Towards lactic acid bacteria-based biorefineries

UNIVERSITÀ DEGLI STUDI DI TORINO

Towards lactic acid bacteria-based biorefineries

Roberto Mazzoli[1*], Francesca Bosco[2], Itzhak Mizrahi[3], Edward A. Bayer[4], and Enrica Pessione[1]

[1]Laboratory of Biochemistry: Proteomics and Metabolic Engineering of Prokaryotes. Department of Life Sciences and Systems Biology, University of Torino. Via Accademia Albertina 13, 10123 Torino, Italy. e-mail: roberto.mazzoli@unito.it; enrica.pessione@unito.it

[2]Department of Applied Science and Technology (DISAT), Politecnico of Torino, Corso Duca degli Abruzzi, 24, 10129 Torino, Italy. e-mail: francesca.bosco@polito.it

[3]Institute of Animal Science, ARO, Volcani Research Center, P.O. Box 6Â, Bet Dagan 50-250 Israel. e-mail: itzhakm@volcani.agri.gov.il

[4]Department of Biological Chemistry, the Weizmann Institute of Science, Rehovot 76100 Israel. e-mail: ed.bayer@weizmann.ac.il

Corresponding author: Mazzoli, R. (roberto.mazzoli@unito.it). Laboratory of Biochemistry: Proteomics and Metabolic Engineering of Prokaryotes. Department of Life Sciences and Systems Biology, University of Torino. Via Accademia Albertina 13, 10123 Torino, Italy. Phone: +39 011 6704644.

Running title: LAB-based biorefineries

Key words: metabolic engineering, polylactide, cellulose, ethanol, butanol, polyhydroxyalkanoates, mannitol, GABA, bacteriocins

Summary

Lactic acid bacteria (LAB) have long been used in industrial applications mainly as starters for food fermentation or as biocontrol agents or as probiotics. However, LAB possess several characteristics that render them among the most promising candidates for use in future biorefineries in converting plant-derived biomass – either from dedicated crops or from municipal/industrial solid wastes – into biofuels and high value-added products. Lactic acid, their main fermentation product, is an attractive building block extensively used by the chemical industry, owing to the potential for production of polylactides as biodegradable and biocompatible plastic alternative to polymers derived from petrochemicals. LA is but one of many high-value compounds which can be produced by LAB fermentation, which also include biofuels such as ethanol and butanol, biodegradable plastic polymers, exopolysaccharides, antimicrobial agents, health-promoting substances and nutraceuticals. Furthermore, several LAB strains have ascertained probiotic properties, and their biomass can be considered a high-value product. The present contribution aims to provide an extensive overview of the main industrial applications of LAB and future perspectives concerning their utilization in biorefineries. Strategies will be described in detail for developing LAB strains with broader substrate metabolic capacity for fermentation of cheaper biomass.

Contents

1. Introduction

Lactic acid bacteria (LAB) include a wide group of Gram-positive fermenting bacteria which are generally non-sporulating and non-motile. They comprise both cocci and bacilli belonging to *Carnobacterium*, *Enterococcus* (*Ent*), *Lactobacillus* (*Lb*), *Lactococcus* (*Lc*), *Leuconostoc* (*Leu*), *Oenococcus*, *Pediococcus* (*Ped*), *Streptococcus* (*Str*), *Tetragenococcus*, *Vagococcus* and *Weissella* genera (Hofvendahl and Hahn-Hägerdal, 2000).

LAB are among the most promising microorganisms for biorefineries converting waste biomasses into industrially important products (Berlec and Strukelj, 2009). Currently, the main application of LAB in industrial processes is as food starters (e.g., for dairy products, pickles, meat and wine) (Papagianni, 2012). However, several physiological characteristics of the LAB render these bacteria highly suitable for much wider industrial application:

i) Except for some pathogenic streptococci strains, LAB are considered GRAS (generally regarded as safe);

ii) LAB are robust organisms already adapted to stress conditions in industrial processes, since they generally show high acid tolerance (they can survive at pH 5 and lower) and broad optimal growth temperatures (ranging from 20 to 45°C, depending on the genus and strain) (Hofvendahl and Hahn-Hägerdal, 2000);

iii) LAB are able to metabolize numerous mono- (both hexose and pentose) and di-saccharides (Kandler, 1983);

iv) LAB naturally produce many metabolites with confirmed applications in both the food and non-food industries (Fig. 1), such as: antimicrobial molecules (e.g., bacteriocins) (Settanni and Corsetti, 2008); food aromas and flavors (e.g., diacetyl and acetaldehyde) (Papagianni, 2012); food complements (e.g., vitamins) (Sybesma et al., 2004); food texturing agents (e.g., exopolysaccharides) (Chapot-Chartier et al., 2011); sweeteners (e.g., mannitol) (Hugenholtz et al., 2011); nutraceutical molecules, e.g., γ-aminobutyric acid (GABA) opioid peptides and seleno-metabolites (Lamberti et al., 2011; Mazzoli et al., 2010; Mazzoli, 2014); bulk chemicals (e.g., lactic acid and ethanol) with applications for plastic polymeric manufacturing, e.g., polylactic acid (PLA) or polyethylene terephthalate (PET), respectively (Madhavan Nampoothiri et al., 2010; Singh and Ray, 2007) or as solvents or biofuels (e.g., ethyl lactate, ethanol) (Ohara, 2003); as well as biodegradable plastics (i.e., polyhydroxyalkanoates, PHA) (Aslim et al., 1998).

Although it is not within the scope of this review, it is worth mentioning LAB properties as extensively used probiotics (Settanni and Moschetti, 2010; Lamberti et al., 2011).

*Fig. 1

Depending on which metabolic pathway(s) is (are) used by a LAB strain to catabolize sugars, a given strain can show either homo-, hetero- or mixed acid fermentation phenotype. Homofermentation virtually produces lactic acid (LA) as the sole end product. Sugars are catabolized through the Embden-Meyerhof pathway, and its end product, i.e., pyruvic acid, is enzymatically reduced either by D-lactate or L-lactate dehydrogenase (D-LDH and L-LDH, respectively) giving rise to the two LA enantiomers. The stereospecificity of the LA produced depends on the specific LAB strain, that is on the presence of the genes encoding L-LDH and/or D-LDH and their relative level of expression. Furthermore, the D-LA/L-LA balance can be affected by the presence of lactate racemase (E.C. 5.1.2.1) that catalyzes their interconversion (Goffin et al., 2005). In heterofermentative metabolism, equimolar amounts of LA, carbon dioxide and ethanol or acetate are formed from glucose via the phosphoketolase pathway. This pathway is used by facultative heterofermenters, such as *Lb. casei*, to metabolize pentoses and for the fermentation of hexoses and pentoses by obligate heterofermenter organisms such as *Leuconostoc*. Almost all LAB, except some lactobacilli, are able to ferment pentoses, i.e., they are facultative heterofermenters (Kandler, 1983). Mixed acids are formed by homofermenters such as lactococci during glucose limitation and during growth on other sugars, e.g., *Lc. lactis* growing on maltose, lactose and galactose, or at increased pH and temperature (Papagianni, 2012). Ethanol, acetate and formate are formed in addition to LA. Here, monosaccharides are catabolized through the Embden-Meyerhof pathway as in homofermentative metabolism, but pyruvate is partly reduced to LA by LDH and partly converted to formate and acetyl-CoA by pyruvate formate lyase (PFL). In the presence of oxygen, PFL is inactivated and an alternative pathway of pyruvate metabolism becomes active via pyruvate dehydrogenase (PDH), resulting in the production of carbon dioxide, acetyl-CoA and NADH (Hofvendahl and Hahn-Hägerdal, 2000).

Although the ability of LAB to synthesize a large panel of highly valuable compounds renders them good candidates for biorefinery application, economic feasibility of such LAB-based industrial fermentations (especially for the production of bulk chemicals) remains problematic. Since LAB have limited potential to biosynthesize amino acids, nucleotides, and/or vitamins, supplementation of these nutrients is necessary for optimal growth. These complex nutritional requirements increase the costs

of both growth medium preparation and product separation and purification (John et al., 2007; Okano et al., 2010a). Furthermore, LAB are generally unable to directly ferment (i.e., without previous saccharification by physico-chemical and/or enzymatic treatments) complex carbohydrates, e.g., starch and cellulose, which comprise the most abundant and least expensive feedstocks for biorefineries (John et al., 2007; Okano et al., 2010a).

Metabolic engineering helps solve defined problems, e.g., broadening the range of carbon sources used by a microorganism or improving its product yield and productivity. The small genomes (about 2-3 Mb) and the relatively simple physiology of LAB make them suitable organisms for metabolic engineering (De Vos and Hugenholtz, 2004). Many genetic tools, including gene expression vectors, are available for LAB (de Vos, 1999; Sorvig et al., 2005). Advantageously, a number of chromosomal integration systems for LAB have been developed since the 1980s and are continuously optimized (for an extensive review please refer to Gaspar et al., 2013). Tools for unlabelled (i.e., without insertion of antibiotic resistance markers) gene integration into the chromosome by either homologous recombination (e.g., pORI, pSEUDO and Cre-lox systems) or single-stranded DNA recombineering currently allow manipulation of lactobacilli and *Lc. lactis* (Douglas et al., 2011; Lambert et al., 2007; Pinto et al., 2011; van Pijkeren and Britton, 2012). Such strategies are essential for engineering microorganisms suitable for application in industrial fermentations, since they circumvent genetic instability of plasmid-bearing strains and avoid the need for antibiotic pressure, which is too costly at the industrial scale and not applicable in food-oriented processes.

To date, *Lc. lactis* is still by far the most extensively studied species among LAB, and many examples of successful metabolic engineering of this species are available (Hugenholtz et al., 2011; Kleerebezem and Hugenholtz, 2003). So far, the main efforts in genetic modification of LAB have been concentrated in the development of LAB with enhanced qualities for food grade applications, e.g., with improved production of flavors or nutritional (health-promoting) components, or increased resistance to bacteriophages (Hugenholtz et al., 2011; Papagianni, 2012; Singh et al., 2006). Only a few attempts have been made to improve LA production by metabolic engineering in LAB. Most were attempts to obtain optically pure LA isomer by cloning or deletion of genes encoding either D- or L-LDH or by deletion of racemase genetic determinant(s) (Singh et al., 2006; Okano et al., 2009b; 2010b). In the meantime, an increasing number of LAB has been the object of extensive fundamental research. Whole genome sequences of about 40 LAB strains have been determined. Moreover, sequencing of more than 100 LAB genomes is currently underway as reported by a number of publicly available databases (http://www.genome.jp/kegg/; http://www.jgi.doe.gov/;

http://www.ncbi.nlm.nih.gov/genome) (Gaspar et al., 2013). The metabolism of several LAB has been investigated by a great number of studies through trancriptomic, proteomic and/or metabolomic approaches (Bron et al., 2012; Carvalho et al., 2013; Gaspar et al., 2013; Pessione et al., 2014). Such information will contribute to expand the potential of systems metabolic engineering of LAB.

The present paper intends to provide an extensive overview of the main current industrial applications of LAB and future perspectives concerning their utilization in biorefineries for converting waste biomass by fermentation. Strategies for improving production of high-value compounds by LAB or expanding their substrate metabolic capacity for cheaper forms of biomass, such as starch or lignocellulose, will be described in detail with examples of both fermentation process optimization and strain engineering through genetic manipulation techniques.

2. Agro-industrial biomass for LAB-catalyzed bioconversion processes: past, present and future

Today, LA production is the most significant application of LAB for large-scale industrial fermentation. In 2002, of the 150,000 tons of LA that were produced worldwide, about 90% was generated via LAB fermentation using glucose as the carbon source (Sauer et al., 2008). The higher the purity of the sugar(s) used as substrate, the higher the purity of the fermentation product(s) (e.g., LA) obtained. This greatly reduces the costs for down-stream product purification. However, this approach is economically unfavourable, since pure sugars are expensive. Therefore, different types of biomass, such as energy crops, forestry residues, or by-products from agro-industrial activities (e.g., milk whey, molasses, starch, wheat bran and flour, and lignocellulose), featuring both low purchase cost and renewability, have either been proposed or have already been tested as fermentative substrates for LAB (Hofvendahl and Hahn-Hägerdal, 2000; Okano et al., 2010a). It is worth noting that several parameters affect product yield and productivity from renewable resources, including the carbon source, the nitrogen source, the fermentation mode, the pH and temperature (Hofvendahl and Hahn-Hägerdal, 2000). Notably, the heterogeneous chemical and physical nature of the different available feedstocks limits the possibility to develop generally applicable bioconversion processes. In particular, the bioconversion of the most abundant and promising biomass types, i.e., starch and lignocellulose, by LAB is currently the most problematic, as described in the section 2.2.

