FOOD INGREDIENTS, PLATFORM CHEMICALS AND BIOFUELS

Engineering at the pyruvate node

Pyruvate is a key metabolic intermediate in the central carbon metabolism in all organisms. The significance of pyruvate as an electron acceptor and of LDH catalyzed reduction to lactate in LAB have motivated many studies to understand the effect of inactivating LDH on the cells and also to reroute the carbon flow from pyruvate to another product through metabolic engineering of an alternative cofactor regeneration pathway. Deletion of the *ldh* gene has often turned out to be a difficult task as the mutants generated were unstable because of the activation of an alternative *ldh* gene via a genetic insertion event (Gaspar *et al.* 2007). The alternative LDH enzymes compete with other native dehydrogenases in the regeneration of reduced cofactor and as a result diverting pyruvate to form a mixture of other products (Viana *et al.* 2005; Gaspar *et al.* 2013). Deletion of at least three of the four *ldh* genes was required to obtain a genetically stable strain for production of alternative reduced compounds (Gaspar *et al.* 2011).

Several LAB have the potential to establish an aerobic respiratory chain, which reduces O_2 to H_2O in the presence of H^+ when grown in an aerobic environment in the presence of exogenous heme (and menaquinones for some bacteria) (Lechardeur *et al.* 2011). This respiratory metabolism improves significantly the growth and survival of the bacteria and finds industrial applications in the production of dairy starter cultures (Garrigues *et al.* 2006). Under aerobic conditions, *L. lactis* was found to consume lactate in the stationary phase with concomitant accumulation of diacetyl and acetoin, the reaction being brought about by reversal of the LDH activity when NADH and pyruvate concentrations were extremely low while NAD^+ and lactate were abundant (Zhao *et al.* 2013).

Fig. 3A shows some of the electron recycling systems introduced for transformation of pyruvate to different organic compounds. Cloning and expression of *Bacillus sphaericus* alanine dehydrogenase gene under the control of nisin A promoter resulted in rerouting of 30%–40% from lactate to L-alanine, an amino acid used as a food sweetener. Introducing the gene in LDH-deficient mutant resulted in complete conversion of glucose to L-alanine (Hols *et al.* 1999) (Fig. 3A).

Diacetyl is a strong buttery flavor formed in many dairy products like fresh cheeses, butter-milk, etc. by specific strains of *L. lactis* and *Leuconostoc* species through spontaneous oxidative decrboxylation of α-acetolactate (AL) produced from citric acid, a minor component of milk (Hugenholtz 1993). AL is also formed by condensation of two molecules of pyruvate by the enzyme AL synthase (Swindell *et al.* 1996), and is subsequently converted by α-AL decarboxylase (ALDB) to acetoin (Fig. 3A).