2.1. Suitable biomasses for LAB biorefinery processes

2.1.1 Milk whey. Milk whey represents a cheap raw material that is available in large amounts (13.500.000 tons/year in the EU) as a by-product of the caseification process (Koller et al., 2007). Furthermore, this surplus product causes a huge and expensive disposal problem for the dairy industry (Koller et al., 2007). Although several possibilities of cheese whey utilization have been explored, a major portion of the world cheese whey production is currently discarded as effluent. The major constituents of whey are lactose (45-50 g/L), proteins (6-8 g/L) and salts. The main components of the whey protein fraction are β-lactoglobulin (2.7 g/L), α-lactalbumin (1.2 g/L), immunoglobulins (0.65 g/L), bovine serum albumin (0.4 g/L), lactoferrin (0.1 g/L) and lactoperoxidase (0.02 g/L) (Wong et al., 1996). The high content of lactose in whey permeate (about 80% of the original lactose in milk) and the presence of other essential nutrients (e.g., vitamins) for the growth of microorganisms would potentially enable milk whey to be used directly as a medium for LAB fermentation, e.g., for LA production.

2.1.2 Molasses. Molasses is a by-product of the sugar manufacturing process and contains sucrose as the most abundant sugar. Its high carbohydrate concentration makes molasses a highly viscous liquid which needs dilution before using it in microbial growth media, so as to avoid osmotic problems for the cells. Molasses has been traditionally used as animal feed and for ethanol and yeast production, but applications for LA production by LAB have also been reported (Hofvendahl and Hahn-Hägerdal, 2000).

2.1.3 Starch. Starch consists of a mixture of glucans, i.e., amylose and amylopectin. Both amylose and amylopectin possess a linear backbone of glucose residues linked by α(1-4) linkages. In addition, in amylopectin branching takes place through α(1-6) bonds occurring every 24 to 30 glucose units of the backbone chain. Starch can be obtained from various plants and is an interesting raw material on the basis of cost and availability (Okano et al., 2010a). However, relatively few LAB strains (mainly lactobacilli) isolated so far have starch-degrading properties (see also section 2.2.) (Okano et al., 2010a). Furthermore, most of the naturally amylolytic LAB show either low LA yields or poor enantioselectivity as described in section 3.1.1. (Okano et al., 2010a). Many groups have explored acid/enzyme hydrolysis of starchy substrates (wheat, corn, cassava, potato, rice, rye, sorghum and barley) followed by LAB fermentation or simultaneous saccharification and fermentation by co-culture/mixed culture in LA production processes (Xiaodong et al., 1997; Datta and Henry, 2006; Hofvendahl and Hahn-Hägerdal, 2000).

2.1.4. Wheat bran. Wheat bran is rich in proteins, oil, nutrients, and calories and is among the major by-products of wheat production. Wheat flour or wheat bran has been used to produce LA, as they contain a large percentage of starch and proteins, which can be utilized as the sole sources of carbon and nitrogen, respectively (John et al., 2006; Li et al., 2010b; Naveena et al., 2005a; b; Oh et al., 2005).

2.1.5. Lignocellulose. Lignocellulose is the main component of the plant cell wall and the most abundant renewable source of biomass on Earth. It also includes waste biomass produced by human activities, such as waste paper, by-products from agricultural crops (e.g., wheat straw, corn stalks) and industrial waste streams (e.g., paper mill sludge, wood industry waste). Despite great differences in composition and in anatomical structure of cell walls across plant taxa, lignocellulose typically consists of cellulose (35-50% of the plant dry weight), hemicellulose (e.g., xylans, mannans and β-glucans, 20-35 % of the plant dry weight) and lignin (5-30% of the plant dry weight) as the major components (Lynd et al., 2002). While cellulose is a linear homopolymer of β-glucose units linked by (1-4) glycosidic bonds, hemicellulose is a heterogenous class of polysaccharides which are often branched and consist of both hexose (e.g., glucose and galactose) and pentose (e.g., xylose, which is the most abundant hemicellulose component, and arabinose) sugars (Jordan et al., 2012). Some LAB strains can ferment short cello-oligosaccharides and/or xylooligosaccharides (Adsul et al., 2007a; De Vos, 1997; Ohara et al., 2006; Kowalczyk et al., 2008). However, no natural cellulolytic or hemicellulolytic LAB has been isolated so far as described in section 2.2.

2.2. Expanding LAB substrate metabolization performance: construction of recombinant amylolytic and (hemi-)cellulolytic LAB

The bioconversion of starchy and lignocellulosic biomasses by LAB is currently hampered by major limitations. Relatively few natural LAB strains having starch-degrading properties, e.g., amylolytic strains belonging to *Lactobacillus species* (*Lb. amylophilus*, *Lb. amylovorus*, *Lb. amylolyticus* and certain strains of *Lb. plantarum*), have been isolated so far (Giraud et al., 1994; Guyot et al., 2000; Narita et al., 2004). Most of them have been used for one-step LA production and have exhibited either low yield or low enantioselectivity (John et al., 2007; Okano et al., 2010a). However, screening for useful amylolytic LAB is both time-consuming and difficult.

Currently, pre-treatment of starchy and ligno-cellulosic feedstocks, including polysaccharide hydrolysis into oligo-/mono-saccharides, is in most cases necessary prior to sugar fermentation by LAB (Hofvendahl and Hahn-Hägerdal, 2000, Okano et al., 2010a). Hydrolysis of polysaccharides

can be achieved by either physico-chemical strategies (e.g., steam, diluted acids or alkali) or enzymatic treatment (addition of commercial amylase or cellulase cocktails). Both physico-chemical and enzymatic hydrolysis can be very efficient, but most physico-chemical processes are known to produce toxic compounds, e.g., furfural and hydroxymethylfurfural, which inhibit microbial growth. Moreover, both physico-chemical and enzymatic treatments significantly increase the cost of the bioconversion processes (Okano et al., 2010a). Notably, dedicated production of hydrolytic enzymes is still a major economic constraint for cost-effective bioprocessing of plant-derived lignocellulosic biomasses (Olson et al., 2012). In this context, the cost of cellulases is currently comparable to the purchase cost of the feedstock, i.e., 50 cents per gallon ethanol (Olson et al., 2012). Extensive research has been dedicated to developing consolidated biomass bioconversion processes (CBP), featuring biomass hydrolysis and bioconversion to high-value product(s) in a single fermenter. As far as LAB are concerned, many efforts have been aimed at the construction of recombinant amylolytic or cellulolytic strains by metabolic engineering over the past 25 years.

To date, owing to the less recalcitrant nature of starch with respect to lignocellulose, the most successful studies have been those aimed at the construction of recombinant amylolytic LAB, i.e., secreting heterologous α-amylase (Okano et al., 2007; Van Assendolk et al., 1993) (see Table 1). Increased amounts of α-amylase from *Str. bovis* 148 could be biosynthesized in *Lb. casei* by fusing the 5'-untranslated leader sequence (UTLS) and the ribosome binding site (RBS) of the *slpA* gene from *Lb. acidophilus* with the promoter of the gene encoding LDH of *Lb. casei* (Narita et al., 2006). Maximum α-amylase activities measured in such engineered LAB are 3-6 fold higher than those shown by the native amylolytic *Str. bovis* 148 (Narita et al., 2004). The most impressive performance was reported for a *Lb. plantarum* strain secreting α-amylase from *Str. bovis* 148, which was able to ferment 86 g of raw corn starch to D-LA in 48 h with a similar yield of 0.85 g/g total sugar consumed but a higher maximum volumetric productivity of 3.86 g/L/h, compared to *Str. bovis* 148 (Narita et al., 2004; Okano et al., 2009c) (Table 1).

*Table 1

Enzymatic systems for lignocellulose hydrolysis are much more complicated than those required for starch hydrolysis. Multiple enzymes, having different substrate specificities (e.g., cellulases, xylanases and other hemicellulases) and catalytic mechanisms (i.e., exoglucanases,

endoglucanases, processive endoglucanases, and β-glucosidases), are required to co-operate synergistically for efficient lignocellulose hydrolysis (Bayer et al., 2013; Lynd et al., 2002; Wilson, 2011). Although recent studies have highlighted that strategies for cellulose hydrolysis can be highly diverse in phylogenetically distant cellulolytic microorganisms (Himmel et al., 2010; Wilson 2011), two main cellulase paradigms have been the most extensively studied so far: i) the "free"-cellulase systems (i.e., secreted cellulases that do not form stable complexes) of aerobic microorganisms, such as filamentous fungi (e.g., *Trichoderma reesei*) and actinomycetes (Chandel et al., 2012; Lynd et al., 2002); and ii) the "complexed" cellulase systems, i.e., the cellulosomes of anaerobic bacteria, such as *Clostridium spp.* and *Ruminococcus spp.*, and fungi (i.e., Chytridomycetes) which are generally bound to the cell surface (Bayer et al., 1998; Bayer et al., 2008; Fontes and Gilbert, 2010; Himmel et al., 2010; Lynd et al., 2002). Aerobic microorganisms generally secrete amounts ranging from 1 to 10 g/l up to 100 g/l (in some fungi) of "free" cellulases, while cellulosome-biosynthesizing anaerobic bacteria, such as *Clostridium thermocellum*, produce much lower amounts of cellulases (around 0.1 g/L) because of the lower energy levels in anaerobic versus aerobic bacteria and the higher specific activity of such complexes with respect to "free-enzyme" systems (You et al., 2012).

Examples of recombinant cellulolytic strategies have been applied so as to bestow the ability to grow on lignocellulose biomasses on LAB strains by heterologous cellulase expression (Mazzoli et al., 2012; Yamada et al., 2013) (Table 1). Early examples in LAB, have been mainly aimed at obtaining LAB strains able to improve silage fermentation and storage and/or silage digestibility by cattle (Bates et al., 1989; Ozkose et al., 2009; Rossi et al., 2001; Scheirlinck et al., 1989). *Lb. plantarum* is commonly used for silage fermentation so as to diminish silage pH by means of LA fermentation and improve long-term silage storage (Scheirlinck et al., 1989). However, the soluble carbohydrate concentration in silage is often very low, and since natural *Lb. plantarum* is not able to ferment glucans (either starch or cellulose), the amount of LA produced is too low to inhibit further silage fermentation by spoilage microorganisms. Heterologous endocellulase expression in *Lb. plantarum* (by gene cloning into plasmids or through integration into the genomic DNA), designed to obtain recombinant cellulolytic strains with improved silage fermentation properties, has been received with great interest (Bates et al., 1989; Rossi et al., 2001; Scheirlinck et al., 1989). The *C. thermocellum* endoglucanase Cel8A was successfully expressed in two probiotic lactobacilli (Cho et al., 2000). Later, construction of cellulolytic LAB for industrial production of LA has been considered. *C. thermocellum* Cel8A was introduced into *Lb. plantarum ΔldhL1*, thus obtaining a recombinant strain that was able to grow on cellooligosaccharides up to 5-6 glucose residues (Okano et al., 2009c; 2010a). Very recently, Morais and co-workers (2013) demonstrated that the construction

of simple consortia of recombinant *Lb. plantarum* strains expressing and secreting cellulase-xylanase mixtures could potentially be used for biomass (e.g., wheat straw) bioconversion.

Current cutting-edge strategies aimed at the development of recombinant cellulolytic microbial strains are mainly based on heterologous expression of so-called mini- or designer-cellulosomes, i.e., artificial cellulosomes, which are composed of the minimum number of components enabling them to be active on lignocellulosic substrates (Bayer et al., 1994; Bayer et al., 2007). Because of lower protein secretion ability of bacteria with respect to eukaryotic cells, complexed-cellulases, showing higher specific activity than free cellulases, seem the most promising enzymatic systems for conferring cellulolytic ability to LAB. Moreover, LAB are relatively close to cellulolytic clostridia from a phylogenetical standpoint, and their G+C content is low and similar to those of strains such as *C. cellulovorans*, *C. thermocellum* and *C. cellulolyticum*. This is essential for efficient biosynthesis of heterologous proteins, with particular emphasis on efficient translation, which is often biased by different codon usage in very distant organisms (Mazzoli et al., 2012).

Modulation of mRNA stability can be an alternative tool to optimize heterologous cellulase expression in LAB (Daguer et al., 2005; Komarova et al., 2005; Narita et al., 2006; Okano et al., 2010b). Recent studies suggest that mechanisms of protein secretion in Clostridia and LAB could be similar (Okano et al., 2010b; Mingardon et al., 2011; Wieczoreck and Martin, 2010). In fact, the products of genes encoding cellulosomal components of cellulolytic clostridia, including their original signal peptide, could be efficiently secreted by *Lb. plantarum* (Okano et al., 2010b; Mingardon et al., 2011; Morais et al., 2013). This significantly reduces problems connected with heterologous cellulase expression. In fact, as for secretion of other proteins in heterologous hosts, heterologous cellulase expression may be hampered by saturation of transmembrane transport mechanisms of the host, which causes reduction/loss of cell viability (Mazzoli et al., 2012). Such limitations are still a major bottleneck of recombinant cellulolytic strategies (Mazzoli et al., 2012). Nevertheless, genomic screening for homologous signal peptides could hold the key for fine tuning and balance between high secretion and reduction in cell viability as was nicely demonstrated by Mathiesen and co-workers (2008).

In other cases, the simple replacement of the original signal peptide with that of Usp45, the main secreted protein of *Lc. lactis*, has been used for promoting heterologous protein secretion in *Lc. Lactis* (Morello et al., 2008), e.g., components of the *C. thermocellum* scaffolding protein CipA (Wieczoreck and Martin, 2010). In addition, *Lc. lactis* HtrA mutants, which are defective in the unique exported housekeeping protease HtrA (i.e., the main protein component responsible for quality control of secreted proteins in this species), have been employed for the efficient secretion of

heterologous cellulases (Wieczoreck and Martin, 2010). The construction of LAB strains that display cell-surface-anchored designer cellulosomes or cellulosomal components has also been recently achieved. Fragments of the CipA scaffolding protein of *C. thermocellum* have been functionally displayed on the cell surface of *Lc. lactis* by fusing them with the C-terminal anchor motif of the streptococcal M6 protein, a sortase substrate (Wieczoreck and Martin, 2010; 2012). Surface-anchored complexes were thus displayed with efficiencies that could approach 10^4 complexes/cell (Wieczoreck and Martin, 2010) (Table 1). A non-covalent surface display system for LAB has also been developed by fusing a target heterologous protein, i.e., the α-amylase, with the C-terminal cA peptidoglycan-binding domain, which shows high homology with LysM repeats of the major autolysin AcmA from *Lc. lactis* (Okano et al., 2008).

Cellulolytic activities, measured in recombinant LAB constructed so far, differ widely (Table 1). It can be estimated that hydrolytic activity of native cellulosome-producing strains, such as *C. thermocellum*, on cellulosic substrates, which are commonly used for *in vitro* enzymatic tests such as β-glucan, carboxy methyl cellulose (CMC) or phosphoric acid swollen cellulose (PASC), ranges between 100 and 1000 U/L (Kraus et al., 2012; You et al., 2012). Some recombinant LAB show cellulolytic activities lower than *C. thermocellum* by 10/100 fold (Table 1). However, for other engineered strains reported values are similar to or higher than those of natural cellulosome-producing bacteria (Table 1). It is worth noting that efficient hydrolysis of more recalcitrant polysaccharides, such as crystalline cellulose, do not depend merely on the amount of cellulases but requires mixtures of different enzymatic activities showing high synergism. Cellulase expression levels shown by some recombinant LAB obtained thus far are encouraging for future development of strains ready for industrial application in biomass biorefinery. It will therefore be interesting if future engineering of LAB strains expressing viable designer cellulosomes with similar catalytic activities on recalcitrant substrates will be possible.

Efficient conversion of plant biomass, should take into account that hemicellulose, consisting of both hexose and pentose polymers, can constitute up to 35% of the plant dry biomass (Jordan et al., 2012; Lynd et al., 2002). Microbial strains which can ferment pentose sugars generally suffer from lower yield and productivity with respect to glucose, because of inefficient uptake, redox imbalance, or carbon catabolite repression (Jojima et al., 2010). A number of LAB such as *Lb. pentosus*, *Lb. brevis*, *Lb. plantarum* and *Leu. lactis* are able to metabolize both arabinose and xylose through the phosphoketolase pathway, leading to equimolar amounts of LA and acetic acid or ethanol (Tanaka et al., 2002; Okano et al., 2009a). An additional xylose fermentation pathway with higher LA production yields was discovered in *Lc. lactis* IO-1 (Tanaka et al., 2002). However, even xylose-metabolizing LAB strains such as *Lc. lactis* IO-1, *Leu. lactis* SHO-47 and *Leu. lactis* SHO-54 cannot

ferment xylan or xylooligosaccharides with degrees of polymerization higher than seven (Ohara et al., 2006). Isolation of bacteria from different sources is a promising approach to discover new LAB strains with pentose/hemicellulose metabolization properties. Alternatively, metabolic pathway engineering (e.g., expression of heterologous xylanases) is an effective tool to improve hemicellulose bioconversion by LAB as well as for cellulose degradation (Morais et al., 2013; Raha et al., 2006) (Table 1). Morais et al. (2013) demonstrated that xylanase-expressing *Lb. plantarum* improved cellulose accessibility. A *Lb. plantarum* strain was engineered so as to obtain a recombinant strain with higher conversion yields of both arabinose and xylose into D-LA (Okano et al 2009 a; b) (Table 1). The final modified *Lb. plantarum ΔldhL1-xpk1*::*tkt*/pUC-PXylAB strain could convert both arabinose and xylose into LA with yields of 0.82 and 0.89 g of produced LA per gram of pentose, respectively.

3. Increasing high-value metabolite production of LAB: fermentation and metabolic (genetic) engineering strategies

3.1. Products for environmental-friendly bulk chemicals and polymers

3.1.1. LA and LA-derived chemicals

LA, the main LAB fermentation product, is currently among the most sought-after chemicals. Apart from its traditional use as a preservative (acidifier) and flavor-enhancing agent by the food industry, LA has also been used as an emulsifying and moisturizing agent by the cosmetics industry, in the synthesis of optically pure pharmaceuticals and as an intermediate in pharmaceutical processes, and by the tanning industry (Papagianni, 2012). Another interesting application of LA is in the synthesis of ethyl lactate that is used as a biodegradable solvent (Singh and Ray, 2007; Madhavan Nampoothiri et al., 2010). Recently, worldwide demand for LA has considerably increased because of the use of LA as a building block for the synthesis of plastic polymers, i.e., polylactides (PLA) (John et al., 2007). Worldwide annual PLA production capacity is expected to be as high as 216,000 metric tons in 2015 (www.Iea-bioenergies.task42-biorefineries.com). PLA are linear aliphatic polyesters showing many interesting features, i.e., biodegradability and biocompatibility, thermoplasticity and high tensile strength, which make them highly versatile and attractive for various commodities and for medical applications (Madhavan Nampoothiri et al., 2010). For these reasons,

PLA is considered a general purpose plastic material, which is expected to replace various polymers traditionally derived by oil refinery in applications comprising: i) food and goods packaging and cutlery; ii) biomedical devices such as matrices for tissue regeneration and drug delivery systems, surgical suture thread, orthopaedic fixation (e.g., pins rods, ligaments), cardiovascular applications (e.g., stents, grafts) and devices; and iii) agriculture plastic sheetings (John et al., 2007; Okano et al., 2010a; Singh and Ray, 2007). PLA are obtained by chemical condensation of LA (Cheng et al., 2009). Since LA is a chiral molecule existing as two optical isomers (D-LA and L-LA) and the physical properties of PLA generally depend on the enantiomeric composition of LA, the availability of optically pure LA is essential for the polymerization process. In this respect, biotechnological production of LA is advantageous. Whereas chemical synthesis always results in a racemic mixture of L- and D-LA, selected, natural or engineered microbial strains can produce optically pure products (John et al., 2007). Although other LA-producing microorganisms, such as filamentous fungi, are known and other microbial models have been proposed for this purpose (Okano et al., 2010a), of the 150,000 tons of LA that are produced worldwide every year, about 90% is produced by LAB fermentation (Sauer et al., 2008).

At present, the main portion of LA for PLA synthesis is obtained by bioconversion of dedicated crops (mainly corn) by industries such as Nature works LLC (USA) and Purac (The Netherlands) (Abdel-Rahman et al., 2013). However, optimization of LA production from cheaper and more environmentally sustainable feedstocks using LAB fermentation has been extensively investigated by several research groups worldwide. This is essential so as to reduce the costs associated with the fermentative production of LA, which should be at or below $ 0.8 per kilogram of LA, in order to ensure that PLA will be competitive with fossil-fuel-based plastics (Okano et al., 2010a).

It has been pointed out that the carbon substrate is not the only nutrient that significantly affects LAB growth. In this context, yeast extract is frequently added as a source of nitrogen for LA production with a significant increase of production costs (Hofvendahl and Hahn-Hägerdal, 2000). Corn steep liquor, a by-product from the corn steeping process, represents a cheaper and successful alternative in LA production processes (Wee et al., 2006).

Among the different possible carbon substrates for LAB growth several low-cost sources of biomass have been considered, which include milk whey, molasses, starchy materials, lignocellulose hydrolysates, and wheat bran, as described in Table 2.

*Table 2

Different lactobacilli species (e.g., *Lb. helveticus*, *Lb. delbrueckii* subsp. bulgaricus, *Lb. acidophilus, Lb. casei*) have been employed in LA production from whey (Table 2). Among them, *Lb. helveticus* is the generally preferred organism. This microorganism is a homolactic LAB that produces a D-/L-LA racemic mixture (Roy et al., 1986). Temperature and pH are the key environmental parameters that affect the LA production process. *Lb. helveticus* showed enhanced lactose utilization and LA production at 42°C and pH 5.8 (Tango and Ghaly, 1999). The highest LA production rate was obtained with *Lb. helveticus* grown in whey permeate, with corn steep liquor as the nitrogen source (Amrane and Prigent, 1998). LA productivity of 9.7 g/L/h using *Lb. helveticus* strain milano has been obtained in continuous fermentation of whey-yeast extract permeate medium (Roy et al., 1986; 1987). Because LAB do not have sufficient proteolytic activities to utilize milk proteins in whey, they frequently require supplementation of yeast extract to the medium (Abdel-Rahman et al., 2013). In the work of Vasala et al. (2005) on *Lb. salivarum*, yeast extract supplementation was replaced by *in situ* treatment of the fermentation medium with proteolytic microorganisms. LAB have been immobilized by several methods on different supports (e.g., calcium alginate, κ-carrageenan, agar and polyacrylamide gels) (Panesar et al., 2007a; b), and the immobilized systems have been investigated for LA production from whey. A two-stage process was used for continuous fermentation of whey permeate medium with *Lb. helveticus* immobilized in κ-carrageenan/locust bean gum, which resulted in high LA productivity (i.e., 19–22 g/L/h) (Schepers et al., 2006). In a study by Panesar et al. (2007b) *Lb. casei* was immobilized in Ca pectate gel. A higher level of lactose conversion to LA (32.95 g/L) was achieved (94.37%), and the cell system was found highly stable: no decrease in lactose conversion to LA was observed up to 16 batches.