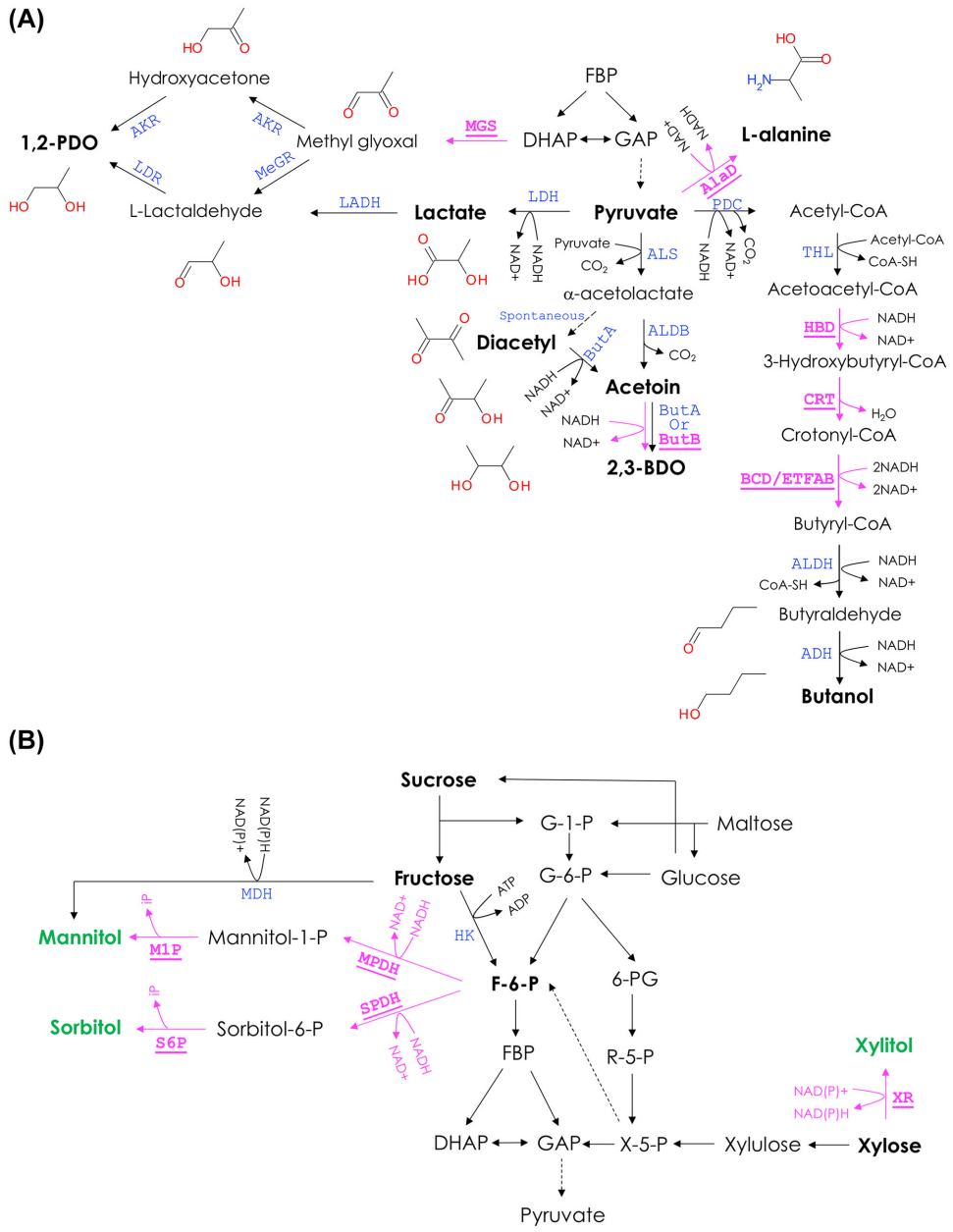


Figure 3. Native and engineered metabolic pathways in lactic acid bacteria to form various products. (A) Pathways diverging from the pyruvate node of central carbon metabolism. Enzyme abbreviations (Blue: native; purple: bold; underlined: heterologous): THL: thiolase, ADH: alcohol dehydrogenase, ALDH: aldehyde dehydrogenase, BCD/ETFAB: butyryl-CoA dehydrogenase complex, CRT: crotonase, HBD: 3-hydroxybutyryl-CoA dehydrogenase, LDH: lactate dehydrogenase, MGS: methylglyoxal synthase, LDH: lactate dehydrogenase, LADH: lactaldehyde dehydrogenase, MeGR: methylglyoxal reductase, AKR: Aldo-keto reductase, LDR: lactaldehyde reductase, PDC: pyruvate decarboxylase complex, ALS: acetolactate synthase, ALDC: acetolactate decarboxylase, ButA: acetoin reductase, ButB: 2,3-butanediol dehydrogenase, AlaD: alanine dehydrogenase. Abbreviations of intermediates: FBP: fructose-1,6-biphosphate, GAP: glyceraldehyde-3-phosphate, DHAP: dihydroxyacetone phosphate, 1,2-PDO: 1,2-propanediol, and 2,3-BDO: 2,3-butanediol. (B) Pathways for transformation of sugars to low calorie polyols. Abbreviations of intermediates: G-1-P: glucose-1-phosphate, G-6-P: glucose-6-phosphate, F-6-P: fructose-6-phosphate, FBP: fructose-1,6-biphosphate, GAP: glyceraldehyde-3-phosphate, DHAP: dihydroxyacetone phosphate, 6-PG: 6-phophogluconate, R-5-P: ribulose-5-phosphate, and X-5-P: xylulose-5-phophate. Enzyme abbreviations (Blue: native, Purple, bold, underlined: heterologous): MDH: mannitol dehydrogenase, MPDH: mannitol-1-phosphate dehydrogenase, M1P: mannitol-1-phosphatase, SPDH: sorbitol-6-phosphate dehydrogenase, S6P: sorbitol-6-phosphatase, and XR: xylose reductase.

Based on the earlier findings on overproduction of *Streptococcus mutans* water-forming Nox in *L. lactis* that resulted in decreased NADH/NAD^+ ratio under aerobic conditions and conversion of pyruvate mostly to acetoin or diacetyl instead of lactate despite the retained LDH activity (Platteeuw *et al.* 1995; de Felipe *et al.* 1998), Hugenholtz *et al.* (2000) designed an efficient scheme for converting glucose to diacetyl by combining Nox overproduction and *aldb* gene inactivation (to prevent formation of acetoin), and using oxygen instead of pyruvate as the electron acceptor.

The AL-acetoin pathway was also engineered using two different cofactor regeneration approaches for converting acetoin into 2,3-butanediol (2,3-BDO), an important chemical for the production of plastics, solvents, pharmaceuticals and

cosmetics (Celinska and Grajek 2009). Overexpression of the native AL synthase and acetoin reductase (ButA) enzymes in *L. lactis* devoid of the *ldh* genes, enhanced pyruvate utilization to form 2,3-BDO at a maximum theoretical yield (67%) from glucose under anaerobic conditions (Gaspar *et al.* 2011) (Fig. 3A). Furthermore, the engineered strain exhibited higher growth rate and biomass yield. In a more recent study, *L. lactis* was transformed into a respiration dependent strain producing large amounts of AL (83.5 mM), which could be converted to diacetyl by metal catalyzed oxidation. The diacetyl was further converted into 2,3-BDO (yield of 0.82 mol/mol glucose) by introducing two additional enzyme activities, diacetyl reductase from *Klebsiella pneumonia* and butanediol dehydrogenase from *Enterobacter cloacae* (Liu *et al.* 2016) (Fig. 3A).