Lb. delbrueckii and *Ent. faecalis* have been used for LA production from molasses (Monteagudo et al., 1997; Göksungur and Güvenç, 1999; Kotzanmanidis et al., 2002; Wee et al., 2004) (Table 2). Monteagudo et al. (1997) studied the kinetics of LA fermentation by *Lb. delbrueckii* grown on beet molasses, whereas production yield of LA from beet molasses by free and immobilized *Lb. delbrueckii* cells has been described in a comparative study by Göksungur and Guvenc (1999). Optimization of LA production on a modified formulation of carbon and nitrogen substrates (using different sugar, yeast extract and calcium carbonate concentrations) from beet molasses by *Lb. delbrueckii* was carried out by Kotzanmanidis et al. (2002). Most of the investigations concerning LA fermentation from molasses were performed using the genus *Lactobacillus*; however, Wee and co-

worker (2004) reported a high yield and productivity of LA from molasses without pretreatment using a strain of *Ent. faecalis*. Continuous production of LA from molasses by perfusion culture of *Lc. lactis* was described by Ohashi et al. (1999).

Many researchers have attempted direct LA production from starchy materials and wild amylolytic LAB have been isolated in different environments (Giraud et al., 1994; Guyot et al., 2000; Narita et al., 2004). Amylolytic LAB utilize starchy biomass and convert it into LA in a single step fermentation (Reddy et al., 2008). *Str. bovis* 148, a common rumen bacterium (Mizrahi, 2013), was found to produce L-LA from raw corn starch with a high yield of 0.88 g/g and a relatively high optical purity of 95.6% (Narita et al., 2004). *Lb. amylophilus* GV6 was found to actively ferment various pure and crude starchy substrates with more than 90% LA yield (Altaf et al., 2005; Vishnu et al., 2000; 2002). *Lb. plantarum A6* showed both a good degree of starch utilization and good yields of LA production in a complex medium composed of free sugars (brown juice) and starch (Thomsen et al., 2007).

An isolated strain of *Lb. brevis* (S3F4) showed LA production capability from lignocellulosic hydrolysates. S3F4 was able to simultaneously utilize xylose and glucose without catabolic repression (Guo et al., 2010). *Lactobacillus* sp. RKY2 was employed in continuous LA fermentations with cell recycling, using lignocellulosic hydrolyzates and corn steep liquor as inexpensive raw materials. The results of this study have indicated that the cell-recycling cultivation method can improve volumetric productivity (Wee and Ryu, 2009). However, a few LAB, such as *Lb. pentosus* (Bustos et al., 2005), *Lb. brevis* (Chaillou et al., 1998), and *Leu. lactis* (Ohara et al., 2006), are known to ferment xylose, producing both LA and acetic acid. *Lc. lactis* utilizes heterofermentative metabolism when metabolizing pentoses, with production of an equimolar mixture of LA and acetate (Doran-Peterson et al., 2008). In the work of Laopaiboon et al. (2010) xylose was obtained as the main fermentable sugar (89%) from hydrolyzate of sugarcane bagasses. The detoxified hydrolysate, supplemented with yeast extract, was found to be a potential substrate for LA production by *Lc. lactis* IO-1. The bioconversion of hemicellulosic sugars (xylose, glucose, and arabinose) from different agro-industrial wastes into LA by *Lb. pentosus* was reported in the work of Moldes et al. (2006). Pure L-LA was produced from sugarcane bagasse cellulose by simultaneous saccharification and fermentation (SSF) in the presence of a cellobiose-utilizing *Lb. delbrueckii* mutant Uc-3 that utilizes both cellobiose and cellotriose efficiently (Adsul et al., 2007a; b). The performance of recycled paper sludge as feedstock for LA production with *Lb. rhamnosus* ATCC 7469 was evaluated by Marques et al (2008): maximum production of LA from this feedstock was obtained by performing the enzymatic hydrolysis and fermentation steps simultaneously.

Plackett–Burman design was employed for screening 15 parameters for production of L(+)-LA from wheat bran by *Lb. amylophilus* GV6 in solid state fermentation (Naveena et al., 2005b). Wheat bran was utilized as both support and substrate in a single-step conversion of raw starch to L(+)-LA (Naveena et al., 2005b). Barley, wheat, and corn were hydrolyzed by commercial amylolytic enzymes and fermented to LA by *Ent. faecalis* RKY1 without additional nutrients. LA productivities of 0.8 g/L/h were obtained from barley and wheat (Oh et al., 2005). In the work of John et al. (2006) protease-treated wheat bran was used for the production of L(+)-LA using a mixed culture of *Lb. casei* and *Lb. delbrueckii.* In L-LA production by *Lb. rhamnosus* LA-04-1, wheat bran hydrolysate combined with corn steep liquor showed a better performance than that without treatment, especially for L-LA yield (0.99 g/g) (Li et al., 2010b).

Regarding the optical purity of the LA product, several examples designed to optimize enantioselective biosynthesis can be cited. L-LA is the most used isomer in both food and pharmaceutical industries, and hence its production by fermentation is a well-established process (Yu and Hang, 1989). Efficient D-LA production with high optical purity (97.2 - 98.3%) from sugarcane molasses by *Lb. delbrueckii* JCM 1148 was reported in the work of Calabia and Tokiwa (2007). A strain of *Lb. lactis* obtained by UV mutagenesis was also employed for the production of D-LA from molasses and hydrolyzed cane sugar (Joshi et al., 2010). Plackett–Burman design and response surface methods were applied for optimization of D(−)-LA production by *Leu. mesenteroides* B512 (Coelho et al., 2011). The production of homo-D-LA from xylose was achieved by using a recombinant strain of *Lb. plantarum* NCIMB 8826 deficient for its L-LDH and whose phosphoketolase gene was replaced by a heterologous transketolase gene (Okano et al., 2009b). Using the same recombinant *Lactobacillus* strain, production of optically pure D-LA from arabinose was achieved (Okano et al., 2009a).

3.1.2. Polyhydroxyalkanoates

Polyhydrohyalkanoates (PHAs) are natural, biodegradable, linear polyesters which are produced as intracellular carbon and energy storage molecules by a great number of bacteria (both Gram-positive – including LAB – and Gram-negative species), but also by some yeasts and plants (Lu et al., 2013). PHAs are particularly versatile biopolymers, since they can consist of different monomeric units (e.g., 3-hydroxybutyrate, 3-hydroxyvalerate, 4-hydroxybutyrate), with properties similar to conventional plastics. PHA applications range from the production of films and containers to biomedical applications, such as in wound management or as cardiovascular devices (e.g.,

pericardial and atrial septal repair patches, scaffolds for regeneration of arterial tissues, vascular grafts, cardiovascular stents and heart valves) and orthopaedic devices (cartilage tissue engineering, bone graft substitutes, etc.), and for drug delivery (e.g., tablets, implants, micro-carriers) (Lu et al., 2013).

Poly-β-hydroxybutyrate (PHB) production was described in LAB belonging to *Lactococcus, Lactobacillus, Pediococcus* and *Streptococcus* genera. Higher yields of PHB were obtained in *Lactobacillus* species reaching 35.8% of cell dry weight. The values for *Lactococcus*, *Pediococcus* and *Streptococcus* species were 20.9%, 8.0% and 17.2%, respectively. The PHB yields obtained with LAB in deMan, Rogosa and Sharpe (MRS) broth were generally lower than the values reported in soil bacteria, e.g., *Alcaligenes* and *Azotobacter* species, in which values higher than 55% have been reported (Aslim et al., 1998).

LA-producing bacteria such as *Lb. lactis* (Tanaka et al., 1995), *Propionibacterium* (Tohyama et al., 2002), *Lb. delbrueckii* (Tohyama et al., 1999; 2000, Patnaik, 2005) and *Cupriavidus necator* have also been used in a co-culture fermentation system. LAB converted sugars into LA which was later taken up *by C. necator* to produce PHAs. In a two-stage system, xylose was converted to LA using *Lc. lactis*, the LA was further converted to 3-hydroxybutyrate by *C. necator* (Tanaka et al., 1995). In another investigation, *Lb. delbrueckii* was used to convert glucose to LA which was later converted to 3-hydroxybutyrate by *C. necator* (Tohyama et al., 1999; 2000). By application of neural optimization, the PHB yield of a co-culture of *Ralstonia eutropha* and *Lb. delbrueckii* could be increased by 19.4% compared with the single cultivation of *R. eutropha* (Patnaik, 2009).

Generally, it has been demonstrated that co-culture fermentations resulted in increased yield with improved control of product qualities. A further advantage in the application of co-cultures is the possibility of utilizing secondary products (e.g., whey, molasses), which are cheaper than glucose as substrates for production of PHAs (Bader et al., 2010). Bacteria that have GRAS status for PHA-production, such as LAB and bacilli belonging to probiotic species (Aslim et al., 1998; Yilmaz et al., 2005), might constitute an added value to these biotechnological processes (Defoirdt et al., 2009).

3.1.3. Biofuels: ethanol, butanol and hydrogen

The demand for renewable energy technologies has initially focused on ethanol – a fermentation product produced by a wide variety of microorganisms – to serve as a potential renewable biofuel. There are many ongoing efforts devoted towards the use of engineered and native microorganisms for use as industrial producers of ethanol (Balusu et al., 2004; Chen et al., 2009;

Jarboe et al., 2007; Ng et al., 1981; Olofsson et al., 2008). LAB have emerged as promising candidates for alcohol (i.e., ethanol and butanol) production by industrial fermentation, which can be competitive alternatives to other types of engineered microbial systems (e.g., *C. thermocellum*, *Saccharomyces cerevisiae*, *Zymomonas mobilis* or *Escherichia coli*) (Morais et al., 2013).

One of the major problems in using bacteria for such endeavors is their low ethanol tolerance. In this context, some LAB species are particularly attractive candidates to serve as ethanol-producing bacteria for the biofuel industry, since they are relatively tolerant to high ethanol concentrations required for commercial alcohol production processes, used in the food industry and for biofuel production (Lucena et al., 2010; Passoth et al., 2007). Notably, Lactobacilli include some of the most ethanol-, butanol- and generally alcohol-tolerant organisms known (Alegria et al., 2004; Knoshaug and Zhang, 2009; Nicolaou et al., 2010). Alegria et al. (2004) demonstrated that *Lb. plantarum* can grow at pH 3.2 with up to 13% ethanol. Interestingly, *Lactobacillus* species are predominant in contaminated ethanol fermentations (Limayem et al., 2011; Roach et al., 2013). Such is the case for *Lb. vini* and *Lb. fermentum*, which were found in a recent study (Lucena et al., 2010) to grow in distilleries used in bioethanol processes in Brazil. In the latter study, the authors reported that a variety of *Lactobacillus* species appeared during the process of bioethanol production, thus competing with the yeast. Towards the end of the harvest season, however, the most frequently found bacterial species were *Lb. fermentum* and *Lb. vini*. Representative isolates of both species had the ability to grow in medium containing up to 10% ethanol. These characteristics, which allow such species to contaminate the yeast cultures in the bioreactors, may thus be used to advantage as an infrastructure for engineering ethanol-producing bacteria.

High tolerance of *Oenococcus oeni* strains was also reported, where these strains retained viability in media of up to 13% ethanol (Alegria et al., 2004).

As mentioned above, ethanol is produced as a product of LAB heterofermentation. Some obligate and facultative LAB heterofermenters, such as *Oenococcus oeni* and *Lb. pentosus*, are capable of fermenting both hexose and pentose sugars to ethanol. In facultative LAB heterofermentors the switch between homofermentation, in which only LA is produced, and heterofermentation, in which a variety of products can be produced, is attributed to the catabolic state of the bacterium, where limiting catabolism such as low glycolytic flux leads to heterofermentation and non-limiting catabolism with high glycolytic flux leads to homofermentative fermentation (Zaunmüller et al., 2006). This phenomenon is ascribed to the regulation of the enzymatic activities of LDH and pyruvate formate lyase, which are subject to control by the catabolic and anabolic flux rates and changes in the $NADH/NAD^+$ ratios (Melchiorsen et al., 2002). Ethanol production is mainly thought to occur when hexose sugars are fermented, due to the reduction of acetyl-CoA to ethanol by

two extra NADPH molecules that are produced. When pentose sugars are fermented, these two NADPH molecules are not produced, thereby resulting in accumulation of LA and acetate. Nevertheless, ethanol production was observed when *Lb. plantarum* – a facultative heterofermenter – was grown solely on pentose sugars (Domagk and Horecker, 1958). The ethanol-producing enzymes of the phosphoketolase pathway exhibit slower kinetics than the hexose-fermenting enzymes; therefore when hexoses are the only carbon source this becomes the rate-limiting step of fermentation and bacterial growth in general (Richter et al., 2001).

The low tendency of LAB towards ethanol fermentation can be addressed by metabolic engineering. This approach has been used successfully in other bacteria, such as *Thermoanaerobacterium saccharolyticum,* an anaerobic bacterium that is able to ferment xylan and biomass-derived sugars. High yields of ethanol production as the only detectable organic product was achieved in this bacterium by knockout of genes involved in organic acid formation (Shaw et al., 2008).

Several studies have attempted to improve ethanol production in LAB by over-expression of heterologous genes encoding pyruvate decarboxylase (*pdc*) and/or alcohol dehydrogenase (*adh*). Gold et al. (1996) reported the expression of the *pdc* and *adh* genes from *Zymomonas mobilis* in *Lb. casei 686*. In the latter study, the recombinant strain produced more than twice the ethanol produced by the parental strain (Gold et al., 1996). In a later study, in which the same operon was expressed in several *Lactobacillus* strains such as *Lb. casei* as well as in other species, it was reported that LA was the primary fermentation product formed by all of the strains, indicating that activities of ADH and PDC were insufficient to divert significant carbon flow towards ethanol. Interestingly, the *Lb. casei* transformant in this study did not exhibit increased ethanol production activity (Nichols et al., 2003). A slightly different approach was attempted by Liu et al. (2006). In the latter study, the authors attempted to increase the production of ethanol by introducing the PDC gene from the Gram-positive bacterium *Sarcina ventriculi* into an LDH-deficient strain, *Lb. plantarum* TF103. The authors speculated that by substituting LDH with PDC activity, pyruvate may be re-directed toward ethanol production instead of LA fermentation. Nevertheless, although slightly more ethanol was observed, carbon flow was not significantly improved toward ethanol, suggesting that additional understanding of this organism's metabolism is necessary for effective strain improvement (Liu et al., 2006). Recently Solem and co-workers (2013) were able to redirect *Lc. lactis* carbon flow toward ethanol production, obtaining a strain with ethanol as the sole fermentation product. This was achieved by a knockout strategy of all LDH genes in this bacterium consisting of *ldhX, ldhB,* and *ldh* together with those coding for phosphotransacetylase (*pta*) and the native ADH (*adhE*). In parallel, the authors introduced codon-optimized *Z. mobilis adh* and *pdc* genes.

Another approach for increasing ethanol production could be the selection of and enrichment for more ethanologenic LAB as was reported in a recent study in which a *Lb. pentosus* strain was isolated through a series of selection and enrichment procedures (Kim et al., 2010). This strain, designated *Lb. pentosus* JH5XP5, was able to produce ethanol without acetate. The production yields of ethanol vs LA in this strain were 2.0- to 2.5-fold higher when either glucose, galactose or maltose was used either as a single carbon source or simultaneously with glucose (Kim et al., 2010).

Advantages of LAB over the yeast *S. cerevisiae* in ethanol production from lignocellulosic biomass include the ability of several LAB strains, e.g., *Lb. plantarum*, to metabolize both hexose and pentose sugars (Kleerebezem et al., 2003; Gänzle et al., 2007; Okano et al., 2009a; b). Furthermore, the production of acid together with LAB acid tolerance reduces the risk of contamination by other bacteria and fungi and may enable degradation of substrates directly after acid pretreatments that are commonly used for lignin deconstruction in plant biomass (Morais et al., 2013). Nevertheless, ethanologenic LAB strains, which would be sufficient for the bioethanol industry, are as yet nonexistent. In the future, the combination of the above approaches may eventually be an effective solution for designing an efficient ethanologenic LAB strain.

As far as butanol production is concerned, it is noteworthy that all natural butanol producers belong to the genus *Clostridium* (Mazzoli, 2012). The highest amounts of butanol are synthesized by *C. acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* (Mazzoli, 2012). Nonetheless, the expression of the clostridial butanol biosynthetic pathway in heterologous hosts, such as in *E. coli*, *S. cerevisiae*, and *Lb. brevis*, has been extensively explored as a means to engineer recombinant butanol-producing strains (Mazzoli, 2012). Transformation of *crt*, *bcd*, *etfB*, *etfA*, and *bcd* from *C. acetobutylicum* into *Lb. brevis*, in concert with autologous thiolase, aldehyde and alcohol dehydrogenase enabled the recombinant strain to produce butanol (Berezina et al., 2010). The final butanol titer by this recombinant *Lb. brevis* was very low (i.e., 300 mg/L), compared to natural or engineered clostridia (i.e., titers up to 19 g/L were reported for strains optimized by random mutagenesis or rational metabolic engineering) (Nicolaou et al., 2010; Tomas et al., 2003). However, recent studies have demonstrated that highly efficient butanol production can be obtained in engineered heterologous hosts (e.g., 15 g/L in recombinant *E. coli*) by the construction of chimeric biosynthetic pathways (Shen et al., 2011). Metabolic engineering has also served towards the development of a *Clostridium cellulolyticum* strain for isobutanol synthesis directly from cellulose (Higashide et al., 2011).

Formate is another LAB metabolite with high potential as a precursor of biofuel, i.e., molecular hydrogen (H_2) (Oh et al., 2011). LAB produce formate during mixed-acid fermentation in anaerobic conditions through pyruvate conversion by pyruvate formate lyase (Fig. 1). Industrial

production of H_2 could be envisaged by coupling such LAB fermentation with a second anaerobic fermentation employing either enteric bacteria such as *E. coli* which are equipped with formate-hydrogen lyase (Oh et al., 2011) or photofermenting bacteria (e.g., purple non-sulfur bacteria such as *Rhodobacter sphaeroides*) (Keskin et al., 2011) which are able to oxidize formate with concomitant H_2 evolution.

3.2. Products for food application and human health promotion

3.2.1. Food aromas and flavors

Production of food aromas such as diacetyl and acetaldehyde by LAB has been extensively reviewed in 2012 by Papagianni and will be only briefly summarized here.

Diacetyl is naturally produced by LAB and is responsible for the typical butter aroma of several dairy products such as butter, buttermilk and a number of cheeses (Papagianni, 2012). *Lc. lactis* biovar. diacetylactis has been extensively employed to produce diacetyl from citrate in co-fermentation with lactose (Papagianni, 2012). Diacetyl is generated by oxidative decarboxylation of the intermediate product of the fermentation α-acetolactate. Because of its value as an aroma compound, efficient production of diacetyl from lactose rather than citrate has been the aim of several metabolic engineering strategies (Fig. 2) (Papagianni, 2012). Under aerobic conditions pyruvate metabolism in LAB strains such as *Lc. lactis* is strongly shifted towards acetate and α-acetolactate biosynthesis (Guo et al., 2012). Pyruvate conversion to α-acetolactate can be catalyzed by either α-acetolactate synthase (ALS) or acetohydroxy acid synthase (ILVBN) (Fig. 2). ILVBN is an anabolic synthase involved in branched chain amino acid synthesis. α-Acetolactate can then be decarboxylated to acetoin or decarboxylated and oxidized to diacetyl (Guo et al., 2012). Furthermore, aerobic conditions also strongly increase NADH-oxidase activity (NOX) which is thought to replace the role of LDH in the re-oxidation of NADH which is generated by glycolysis (Guo et al., 2012). Several attemps to increase diacetyl production through metabolic engineering by genetic manipulation techniques have been performed so far in which i) LDH was inactivated and/or; ii) either ALS or ILVBN has been overexpressed and used, and/or; iii) α-acetolactate decarboxylase (ALDB), i.e., the enzyme which catalyzes the conversion of α-acetolactate to acetoin, was inactivated (Guo et al., 2012; Papagianni, 2012). However, these approaches have been relatively unsuccessful in significantly increasing diacetyl production. In fact, Hoefnagel and co-workers (2002) demonstrated that the enzymes with the greatest effect on the flux to diacetyl reside outside the ALS branch itself, i.e., LDH

and NOX. Recently, Guo and co-workers (2012) have been able to increase NOX activity by 58-fold in *Lc. lactis* by using selected strong promoters for the constitutive expression of the NADH oxidase gene. Such engineered strains showed an altered $NADH/NAD^+$ ratio which led to re-routing of pyruvate flux from LA to diacetyl whose final titer increased from approximately 1.07 mM to 4.16 mM.

***Fig. 2**

Acetaldehyde, an important aroma compound in dairy products, can be produced by LAB through at least two pathways: i) pyruvate decarboxylation by PDC; ii) threonine conversion (i.e., giving rise to acetaldehyde and glycine) catalyzed by threonine aldolase (Papagianni, 2012). Actually, the latter reaction seems to play the main role in acetaldehyde biosynthesis in LAB (Papagianni, 2012). Successful paradigms of an engineered strain with increased acetaldehyde production have been performed by improvement of either threonine aldolase or PDC activity (Papagianni, 2012).

3.2.2. Polyols and other sweeteners

Several successes were obtained in the field of production of low-calory sweeteners, such as polyols (i.e., mannitol and sorbitol) and alanine, by LAB fermentation (Fig. 3).

Both mannitol and sorbitol are six-carbon sugar alcohols which are traditionally produced by catalytic hydrogenation from glucose or glucose/fructose mixtures and are used in the food and pharmaceutical industries, as well as in medicine (Papagianni, 2012). Using this process, mixtures of mannitol and sorbitol are often produced, which are then relatively difficult to separate, thus adding supplemental cost to this mode of production (Papagianni, 2012).

Mannitol production without co-formation of sorbitol by heterofermentative LAB is well known (Hugenholtz et al., 2011). These LAB divert a part of fructose-6-phosphate (fructose-6P) from the glycolytic flux to mannitol by using mannitol-1-phosphate dehydrogenase (Wisselink et al., 2002) (Fig. 3). Mannitol production is increased in this reaction if fructose is co-fermented with glucose (Wisselink et al., 2002). Increased mannitol yields have been achieved by optimizing the mannitol fermentation of heterofermentative LAB (Racine and Saha, 2007). Although fructose to mannitol conversion yields of up to 66% were obtained by natural heterofermentative LAB, nonetheless co-production of other metabolites (e.g., LA and acetic acid) occurs in these strains. Rational metabolic engineering (Aarnikunnas et al., 2003) or random mutagenesis (Helando et al., 2005) approaches have been used to reduce the amounts of such co-products and improve mannitol production yield. Homofermentative LAB usually produce but very low amounts of mannitol (Papagianni, 2012).

However, several strategies have been reported for enhancing mannitol production in homofermentative strains such as *Lc. lactis* and *Lb. plantarum* (Wisselink et al., 2002; 2005). Construction of L-LDH-deficient *Lc. lactis* strains, together with the inactivation of *mtlA* and/or *mtlF* mannitol transport systems and overexpression of the mannitol-1-phosphate dehydrogenase gene (*mtlD*) of *Lb. plantarum* and the mannitol-1-phosphate phosphatase (Mtl1Pase) gene of the protozoan parasite *Eimeria tenella*, led to improved glucose conversion to mannitol with yields (50%) close to the theoretical maximum (67%) (Wisselink et al., 2002). By a similar strategy *Lb. plantarum* strains showing up to 50% carbon flux re-routing toward mannitol were obtained (Wisselink et al., 2005).

Only a few organisms have been described as able to naturally produce sorbitol, e.g., the Gram-negative bacterium *Zymomonas mobilis* (Silveira and Jonas, 2002). In LAB, high sorbitol production through metabolic engineering has been reported with *Lb. plantarum*. A strategy including inactivation of both LDH and mannitol-1-phosphate dehydrogenase in a *Lb. plantarum* strain overproducing a sorbitol-6-phosphate dehydrogenase led to efficient re-routing of fructose-6P towards sorbitol with a near-to-theoretical yield of 0.65 mol/mol (Ladero et al., 2007) (Fig. 3).