The cofactor engineering strategy was also successfully employed for the production of acetaldehyde, yet another important aroma compound present in dairy products. It is also a commodity chemical used for the industrial production of a range of chemicals for paints, plasticizers, cosmetics and pharmaceuticals. Limited amounts of acetaldehyde are currently produced from bioethanol (http://www.agrobiobase.com/en/database/bioproducts/consumers-goods/acetaldehyde). Acetaldehyde is normally formed in heterofermentative bacteria as an intermediate during ethanol production from acetyl-CoA by the two-domain aldehyde/alcohol dehydrogenase (ADHE) (Bosma, Forster and Nielsen 2017), but not as an end-product of fermentation. Re-routing of pyruvate towards acetaldehyde in *L. lactis* was achieved by nisin-controlled overexpression of *Zymomonas mobilis* pyruvate decarboxylase (*pdc*) gene with that of endogeneous *nox* gene (Bongers, Hoefnagel and Kleerebezem 2005). Nox overproduction helped to maintain a very low ratio of $NADH/NAD^+$ even in the absence of molecular oxygen, hence favoring the utilization of pyruvate by NADH independent PDC for acetaldehyde production rather than for lactate or ethanol production.

Attempts have been made to develop LAB strains for production of biofuel candidates, ethanol, propanol and butanol production. Some LAB exhibit relatively high tolerance to the solvents, and are hence considered as promising hosts for their production. Although some increases in ethanol levels have been reported by expression of a heterologous PDC in LDH-negative *Lb. plantarum*, lactate has often turned out to be the main product due to activation of alternative LDHs (Liu *et al.* 2006). The potential of developing LAB for production of ethanol as the main product was demonstrated by engineering of a *L. lactis* strain involving codon-optimized expression of *Z. mobilis* PDC and ADHE genes using synthetic promoters and knockout of genes encoding three LDHs, phosphotransacetylase and native ADHE (Solem, Dehli and Jensen 2013). For production of *n-propanol* by LAB, glucose was initially converted into 1,2-propanediol (1,2-PDO) (see next section), which is further converted into the desired alcohol by the action of enzymes encoded by genes of the propanediol-utilization (Pdu) operon (Christensen *et al.* 2014). The feasibility of butanol production has been investigated by reconstructing butanol synthesis pathway of *Clostridium acetobutylicum* in *Lb. brevis* that possesses the genes and several enzyme activities crucial for the formation of butanol (Berezina *et al.* 2010). A recombinant *Lb. brevis* strain carrying the clostridial *bcs* operon (comprising genes encoding 3-hydroxybutyryl-CoA dehydrogenase, crotonase, butyryl-CoA dehydrogenase and two subunits of the electron transfer flavoproteins) was able to produce 300 mg/L butanol from glucose, with participation of its own thiolase, aldehyde dehydrogenase and ADHE.

Engineering enhanced polyol production

Low-calorie polyols including mannitol and sorbitol are among the high value products produced naturally by several heterofermentative LAB (Fig. 3B), with applications in food and pharmaceutical industries, as well as in medicine. D-Sorbitol is also regarded as one of the top 10 renewable value added chemicals from biomass (Bozell and Petersen 2010); being an important precursor for L-ascorbic acid and long chain polyols. Furthermore, isosorbide, a dehydration product of sorbitol, is currently of great interest as a building block of polycarbonates, polyesters, polyurethanes and epoxides (Dussenne *et al.* 2017).

In some LAB, fructose is used as a carbon source for growth as well as an electron acceptor, getting converted into mannitol using mannitol dehydrogenase (Fig. 3B) (Saha and Racine 2011). Although produced in significant quantities, mannitol is normally formed along with lactic acid, acetic acid and other metabolites. Inactivation of *ldhD* and *ldhL* genes in *Lb. fermentum* led to the production of mannitol together with pyruvate, and under anaerobic conditions 2,3-BDO was formed as a co-product (Aarnikunnas *et al.* 2003). In *Leuconostoc pseudomesenteroides*, the mannitol yield from fructose was increased from 74% to 85% by random mutagenesis that led to inactivation of fructokinase activity to about 10% of that in the parent strain, with a resultant reduced leakage of fructose into the PK pathway (Helando *et al.* 2005). On the other hand, in *Lb. reuteri*, a truncated version of fructokinase from *Aspergillus niger* along with its activator was expressed, resulting in a strain with enhanced flux through EM pathway and improved ability to handle elevated glucose concentrations, resulting in improved biomass yield and NADH availability for the fructose to be more efficiently transformed to mannitol (Papagianni and Legisa 2014). Attempts have also been made to enhance mannitol production by homofermentative LAB. Highest mannitol yield of 50% from glucose was reported in *ldh* deficient *L. lactis* overexpressing mannitol-1-phosphate dehydrogenase and mannitol-1-phosphatase genes from *Lb. plantarum* and protozoan parasite *Eimeria tenella*, respectively (Fig. 3B) (Wisselink *et al.* 2005).

For sorbitol production, mannitol phosphate dehydrogenase and LDH enzymes were inactivated, and a sorbitol dehydrogenase gene was overexpressed in *Lb. plantarum*, allowing more fructose 6-phosphate to be reduced to sorbitol 6-phosphate (Fig. 3B) (Ladero *et al.* 2007). This resulted in near theoretical yield of 0.65 mol sorbitol/mol glucose by resting cells. In *Lb. casei*, sorbitol 6-phosphate dehydrogenase gene was integrated into the *lac* operon; while the resting cells pre-grown on lactose produced sorbitol at low concentration from glucose, the production was increased by inactivation of *ldhL* gene (Nissen, Perez-Martinez and Yebra 2005).