LAB are not reported to produce xylitol naturally although strains of *Str. avium* and *Lb. casei* are able to metabolize it (London, 1990). Nyyssölä et al. (2005) constructed a recombinant *Lc. lactis* strain in which the xylose reductase (XR) gene from *Pichia stipites* and a xylose transporter from *Lb. brevis* were expressed. This co-expression however did not improve xylitol production. Nevertheless, an increased productivity level, comparable to that of the more efficient yeast producers, was achieved in fed-batch fermentation by using non-growing *Lc. lactis* cells (Papagianni, 2012).

L-Alanine is used as a food sweetener and in pharmaceutical applications (Papagianni, 2012). Conversion of pyruvate into alanine occurs in various anaerobic bacteria and involves a single enzymatic reaction catalyzed by alanine dehydrogenase. Homo-L-alanine from sugar fermentation was obtained with an engineered *Lc. lactis* (Hols et al., 1999). Metabolism shift from homolactic to homo-alanine in this strain was obtained by functional replacement of autologous L-LDH with alanine dehydrogenase from *Bacillus sphaericus* and growth in the presence of excess of ammonium (which is required for the conversion of pyruvate to alanine by alanine dehydrogenase) (Fig. 3). Under these conditions, pyruvate obtained through glycolysis was completely converted to alanine, with NADH consumption, thus maintaining the glycolytic redox balance. Furthermore, the inactivation of the alanine racemase gene led to complete conversion of glucose into L-alanine.

***Fig. 3**

3.2.3. Exopolysaccharides

LAB are able to synthesize a large spectrum of structural polysaccharides that are either integral constituents of their cell wall, e.g., peptidoglycan and lipoteichoic acids, or linked to the cell wall *via* covalent, as in the case of capsular polysaccharides, or non-covalent bonds, or released into the extracellular environment (i.e., exopolysaccharides, EPS) (Chapot-Chartier et al., 2011). Certain EPS can be loosely associated with the cell wall (Chapot-Chartier et al., 2011). The exact role of EPS in LAB physiology is not clear and is probably multiple and complex for different EPS and strains and likely includes: i) protection against dessication and osmotic stress, phage attack, toxic compounds (e.g., ethanol, sulphur dioxide and toxic metal ions), antibiotics and host immune system (especially phagocytosis); ii) adhesion to solid surfaces (e.g., adhesion to eukaryotic cells) and biofilm formation (Chapot-Chartier et al., 2011).

LAB EPS have been subjected to continuous investigation because of their enormous potential application. This includes i) for improving the rheological properties, smoothness, creaminess, mouth feel, texture, stability (thus replacing other food stabilizers such as pectin, starch, alginate, or gelatin) and water retention capacity of dairy products (e.g., yogurt, cheese, sour cream, ice cream) and notably in the case of low-fat versions and other food (e.g., bakery) products; ii) for clinical (e.g., in microsurgery), pharmaceutical, and other biotechnological use (e.g., for the manufacture of chromatographic media); iii) for their properties as prebiotics; iv) for other health-promoting effects such as possible anti-tumor, anti-ulcer, immunomodulating, or cholesterol-lowering activities (Chapot-Chartier et al., 2011).

Nonetheless, natural production of EPS by LAB is very low compared to that of other food-grade EPS (e.g., xanthan, gurdlan) produced by non-dairy bacteria. Metabolic engineering has therefore been used for improving EPS production by LAB and/or for structural engineering of EPS produced by LAB (Papagianni, 2012).

EPS are classified in homopolysaccharides (homo-EPS) and heteropolysaccharides (hetero-EPS). Homo- and hetero-EPS differ in their composition, biosynthesis, yields, molecular organization, rheological properties, and applications (Fig. 4).

***Fig. 4**

Homo-EPS are composed of either D-glucose (glucans) or D-fructose (fructans) units, and differ regarding the type of glycosidic linkages, type and degree of branching, length of the polysaccharide chains, and conformation. Homo-EPS are biosynthesized extracellularly by only one transglycosylase or glycansucrase by using sucrose as the substrate (Fig. 4A). These enzymes hydrolyze the glycolytic bond in sucrose and use either the glycosyl or the fructosyl moiety for the polymerization of α-D-glucans or β-D-fructans, respectively (Chapot-Chartier et al., 2011). These features render homo-EPS biosynthesis independent of central carbohydrate catabolism. Apart from glucans (e.g., alternan, dextran, mutan, reuteran) and fructans (e.g., inulin and levans), glycansucrases can also biosynthesize low-molecular mass oligosaccharides such as fructooligosaccharides (FOS) and glucooligosaccharides (GOS). FOS and GOS have prominent commercial importance as prebiotics, i.e., compounds supporting growth of probiotic organisms.

Hetero-EPS are biosynthesized by the polymerization of oligosaccharidic, ranging from di- to octasaccharide, repeating units. Hetero-EPS repeating units are biosynthesized intracellularly and then exported and polymerized in the extracelullar environment (Fig. 4B). Glucose, galactose, xylose, mannose, arabinose and rhamnose are the most represented constituent monosaccharides, but amino-sugars and polyols can also be occasionally present as well as glucuronic acid. A huge diversity of hetero-EPS is produced by LAB with respect to monosaccharide composition and ratio, branching type and degree, molecular structure and mass, conformation and rigidity. The biosynthesis of hetero-EPS is a process that demands high-energy consumption and consists of four reactions: 1) sugar uptake; 2) synthesis of high-energy sugar-nucleotide precursors (e.g., UDP-glucose, UDP-galactose); 3) glycosyltransferase-catalyzed biosynthesis of the oligosaccharide repeating unit; 4) oligosaccharide export and extracellular polymerization of the EPS (Fig. 4B). The biosynthesis of UDP-glucose and dTDP-glucose generally uses glucose-6-phosphate as the substrate, which is then diverted from glycolysis. As an alternative, the conversion of galactose to UDP-Gal and UDP-Glu can be obtained through the Leloir pathway (Welman and Maddox, 2003) (Figs 1, 4B).

Because of their very different biosynthetic pathways, homo- and hetero-EPS biosynthetic yields generally differ greatly, with homo-EPS being produced in much higher amounts than hetero-EPS (Chapot-Chartier et al., 2011). As more information about EPS biosynthetic enzyme-encoding genes and their regulation becomes available, the possibility of recombinant production of either natural or “designer” hetero-EPS at high yields becomes possible (Welman and Maddox, 2003). Different strategies for enhanced EPS production in LAB have been employed or suggested, including reduction of LDH activity, and overexpression of genes encoding the enzymes that catalyze conversion of glucose-6-phosphate to sugar nucleotide precursors or specific glycosyl transferases

(Welman and Maddox, 2003). Overexpression of GalU, catalyzing the synthesis of the EPS precursor UDP-glucose from glucose-1-phosphate, under the control of a nisin-inducible promoter, increased the specific activity of the enzyme by 20-fold in *Lc. Lactis,* which in turn increased both UDP-glucose and UDP-galactose synthesis by 8-fold, although EPS synthesis was not significantly enhanced (Boels et al., 2001a). However, overexpression of both GalU and phosphoglucomutase, which catalyzes glucose-6-phosphate isomerization to glucose-1-phosphate in *Str. thermophilus,* led to a 2-fold increase in EPS synthesis (Levander et al., 2002). Yet, to date, all these metabolic engineering strategies resulted only in modest increase in EPS production (Hugenholtz et al., 2011). In fact, an inherent limitation in high-yield hetero-EPS biosynthesis is that it involves high energy-demanding pathways (Welman and Maddox, 2003). It has been calculated that at least two glucose molecules should be catabolized through glycolysis to obtain enough energy for the incorporation of one glucose molecule in EPS and that EPS yield cannot be higher than 33% of a given substrate (Welman and Maddox, 2003). Therefore, strategies for improving hetero-EPS production should also comprise energy-saving re-arrangements of LAB metabolism.

A different approach aims at structural engineering of EPS produced by LAB. This can be achieved either by controlling the culture conditions (e.g., the type of sugar source) or by genetic engineering strategies (e.g., by introducing new heterologous, or engineered glycosyltransferases into LAB) (Boels et al., 2001b; Welman and Maddox, 2003).

3.2.4. Nutraceuticals: bio-active amines, conjugated linoleic acids, seleno-metabolites and vitamins

LAB biosynthesize a large spectrum of molecules with recognized health-promoting properties such as γ-aminobutyrric acid (GABA), β-phenylethylamine, bioactive peptides, short chain fatty acids, conjugated linoleic acids, selenometabolites, and vitamins (Pessione 2012; 2014). The *in situ* and *ex-situ* production of these molecules by LAB is currently used or has been proposed for the manufacturing of “functional foods” or “nutraceuticals”, i.e., foods that provide the consumer with an “added benefit” over and above the nutrient content, and possibly reduce the risk of specific chronic diseases (Mazzoli, 2014). Although at present the use of probiotic LAB is preferable to supplying the purified molecules as nutraceutical food supplements, the question remains with respect to vitamins and amino acid derivatives.

Bioactive amines, such as GABA, β-phenylethylamine, and tryptamine, are produced by LAB as well as by eukaryotes (including humans) by decarboxylation of their precursor amino acid (i.e.,

glutamate, phenylanaline, tryptophan, respectively). Amino acid decarboxylation is a general strategy used by LAB and other bacteria to supply cells with additional metabolic energy, through functional coupling with an electrogenic amino acid/amine antiport system which generates a proton gradient across the cytoplasm membrane (Konings, 2006; Mazzoli et al., 2010). Furthermore, it is a defense mechanism against environmental acidity, since amino acid decarboxylation produces a compound which is less acidic than the substrate (Mazzoli et al., 2010).

While some amino acid decarboxylation products, e.g., histamine, tyramine, putrescine and cadaverine, are considered spoilage molecules that can be found in fermented food and have negative effects on human health (e.g., headaches, smooth muscle contraction, hypertension, brain hemorrage, allergies, and enteric histaminosis) (Pessione et al., 2005; 2009), GABA and β-phenylethylamine have desirable properties (Mazzoli et al., 2010). Several studies have reported that β-phenylethylamine is a mood elevator (Mazzoli, 2014).

GABA, together with its antagonist, i.e., glutamate, is the major neurotransmitter of the central nervous system of vertebrates. It also acts as a blood pressure modulator in mild hypertensive patients having diuretic and tranquillizer effects (Li and Cao, 2010; Mazzoli et al., 2010). Furthermore, GABA plays a regulatory and trophic role on the pancreas and in immunological processes, such as the down-regulation of pro-inflammatory cytokine release (Mazzoli, 2014).

A number of studies have investigated factors that promote glutamate decarboxylase biosynthesis or catalysis, leading to GABA accumulation in the fermentation medium. In *Lc. lactis* NCDO 2128, GABA is biosynthesized only by cultures in stationary phase and in acidic media (pH lower than 5.7), while the presence or absence of a high glutamate concentration did not have a significant effect (Mazzoli et al., 2010). Actually, in this strain, catalytic activation of glutamate decarboxylase by glutamate seems more important than its biosynthetic regulation.

Recently, simple and effective fermentation methods have been developed for several LAB strains, including *Lb. brevis* NCL912, *Lb. brevis* GABA100, *Lb. buchneri*, and *Ent. avium* G-15, in order to produce high amounts of GABA (Cho et al., 2007; Kim et al., 2009; Li et al., 2010a; Park and Oh, 2007; Siragusa et al., 2007; Tamura et al., 2010). All these strategies rely on exogenous addition of high amounts of glutamate, which is not economically viable at the industrial scale. The future in this research area is therefore either i) the development of co-cultures of GABA-producing strains and glutamate-producing microbes (e.g., *Corynebacterium glutamicum*) or ii) the engineering of strains which can achieve high-level GABA production directly from glucose (Adkins et al., 2012).