There has also been interest in the production of xylitol, a five-carbon low calorie sugar alcohol, which is not naturally produced by LAB. Xylitol production was demonstrated in a *L. lactis* strain engineered with xylose reductase from *Pichia stiptis* and a xylose transporter from *Lb. brevis*, and grown in a glucose-limited fed-batch cultivation mode with high xylose concentration (Nyyssola *et al.* 2005) (Fig. 3B).

Lb. buchneri, *Lb. brevis* and *Lb. fermentum* are able to convert lactate into 1,2-propanediol (1,2-PDO), a polyol widely used in anti-freeze fluids, polyester resins, non-ionic detergents, coolants and additive in cosmetics, nutritional products, pharmaceuticals and dyes (Bennett and San 2001). The two-step conversion occurs at low pH and involves hydrogenation to lactaldehyde catalyzed by lactaldehyde dehydrogenase followed by reduction to 1,2-PDO by 1,2-propanediol dehydrogenase,

(Elferink et al. 2001; Nishino et al. 2003; Bosma, Forster and Nielsen 2017). Two moles of lactate are required for this reaction, one being converted to 1 mol 1,2-PDO and the second to 1 mol acetic acid maintaining cofactor balance (Elferink et al. 2001). The titers are however too low. Introduction of methylglyoxal synthase gene in *Lb. reuteri* converted it into 1,2-PDO producer from glucose mediated through dihydroxyacetone phosphate, methylglyoxal and lactaldehyde, respectively, as intermediates. Simultaneously, *n*-propanol was obtained from 1,2-PDO by the action of the Pdu pathway (Christensen et al. 2014).

ENGINEERING ENHANCED PRODUCTION OF SPECIALTY PRODUCTS

Production of EPS of different compositions by wide range of LAB is well known; they alter the rheological properties of the matrix in which they are dispersed and also provide prebiotic effect, stress tolerance and facililtate biofilm formation (Caggianiello, Kleerebezem and Spano 2016; Zeidan et al. 2017). Increasing demand for EPS with interesting properties has led to efforts in improving the level of production by natural or metabolic engineering approaches. The latter has focused on synthesis of precursors such as UDP-glucose, UDP-galactose, etc. Overexpression of the enzymes involved in the synthesis of intermediates such as phosphoglucomutase and UDP-glucose phosphorylase has led to varying levels of improvements in different LAB (Boels et al. 2001; Levander, Svensson and Rådström 2002). The biosynthetic routes of EPS have also been used for heterologous expression of polysaccharides in LAB for which the GRAS status is an advantage over other microbial hosts. An interesting example is that of hyaluronic acid (HA), a copolymer of UDP-glucuronic acid and UDP-acetylglucosamine, with applications in medicine, cosmetics and specialty foods. Combining the expression of a streptococcal HA synthase with UDP-glucose dehydrogenase and pyrophosphorylase in *L. lactis* resulted in good production of HA (Prasad, Ramachandran and Jayaraman 2012), and even the size of the polymer could be controlled by regulating the ratio of HA synthase and the dehydrogenase enzyme (Sheng et al. 2009).

Even though several LAB lack the ability to synthesize many vitamins, certain strains have been shown to produce or possess genes related to the biosynthesis of water soluble vitamins belonging to the B groups such as riboflavin, folate and Vitamin B12 that are essential cofactors for important metabolic activities. (Capozzi et al. 2012). Riboflavin production has been noted in strains of *Lb. fermentum*, *L. plantarum*, *L. lactis* and *L. acidophilus* (Thakur, Tomar and De 2016), while folate is produced by *L. lactis* and other LAB. Pseudovitamin B12, a corrinoid like molecule, was isolated (Santos et al. 2007), and also a complete biosynthetic gene cluster of Vitamin B12 was identified in *Lb. reuteri* CRL 1098 (Taranto et al. 2003; Santos et al. 2008a). Subsequently, production of active Vitamin B12 was demonstrated in two *L. reuteri* strains by supplementing the culture medium with 5,6-diemthylbenzimidazole and δ-aminolevulinic acid (ALA) and optimizing the fermentation conditions (Mohammed et al. 2014). More recently, isolation of two *Lb. plantarum* with high extracellular yields of Vitamin B12 has been reported (Li et al. 2017). LAB producing vitamins have primarily attracted interest due to the possibility of *in situ* fortification of the foods and for production of novel vitamin-enriched foods (Hugenholtz 2008; LeBlanc et al. 2011; Li et al. 2017). Overexpression of the genes involved in folate biosynthesis and of *p*-aminobenzoic acid has led to increased folate production in *L. lactis* (Wegkamp et al. 2007). Different strategies have been applied to transform riboflavin consuming strains to producer strains by genetic engineering (by overexpression of riboflavin biosynthetic genes) (Burgess et al. 2004; Sybesma et al. 2004) or by exposure to purine analogues and/or toxic riboflavin analogues roseoflavin (Burgess et al. 2004, 2006). LAB producing more than one vitamin were constructed by overexpressing folate biosynthesis genes in *L. lactis* overproducing riboflavin (Sybesma et al. 2004), and in *Lb. reuteri* producing Vitamin B12, respectively (Santos et al. 2008b).