Possible health benefits can be ascribed to some isomeric forms of linoleic acid, currently called conjugated linoleic acids (CLA). These molecules, used as dietary supplements, are generally produced by isomerization of linoleic acid by chemical processes (Ogawa et al., 2005). Nevertheless,

this process often results in the by-production of undesired isomers having poor biological activity. Conversely, biological CLA production is more isomer-selective and it is possible to control the isomer ratio by acting on the culture condition (Ogawa et al., 2005). Both bifidobacteria and several LAB genera like *Streptococcus* and *Lactobacillus* are able to synthesize CLA when they are grown in presence of linoleic acid (i.e., cis,cis-9,12-octadienoic acid) (0.5 g/L) (Coakley et al., 2003). The highest biological activities are currently ascribed to either the cis,trans-9,11 or, alternatively, the trans,cis-10,12 isomer, and LAB produce them generally by biohydrogenation and oxidation processes (Wahle et al., 2004) but also by hydration and dehydration reactions (Ogawa et al., 2005). Several positive effects exerted by CLA on human health have been described in the literature. Among these, it is worth mentioning a modulation of sugar metabolism, resulting in attenuation of insulin resistance and improvement of metabolic syndrome and diabetes (Wahle et al., 2004), body weight loss with an increase of lean body mass (Terpstra, 2004), and induction of apoptosis in cancer cells (Ewaschuk et al., 2006). Nevertheless, some experimental evidence obtained in mice, like body fat reduction, has never been confirmed in humans, and either tumor-promoting activity or deleterious effects on lipid balance (i.e., increase in oxidative lipid products, HDL-cholesterol lowering effect) has been described using several animal models (Ewaschuk et al., 2006; Terpstra, 2004; Wahle et al., 2004). As for many other pharmaceutical treatments, it has been hypothesized that CLA concentration is a key factor in defining limits between beneficial or negative effects: an anti-carcinogenic action has been demonstrated using CLA concentrations of 0.5%-1% (w:w) of the total diet (Ewaschuk et al., 2006). Considering that high linoleic acid dietary intake can be detrimental (Ewaschuk et al., 2006), the CLA dosage has to be maintained at the minimal active concentration to avoid undesired consequences. On the other hand, some experimental evidence suggests that somewhat opposite effects likely correlate with different CLA isomers, due to the fact that they can act through different cell signalling pathways. For instance, the trans,cis-10,12 isomer has been considered dangerous for human health, causing decreased plasma leptin concentrations and insulin resistance (Terpstra, 2004; Wahle et al., 2004). For all these reasons, it is very important, on the one hand, to screen the appropriate strains producing the beneficial isomers, but on the other, to check the optimal dosage of the purified molecules to be administered as supplements. In view of nutraceutical applications in humans, Ogawa and co-workers (2005) set up a safe isomer-selective process for the production of CLA by *Lb. plantarum* strain AKU 1009a. These authors suggested that the substrate (i.e., linoleic acid) has to be dispersed with albumin or with a surfactant so as to be more bio-available for the bacterial cells: a final concentration of 40 g/L of CLA was obtained from linoleic acid by using this fermentation strategy. Furthermore, the use of anaerobic conditions allowed the authors to avoid interfering oxidative metabolism of linoleic acid, thus improving CLA yields. Finally, since it was

previously observed that free unsaturated fatty acids inhibit bacterial growth and trigger defense mechanisms involving "undesired" saturation reaction, these authors used for CLA production resting washed cells instead of actively growing bacteria. With this approach it was possible to enhance productivity by 100-fold. The most interesting finding of this study was the development of a method to control the ratio of isomer production between cis,trans-9,11 (i.e., biologically active) and trans,trans-9,11 (i.e., reduced biological activity) octadecadienoic acid. The addition of L-serine, glucose, NaCl or $AgNO_3$ to the growth medium strongly improved the cis,trans-9,11 production (about 75% selectivity) (Kishino et al., 2003). The possibility of producing CLA by *Lb. plantarum* fermentation of less expensive substrate, i.e., castor oil which is currently used in cosmetics, has also been investigated by Ogawa et al. (2005). These authors reported that production of cis,trans-9,11 CLA could be obtained with a selectivity yield of about 50%. However, the final titer of total CLA produced was much lower (i.e., 7.5 g/L only) as compared with amounts (i.e., 40 g/L) produced by using linoleic acid as the substrate. Moreover, pre-treatment with lipases was necessary in order to hydrolize ricinoleic acid from its esters in castor oil (Ogawa et al., 2005). Further application of LAB as biocatalysts has also been proposed, e.g., in production of regioselective partially hydrogenated oils (Ogawa et al., 2005).

The ability to produce metal-fixing enzymes is a further metabolic feature of LAB which can be exploited for nutraceutical applications (Pessione, 2012). Since several *Lactobacillus* species can fix intracellularly sodium selenite into selenocysteines they are potential sources of selenium-containing molecules which are more bio-available (and therefore more easily adsorbed by human gut cells) than inorganic Se (Calomme et al., 1995). Nowadays, selenocysteine is considered as the 21^{st} standard amino acid. Selenocysteine is encoded by the UGA codon, which usually corresponds to a STOP codon, but can be recognized by specific Se-cysteine-tRNA in suitable genetic environment and physiological conditions (Pessione, 2012). Selenomethionine is incorporated into proteins non-specifically in place of methionine. In both bacteria and eukaryotic cells, including human cells, several enzymes containining selenocysteines in their active site have been identified so far, almost all of which belong to the oxidoreductase class. Glutathione peroxidase, a key enzyme for control of oxidative stress and related diseases in both bacteria and eukarya, is but one of many studied proteins in which seleno amino acids are incorporated into the active site. Lamberti and co-workers (2011) have recently identified a selenocysteine lyase in *Lb. reuteri*. Selenocysteine lyase is a PLP-dependent enzyme which is essential for the biosynthesis of new seleno-proteins from selenide (Lacourciere and Stadtman, 1998). Very recently, it has been shown that by growing a Se-fixing LAB strain in sodium selenite-supplemented medium some of the selenium-containing proteins (i.e., containing selenocysteine) were released extracellularly, thus rendering selenium more bioavailable

(Galano et al., 2013). This finding has opened new perspectives in both probiotic-nutraceutical applications and in industrial production of selenoproteins to be used as food supplements.

Production of B-vitamins, especially folate and riboflavin (B2), by LAB has been another very active research area as described extensively in a recent review (Papagianni, 2012). These vitamins are produced by several LAB species (e.g., *Lc. lactis*, *Lb. gasseri* and *Lb. reuteri*), often in large quantities, and are therefore found in fermented foods (Papagianni, 2012). Moreover, increased vitamin biosynthesis has been obtained by metabolic engineering (Burgess et al., 2004; Hugenholtz et al., 2002). Folate biosynthetic genes and riboflavin biosynthetic operon have been overexpressed in *Lc. lactis* leading to strains with significantly increased folate (Hugenholtz et al., 2002) or riboflavin (Burgess et al., 2004) production, respectively. By directed mutagenesis followed by selection and metabolic engineering, Sybesma and co-workers (2004) modified the biosynthetic pathways of folate and riboflavin in *Lc. lactis*, resulting in the simultaneous overproduction of both vitamins.

3.2.5. Antimicrobial molecules: LAB bacteriocins

A promising feature of LAB is the production of interference molecules, i.e., bacteriocins. Bacteriocins are proteinaceous compounds (peptides or small proteins), synthesized at the ribosomal level (and not as secondary metabolites) specifically interfering with the growth of other bacteria. They have bactericidal action and are selective for prokaryotes. These compounds have found application both in the food industry, i.e., to counteract both spoilage and pathogenic bacteria, and as antibiotic substitutes to treat bacterial infections in humans and animals (Cotter et al., 2005; 2013; Papadimitriou et al., 2014).

LAB are particularly prolific in bacteriocin production and can biosynthesize different types of antagonistic molecules. Due to the urgent necessity of new antimicrobial compounds, research is proceeding rapidly, and natural and food environments have been screened thus leading to isolation and characterization of new molecules every year. However, the continuous discovery of new bacteriocins makes it necessary to frequently revise previous classification based on bacteriocin structure, mode and spectrum of action. A very recent and detailed classification has been reported by Papadimitriou and co-workers (2014). Traditionally, bacteriocins were divided into three classes. Class I consists of the lantibiotics, because they contain post-translationally modified amino acids such as lanthionine (i.e., two alanines linked by a sulphur), β-methyl-lanthionine, dehydroalanine and dehydrobutyrine. Lantibiotics are thermo-resistant small peptides (19-38 amino acids in length) active mainly against Gram-positive bacteria. They can damage cell-envelope structure and function

through different mechanisms, e.g., pore formation and inhibition of peptidoglycan synthesis. The class II bacteriocins are very small (<10 kDa) heat-stable peptides, without extensive post-translational modifications, although they may contain D-amino acids. The best-known class II "pediocin-like" bacteriocin has a narrow but very specific activity against the food pathogen *Listeria monocytogenes*. Finally, bacteriolysins are large, heat-labile antimicrobial enzymatic proteins causing the lysis of sensitive cells by catalyzing cell-wall hydrolysis.

Although pore formation seems to be a shared property by divergent bacteriocins, we now know that the precise mechanism of bacteriocin action is more complicated than initially suspected. For example, the existence of docking molecules (receptors) that may be necessary for the initial binding of the bacteriocin to the cell surface is now recognized (Hassan et al., 2012), although some bacteriocins like enterocin AS-48, gassericin A, subtilosin A and carnocyclin A can exert their activity without binding to any receptor (Nishie et al., 2012).

Bacteriocins have been applied to the control of spoilage and pathogenic bacteria in food. Since bacteriocins are sensitive to proteases and peptidases, which are often present in the food matrix (notably in cheese), it is preferable to purify them and to immobilize them into the food packaging instead of directly adding them into the food itself (Jin et al., 2010). By this approach it is possible to extend the shelf-life of food products by inhibiting the growth of spoilage microorganisms such as *Bochotrix sp.* or *Clostridium tyrobutyricum*, but also to prevent food-born infections by pathogenic bacteria such as *Listeria monocytogenes* and *Staphylococcus aureus*, which are currently responsible for 9000 cases of death per year (Lamberti et al., 2014). Furthermore, the use of bacteriocins allows to reduce the use of: i) sugar and salt in food with positive effects on diabetic and hypertensive patients; ii) other food preservatives and the need of a constant and stringent cold-chain. Application of bacteriocins in the food domain brings potential benefits for the whole population, from both health and energy-saving aspects.

A cutting-edge area of research is the use of bacteriocins as antibiotic substitutes. The list of multidrug resistant (MDR) bacterial strains, causing death chiefly in the hospital environment and more recently triggering severe illnesses in previously non-vulnerable patients in the community, is expanding fast. Nowadays, the number of options to counteract infectious deseases by "traditional" antibiotic molecules is progressively lower (Alanis, 2005). The necessity to find new molecules for the treatment of severe infections is crucial (Siegel, 2008). Recent evidence suggests that LAB bacteriocins could provide this function, as supported by findings from animal and human trials (Sang and Blecha, 2008). The spectrum of bacteriocin action can vary depending on the species producing them (Montalbán-Lopez et al., 2011). Appreciated features of LAB bacteriocins include their activity

at very low concentrations (nanomolar range) and for extended time periods. In spite of the fact that some of them, like nisin, have been used for several decades in food, no resistant mutants have been described so far (Nishie et al., 2012). Actually, the rise of naturally appearing bacteriocin-resistant mutants appears to take place at a very low frequency. For example, in *L. monocytogenes*, nisin-resistant mutants appeared at a frequency of 10^{-6} to 10^{-8} (Harris et al., 1991).