Several LAB isolated from traditional fermented foods including *Lb. paracasei*, *Lb. buchneri* and *Lb. brevis* have been found to produce gamma-aminobutyric acid (GABA), a non-protein amino acid (Li and Cao 2010; Lim et al. 2017), which acts as an inhibitory neurotransmitter, and is used in functional foods and pharmaceuticals (Dhakal, Bajpai and Baek 2012). It is produced by decarboxylation of L-glutamate in a reaction catalyzed by glutamate decarboxylase, and high yields have been reported by fed-batch cultivation of *Lb. brevis* (Li et al. 2010).

Production of the biodegradable polymer, poly-β-hydroxybutyrate (PHB) has been observed in several LAB although in low concentrations (6%–35.8% of cell dry weight), the *Lactobacillus* species, in general, accumulating more PHB than the others (Aslim et al. 1998).

Although expression of plant genes in LAB is still relatively scarce as compared to that in *E. coli* and yeasts, some examples of attempts on expression of biosynthetic pathways of plant metabolites including isoprenoids and phenylpropanoids in *L. lactis* are known (Hernández et al. 2007; Song et al. 2012).

THE PROPANEDIOL-UTILIZATION PATHWAY AND PLATFORM CHEMICALS

Glycerol acts as an electron acceptor (and not as a carbon source) in several heterofermentative LAB like *Lb. reuteri*, *Lb. brevis*, *Lb. buchneri* and *Lb. diolivorans* when present together with glucose and enables regeneration of NAD(P)H, being itself converted to 1,3-propanediol as the end product and also resulting in increased growth rate and biomass yield (Pflügl et al. 2012). Industrial production of biobased 1,3-PDO is currently done from glucose using engineered *E. coli* (Sauer, Marx and Mattanovich 2008) and the product is used to produce a polyester fiber, polytrimethylene terephthalate under the tradename Sorona® (sorono.com/our-story/). 1,3-PDO is also used in solvents, adhesives, resins, detergents and cosmetics (Zeng and Sabra 2011). Production of 1,3-PDO from glycerol by wild type microbes including the *Lactobacillus* species and *Klebsiella pneumonia* led to an increasing interest in understanding the metabolic pathway involved. It is now known that several LAB metabolize glycerol as well as 1,2-PDO through Pdu pathway (Fig. 4), which has been known for decades in *Salmonella enterica* but has remained elusive in LAB despite its presence in some *Lactobacillus* spp., *Streptococcus sanguinis*, *Enterococcus malodoratus* and *Listeria monocytogenes* (Chen and Hatti-Kaul 2017).

Studies with *Lb. reuteri* have shown that the the first step of glycerol metabolism is dehydration catalysed by a Vitamin B12 dependent diol dehydratase (PduCDE) to form 3-hydroxypropionaldehyde (3-HPA), which is reduced to 1,3-PDO by 1,3-PDO oxidoreductase (PduQ) (Sriramulu et al. 2008) (Fig. 4). By producing *Lb. reuteri* mutants with individual deletions of PduQ and other cytosolic ADHEs, it was demonstrated by Chen et al. (2016) that PduQ is more active in generating NAD^+ during glycerol metabolism by the resting cells, while ADH7 is

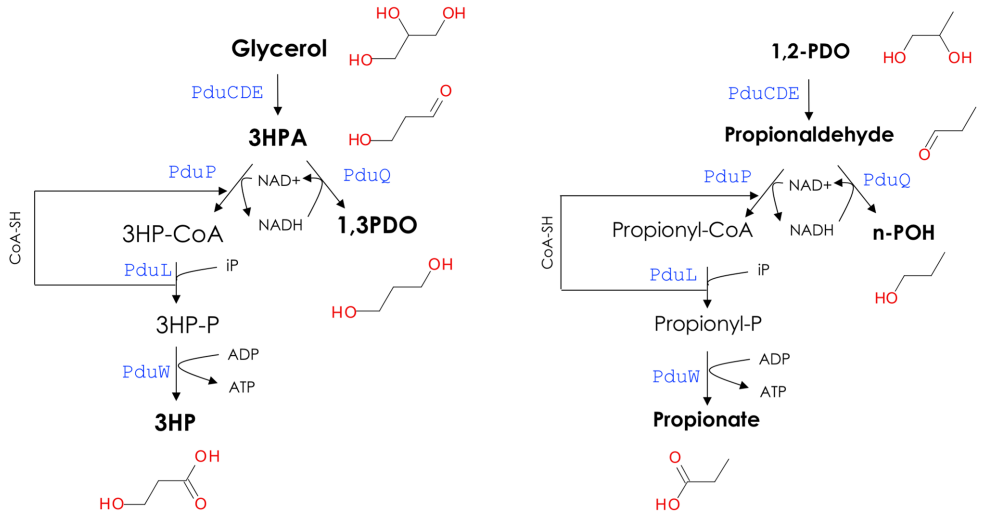


Figure 4. Overview of the propanediol-utilization pathway. (Left) Dehydration of glycerol to 3-hydroxypropionaldehyde (3-HPA) followed by reduction to 1,3-propanediol (1,3-PDO) and oxidation to 3-hydroxypropionic acid (3-HP) via 3-hydroxypropionyl-CoA (3-HP-CoA) and 3-hydroxypropionyl phosphate (3-HP-P) as intermediates. (Right) Dehydration of 1,2-propanediol (1,2-PDO) to propionaldehyde followed by reduction to n-propanol (n-POH) and oxidation to propionic acid via propionyl-CoA and propionyl phosphate as intermediates. Enzyme abbreviations (Blue capitals): PduCDE: glycerol dehydratase, PduQ: 1,3-propanediol oxidoreductase, PduP: propionaldehyde dehydrogenase, PduL: Phosphotransacylase, and PduW: propionate kinase.

responsible for maintaining the cofactor balance by converting 3-HPA to 1,3-PDO outside the microcompartment (MCP) in the growing cells. The genome sequence of *Lb. reuteri* revealed a parallel oxidative branch of the Pdu pathway for 3-HPA metabolism involving 3 consecutive reactions catalysed by coenzyme-A acylating propionaldehyde dehydrogenase (PduP), phosphotransacylase (PduL) and propionate kinase (PduW), respectively, to give 3-hydroxypropionic acid (3-HP), which has also been confirmed experimentally (Sabet-Azad *et al.* 2013; Dishisha *et al.* 2014, 2015). 3-HPA and 3-HP are not commercially available and are ranked as important platform chemicals for the biobased industry (Bozell and Petersen 2010). 3-HPA, also known as *reuterin*, can also be used as an antimicrobial agent in food and health products (Vollenweider and Lacroix 2004).

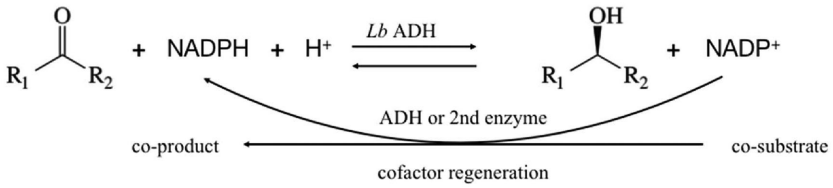
The enzymes of the Pdu pathway are encoded by a *pdu* operon that also codes for the structural proteins making up the MCP housing the pathway (Sriramulu *et al.* 2008). The MCP protects the cells from the toxic effects of the aldehyde intermediate. The diol dehydratase (PduCDE) is optimally active with glycerol and 1,2-PDO (C3 polyols) as substrates, and displays some activity with ethane-1,2-diol (C2) and 1,2-butanediol (C4) to produce corresponding aldehydes. Construction of a double mutant of the *pduC* gene (PduC-Ser302Ala/Gln337Ala) of the dehydratase extended the substrate range up to C6-diols. On the other hand, the enzymes PduQ and PduP of the reductive and oxidative branches of the Pdu pathway were optimally active with 3-HPA but also showed activity with C3-C10 aliphatic aldehydes, suggesting a broad substrate scope (Chen and Hatti-Kaul 2017).

Efficient production of 1,3-PDO by co-feeding glucose or lignocellulose hydrolysate to *Lb. diolivorans* has been reported; optimized feeding resulting in yields up to 95% based on glycerol and titers of 92 g/l (Pflügl *et al.* 2012, 2014; Lindibauer, Marx and Sauer 2017). A limitation with such a process however is the complex, expensive downstream processing for recovery of the pure polyol from the product mixture containing also organic acids and ethanol. This problem can be overcome by the use of resting cells, which requires first the production of cell biomass in which the Pdu pathway is induced by including low concentration of glycerol in the medium and then using the harvested cells to transform glycerol in aqueous solution. Such a two-step process has been used for production of the important metabolites, 3-HPA, 3-HP and 1,3-PDO, of the Pdu pathway by *Lb. reuteri*.

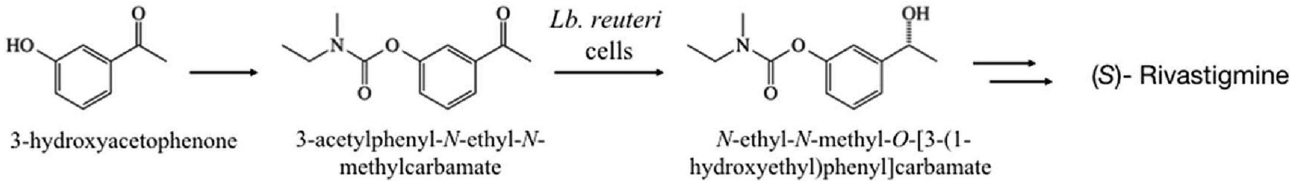
Production of 3-HPA was achieved by trapping the aldehyde *in situ* to resins functionalised with bisulfite and semicarbazide, respectively, which helped to minimise the product inhibition and also its further conversion to 1,3-PDO/3-HP (Sardari *et al.* 2013, 2014). Dishisha *et al.* (2014) demonstrated clean and efficient transformation of glycerol using the resting cells to an equimolar mixture of 3-HP and 1,3-PDO through the two branches of the Pdu pathway, confirming that the two routes maintained the cofactor balance and the process would continue as long as the Pdu enzymes were active. As 3-HP and 1,3-PDO can be easily separated from each other, their co-production is interesting even from an industrial perspective. Production of 1,3-PDO and 3-HP by *Lb. reuteri* was further integrated with another biotransformation step using *Gluconobacter oxydans* for selective oxidation of 1,3-PDO in the mixture to 3-HP, which, in turn, was catalytically dehydrated using TiO_2 to acrylic acid (99 mol% from glycerol), thus providing a biobased route to a number of highly important industrial chemicals (Dishisha, Pyo and Hatti-Kaul 2015).

LAB AND THEIR ENZYMES AS BIOCATALYSTS FOR SELECTIVE TRANSFORMATIONS

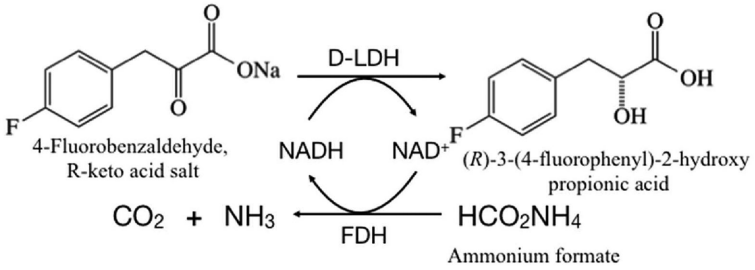
The biocatalytic potential of LAB has been demonstrated in several reports, wherein the activity of an enzyme in the cells is utilized for transformations of natural/synthetic substrates to high value products such as pharmaceutical intermediates, nutraceuticals, specialty chemicals, etc. The whole cells are used as the biocatalyst, which lowers the cost of enzyme isolation and purification, and moreover the enzymes are more stable in the intracellular milieu. The GRAS and non-GMO status of these bacteria is of course an added advantage because of higher



Scheme 1. Enantioselective reduction of a ketone to a corresponding *R*-alcohol by *Lactobacillus* sp. alcohol dehydrogenase. Regeneration of the cofactor NADPH is achieved by oxidation of a co-substrate using a suitable enzyme (Leuchs and Greiner 2011).



Scheme 2. Chemo-enzymatic synthesis of (*S*)-Rivastigmine (Vitale *et al.* 2018). The key step is the bioreduction of 3-acetylphenyl-*N*-ethyl-*N*-methylcarbamate, obtained from commercially available 3-hydroxyacetophenone, using whole cells of *Lb. reuteri* to the corresponding secondary (*R*)-alcohol, which is finally converted into the final product, S-Rivastigmine by a two-step reaction.



Scheme 3. Synthesis of (*R*)-3-fluorophenyl-2-hydroxy propionic acid using D-LDH as catalyst in aqueous medium. Cofactor regeneration was achieved by oxidation of ammonium formate used as the co-substrate catalyzed by formate dehydrogenase (Tao and McGee 2002).

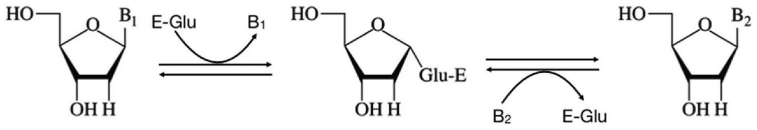
consumer acceptance even if they are not included in the final product. In some cases, however, the enzymes are isolated for use or expressed in a heterologous host for biotransformations.

One of the most successful examples of biocatalysis is the use of ADH activity of different lactobacilli for catalyzing bioreduction of prochiral ketones to optically active alcohols (Scheme 1), which are important pharmaceutical intermediates.

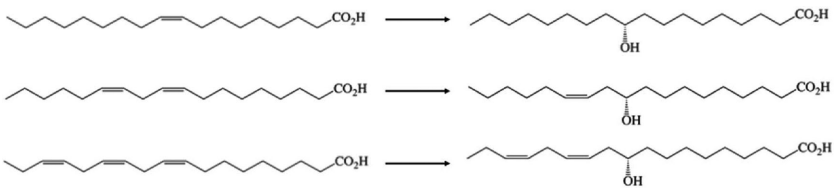
Recombinant ADHs from *Lb. kefir* and *Lb. brevis* have been extensively characterized (Weckbecker and Hummel 2006; Leuchs and Greiner 2011). The enzymes have broad substrate scope with high regio- and enantioselectivity, and are NADPH dependent, which needs to be regenerated for lowering the cofactor costs. *Lb. brevis* ADH has been used both in an isolated form or as a recombinant whole cell biocatalyst (*E. coli*), free or immobilized, with different means of cofactor regeneration, in homogeneous or biphasic reaction media (Leuchs and Greiner 2011). The enzyme remains active in the presence of organic solvents, supercritical fluids or gaseous reactants. *Lb. kefir* whole cells have been used for reduction of a prochiral ketoester to the corresponding (S)- alcohol in high optical purity and the intracellular cofactor (NADP) regeneration was achieved using 2-propanol as the co-substrate, which was oxidized to acetone (Amidjojo and Weuster-Botz 2005). Use of the recombinant *Lb. kefir* ADH has also been reported for stereoselective reduction of several aliphatic and aromatic ketones as well as β-ketoesters to their corresponding alcohols with >99% enantiomeric excess (Weckbecker and Hummel 2006). Here, the NADPH was regenerated using a coupled glucose dehydrogenase catalyzed oxidation of the co-substrate glucose. The bioreduction process was improved to give high selectivity and space-time yield by using immobilized *Lb. kefir* cells in a plug flow reactor (Tan *et al.* 2006).

More recently, resting cells of *Lb. reuteri* have been used for asymmetric reduction of prochiral aryl ketones into chiral and optically active alcohols with (*R*)-enantiopreference, which are important building blocks for the synthesis of drugs for neurological and neurodegenerative disorders (Perna *et al.* 2016). The reactions were performed in aqueous medium with high yields, using pure glucose, lactose, spruce lignocellulose hydrolysate and cheese whey, respectively, as the sources of reducing equivalents. This work has been further extended to develop a chemoenzymatic process for the synthesis of (S)-Rivastigmine (Scheme 2), an important drug used for the treatment of mild to moderate dementia of the Alzheimer´s type, in which R-regioselective reduction of an aromatic ketone by whole cells of *L. reuteri* DSM 20 016 constituted an important intermediate step (Vitale *et al.* 2018).

Another interesting example of a dehydrogenase enzyme is that of D-LDH from *Leuconostoc mesenteroides*, which was used as a biocatalyst in an efficient continuous process using a membrane reactor for synthesis of (*R*)-3-(4-fluorophenyl)-2-hydroxypropionic acid (Scheme 3), a building block for a rhinovirus protease inhibitor, with excellent enantiomeric excess (ee > 99.9%) at multikilogram scale (Tao and McGee 2002). Cofactor regeneration was achieved by coupling the reaction with *Candida boidinii* formate dehydrogenase catalyzed oxidation of ammonium formate to CO_2 and NH_3.



Scheme 4. General scheme of nucleoside 2′-DT catalyzed exchange between the purine or pyrimidine base (Fernández-Lucase *et al.* 2010).



Scheme 5. Regio- and stereoselective hydration of oleic, linoleic and linolenic acid using whole cells of *Lb. rhamnosus* (Serra and De Simeis 2018).

As already described above, the H_2O producing NADH oxidase present in lactobacilli provides a clean system for efficient recycling of NAD^+ and is applicable for several oxidation systems. *Lb. brevis* NADH oxidase was used for NAD^+ regeneration in a reaction catalyzed by *Bacillus subtilis* acetoin reductase/2,3-butanediol dehydrogenase expressed in *E. coli* cells for stereospecific oxidation of (2*R*,3*R*)-2,3-butanediol and *meso*-2,3-butanediol to chiral acetoins (3*R* and 3*S*), widely used to synthesize novel optically active α-hydroxyketone derivatives and liquid crystal composites (Xiao *et al.* 2010).

2′-N-deoxyribosyltransferase (NDT) is an enzyme that catalyzes the cleavage of N-glycosidic bond of 2′-deoxyribonucleoside and transfers the glycosyl moiety to a purine or a pyrimidine base, a reaction that can be used for the synthesis of nucleoside analogs (NAs) (Scheme 4), which constitute an important class of antiviral and antitumor drugs. NDTs from *Lb. reuteri* and *Lb. animalis* have been used for the synthesis of natural and non-natural NAs including new arabinonucleosides and halogenated pyrimidine and purine 2′-deoxyribonucleosides (Fernández-Lucas *et al.* 2010; Britos *et al.* 2016). Covalent immobilization of the *Lb. animalis* NDT to a solid support resulted in enhanced process and storage stability of the enzyme (Méndez *et al.* 2018).

Deglycosylation of ginsenosides (ginseng saponins), the primary active components of ginseng roots used as a traditional herb in South East Asia, increases their biological potency. The whole cells of *Lb. rhamnosus* cells, induced for β-glucosidase production using cellobiose, were used for deglycosylation of ginsenoside Rb1 to form ginsenoside Rd, reported to have several beneficial effects for humans including anti-obesity, wound-healing and immunosuppression (Ku *et al.* 2016).

Microbial hydration of unsaturated fatty acids (oleic, linoleic and linolenic acid) present in vegetable oils provides a facile route for the production of hydroxyl fatty acids with a variety of applications as starting materials for industrial- and fine chemicals, cosmetics and pharmaceuticals. Biotransformation using an anaerobic culture of *Lb. rhamnosus* resulted in the formation of only 10-hydroxy derivatives of the fatty acids with very high enantiomeric purity (ee >99%) (Serra and De Simeis 2018) (Scheme 5).

Furthermore, several LAB, particularly the *Lactobacillus* species, catalyze the transformation of linoleic acid through linoleate isomerase activity to conjugated linoleic acid isomers, which are reported to possess beneficial effects on humans including anti-inflammatory, anti-obesity, anti-carcinogenic activities (Kishino *et al.* 2002; Kuhl and Lindner 2016; Yang *et al.* 2017).

CONCLUDING REMARKS

LAB have a unique position in being important tools for combining the production of food and non-food products in the biobased economy. This review has provided a glimpse of the increasing trend in utilizing LAB and their enzymes for the production of platform-, specialty-, fine chemicals, nutraceuticals and pharmaceutical intermediates. LAB have a number of advantages as industrial microbes in terms of high growth rates, tolerance to various stress conditions, uncoupling of growth and production, ability to use diverse feedstocks as carbon sources, ease of scaling up based on their microaerophilic/anaerobic characteristics. With the availability of genome sequences and the high throughput omics tools, their use will benefit from the systems biology approach through combining mathematical modeling techniques with functional genomics data (Teusink and Smid 2006; de Vos 2011). In order to be competitive with other established organisms as industrial production hosts, LAB cells need to be designed for growth on simple media, direct hydrolysis of biomass polysaccharides (Okano *et al.* 2010) and even combine the synthesis of two or more products in a cell biorefinery (Cheirsilp *et al.* 2018). In some cases, two-steps process involving cell growth and product(s) formation, respectively, with dynamic metabolic control can provide an optimal strategy for better control and improved volumetric rates, titers and yields (Dishisha, Pyo and Hatti-Kaul 2015; Burg *et al.* 2016).

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