Today, the main challenge for bacteriocin use in the treatment of bacterial infections is their proteinaceous nature which renders them active *in vitro* but sometimes problematic *in vivo*. When lacticin 3147 from *Lc. lactis* was exposed to the conditions of the GI tract of pigs it was rapidly deactivated indicating that such lantibiotics may undergo proteolytic degradation like nisin F (Gardiner et al., 2007). Attempts have been made to protect bacteriocins from the action of digestive and tissue enzymes (i.e., proteases and peptidases). Recenlty, van Staden and co-workers (2012) have reported that brushite cement-incorporated nisin F (at concentrations ranging from 1 to 5%) maintained its antimicrobial activity both *in vitro* and *in vivo* when implanted in sub-cutaneous pockets on the back of mice previously inoculated with *S. aureus*. No infection could be established and no viable cells of this pathogen could be recovered within a time period of seven days (van Staden et al., 2012)

Some bacteriocins have proved to be effective against Staphylococcus (e.g. enterocin 96), while others are known to target *Str. pneumoniae* (e.g. salivaricin D), which is the major cause of pneumonia (Hammami et al., 2013). The purified bacteriocin E 50-52 produced by *Enterococcus* was unable to provide any protection to mice infected with *Mycobacterium turbeculosis* indicating that it could not reach the mycobacteria intra-cellularly. Conversely, when the bacteriocin was used in complex with phosphatidylcholine–cardiolipin liposomes it was able to inhibit mycobacterium within the cells and to prolong the life of infected mice (Sosunov et al., 2007). An *in vivo* study concerning women affected by staphylococcal mastitis demonstrated that the use of a nisin-containing solution on the infected area for two weeks significantly decreased staphylococcal counts and mastitis symptoms (Fernandez et al., 2008). Mutacin B-Ny266 from the *Str. mutans* is active against both methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) (Mota-Meira et al., 2005). A number of variants of nisin (i.e., nisin F, Q and Z) which differ by up to 10 amino acids from nisin A were tested against MRSA and vancomycin-intermediate-resistant (VISA) *Staphylococcus aureus*, and nisin F proved to be the most effective (Piper et al., 2011).

From a biotechnological standpoint important aspects to be considered in view of large-scale application of LAB bacteriocins are yield optimization and genetic modifications to improve both spectrum of activity and protease resistance.

Regarding the yield optimization of bacteriocin, two aspects should be considered: i) time of growth providing maximum harvesting and ii) modulation of biosynthesis by environmental/endogenous factors. In most LAB species the highest bacteriocin accumulation occurs at the end of the exponential growth phase, because their production is under quorum-sensing control, i.e., connected to the time in which biomass has reached a threshold number of cells (Park et al., 2003). Nevertheless, it has been reported that early bacteriocin production can occur in both *Lb. sakei* (Aasen et al., 2000), *Lc. lactis* subsp cremoris (Lamberti et al., 2014), and *Ent. faecium* (Leroy and DeVuyst, 2002). The hypothesis concerning these results is that the low growth rate typical of the early logarithmic phase led to a gain of energy and nutrients, higher than that required for biomass generation. This condition can support higher bacteriocin biosynthesis due to better utilization of carbon/energy sources and enhanced ATP availability. However, this is a transitional event, since growth must proceed and hence energy and nutrients are soon directed towards biomass formation, and bacteriocin production is then swithched off.

Environmental factors modulating bacteriocin synthesis are generally connected with bacterial stress: a fine tuning of bacteriocin production allows reducing the energy costs and optimizing yields limiting the synthesis to times of stress (Gillor et al., 2008). Oxygen stress-enhanced bacteriocin biosynthesis in *Lb. amylovorus* DCE 417 has been reported by Neysens and co-workers (2005). Regarding endogenous factors, it has to be considered that growth rate and biomass yield of the producer strains are not affected during bacteriocin production, since they are immune to their own bacteriocins. LAB possess genes that encode immunity mechanisms: among which, it is worth mentioning that most bacteriocins are biosynthesized as biologically inactive precursors containing a leader sequence preventing the bacteriocin from being active while located inside the producing cell. This sequence has to be removed to generate the fully active peptide which is then secreted (Willey and van der Donk, 2007). Protection can also be provided either by specific immunity proteins (Fimland et al., 2005) or by means of a specialized ABC-transport system pumping the lethal molecule outside of the cell (Draper et al., 2009).

The strategy of modifying the natural bacteriocins by biotechnological approaches is a promising field of research to obtain more effective molecules. The potential to create salivaricin variants with enhanced resistance to the intestinal protease trypsin has been explored by O'Shea and colleagues (2010; 2013). Eleven variants of the salivaricin P components (a two-component bacteriocin, highly active against *Listeria monocytogenes*), with conservative modifications at the trypsin-specific cleavage sites were created. Eight of such salivaricin P variants were resistant to trypsin digestion while retaining antimicrobial activity. Similarly, in the same research group, Field

and co-workers (2012) obtained nisin variants with increased antibacterial activity towards both Gram-positive and Gram-negative bacteria. An additional bacteriocin feature that has been improved by genetic engineering is the enhanced capability to diffuse through complex polymers, with useful applications in the food industry (Rouse et al., 2012).

4. Concluding remarks and future perspectives

As early as 2003, Ohara preconized LAB-based biorefineries as among the most promising biotechnological strategies for obtaining high-value molecules and commodity chemicals (Ohara, 2003). Some of the LAB showing the highest potential for biorefinery application are summarized in Table 3, where their main growth characteristics (carbon sources, optimal temperature and pH ranges) are compared with those of other attractive candidates for future industrial fermentation processes.

*Table 3

Since then, significant advances have been achieved in the development of molecular tools for engineering the metabolic pathways of LAB, optimization of fermentation processes, as well as more in depth understanding of enzymes and other biochemical systems and metabolic pathways relevant for industrial application. Nonetheless, at least two major problems need to be solved prior to concrete application of LAB in cost-sustainable biorefineries. Complex growth media are currently necessary to complement the limited biosynthetic capacities of LAB for production of amino acids and vitamins, which increase both fermentation and product-purification costs. Search for less expensive nutritional supplements with reduced content of impurities than yeast extract is an active field of study (John et al., 2007; Okano et al. 2010a). Alternative fermentation strategies (e.g., co-cultivation with microbial strains supplying essential nutrients) and metabolic engineering are additional tools to resolve or reduce such nutrient requirements. Recombinant strategies can address another major issue, namely to expand substrate-metabolization abilities of LAB, thus enabling them to directly ferment (i.e., without the need for exogenous addition of enzymes and/or physico-chemical saccharification pre-treatments) cheap and abundant biomass, such as starch and lignocellulose. Recombinant amylolytic LAB showing high yield and productivity have recently been constructed (Okano et al., 2009c).

However, development of recombinant cellulolytic microorganisms is at a much earlier stage, mainly because of the more recalcitrant nature of lignocellulose which requires heterologous expression of multiple proteins. To date, relatively few LAB strains have been engineered with single cellulases/hemicellulases that are able to hydrolyze amorphous substrates or grow on short cello-oligosaccharides (Table 1). Intensive research is still necessary to fully understand the molecular mechanisms to enable native cellulase systems to hydrolyze crystalline cellulose for rational design of efficient minimal enzyme mixtures. Furthermore, genetic engineering strategies need to be improved to promote secretion of sufficient amounts and optimal relative ratios of required multiple enzymatic activities in LAB.

In the near future research progress will likely lead to the application of natural or engineered LAB strains in biorefineries (Fig. 5). Appropriate LAB strains will be grown in industrial plants on inexpensive biomass (e.g., plant-derived biomass) under fermentation conditions promoting the biosynthesis of bulk commodities and/or high-value products, such as LA, ethanol, butanol, PHAs, polyols, EPS, bacteriocins, molecules with nutraceutical properties (e.g., GABA, CLA, selenoproteins and vitamins). Most of these products will be purified from the cell-free fermentation medium by simple and low-cost procedures, while in the case of PHA-related processes, polymers of interest will be extracted and purified from bacterial biomass by a variety of methods as described in detail elsewhere (Dias et al., 2006; Keshavarz and Roy, 2010). Moreover, LAB biomass can be recycled for other fermentation processes or used for probiotic manufacturing or as a protein supplement for food and feed applications (Fig. 5). Purified high-value molecules will be used, either directly or after further chemical-physical processing, for a number of applications, some of which are illustrated in Fig. 5. Both PHA and polymers of LA (i.e., PLA) can be used for the manufacturing of biodegradable plastics with broad application spectrum, as described above. Furthermore, LAB-produced bacteriocins can be used for functionalizing plastic films designed for food packaging, thus obtaining foods with improved safety (i.e., more “recalcitrant” to colonization by pathogenic strains) and longer shelf life (e.g., more “recalcitrant” to colonization by spoilage microorganisms). Among other compounds with food application, EPS, polyols and bioactive compounds (e.g., GABA) can be added to food so as to obtain products with improved properties, such as: i) healthier features (i.e., containing polyols instead of sugars) and therefore suitable for some pathological conditions (e.g., diabetes); ii) increased organoleptic characteristics, e.g., smoother or creamier by EPS addition; iii) nutraceutical properties, i.e., containing molecules such as GABA and other bioactive amines, CLA or vitamins which will provide additional health benefits to consumers. It is not unrealistic to hypothesize that in the future the same food factory will be able to produce dairy product(s) and bioplastic films for packaging of such food products, possibly functionalized with bacteriocins, by

using the same LAB strain(s). Apart from polymerization to PLA, LA can be esterified with ethanol thus producing a highly sought-after biodegradable solvent, i.e., ethyl lactate. Last but not least, LAB are currently considered good candidates for production of biofuels such as ethanol and butanol, as well as for synthesis of formate (as a biofuel precursor) which is a suitable substrate for hydrogen-producing fermentation processes. Hopefully, what is currently an optimistic vision could be realized in the near future, so that such LAB-based biorefineries will become a relevant option for an environmentally friendly and cost-sustainable economy.

***Fig. 5.**

Acknowledgements

This study was financially supported partly by "Action B-Return of Italian researchers from abroad" scheme from University of Torino and "Regione Piemonte" and partly by private financing from Carbios, Biopôle Clermont-Limagne, Saint-Beauzire (France). Support to IM and EAB was generously provided by the Israel Ministry of Science (IMOS), grant number 3620320151. E.A.B. is the incumbent of The Maynard I. and Elaine Wishner Chair of Bio-organic Chemistry.

References

Aarnikunnas J, von Weymarn N, Rönnholm K, Leisola M, Palva A. Metabolic engineering of *Lactobacillus fermentum* for production of mannitol and pure L-lactic acid or pyruvate. Biotechnol Bioeng 2003;82:653–63.

Aasen IM, Morestro T, Katla T, Axelsson L, Storro I. Influence of complex nutrients, temperature and pH on bacteriocin production by *Lactobacillus sakei* CCUG 42687. Appl Microbiol Biotechnol 2000;53:159–66.

Abdel-Rahman MA, Tashiro Y, Sonomoto K. Recent advances in lactic acid production by microbial fermentation processes. Biotechnol Adv 2013;1:877–902.

Adkins J, Pugh S, McKenna R, Nielsen DR. Engineering microbial chemical factories to produce renewable "biomonomers". Front Microbiol 2012;3:313.

Adsul M, Khire J, Bastawde K, Gokhale D. Production of lactic acid from cellobiose and cellotriose by *Lactobacillus delbrueckii* mutant Uc-3. Appl Environ Microbiol 2007a;73:5055–7.

Adsul MG, Varmab AJ, Gokhale DV. Lactic acid production from waste sugarcane bagasse derived cellulose Green Chem 2007b;9:58–62.

Alanis AJ. Resistance to antibiotics: are we in the post-antibiotic era? Arch Med Res 2005;36:697–705.

Alegria EG, Lopez I, Ruiz JI, Saenz J, Fernandez E, Zarazaga M, et al. High tolerance of wild *Lactobacillus plantarum* and *Oenococcus oeni* strains to lyophilisation and stress environmental conditions of acid pH and ethanol. FEMS Microbiol Lett. 2004;230:53–61.

Altaf M, Naveena BJ, Reddy G. Screening of inexpensive nitrogen sources for production of L(+) lactic acid from starch by amylolytic *Lactobacillus amylophilus* GV6 in single step fermentation Food Technol Biotechnol 2005;43:235–9.

Amrane A, Prigent Y. Influence of yeast extract concentration on batch cultures of *Lactobacillus helveticus*: growth and production coupling. World J Microbiol Biotechnol 1998;14:529–34.

Aslim B, Caliskan F, Beyath Y, Guënduëz U. Poly-L-hydroxybutyrate production by lactic acid bacteria. FEMS Microbiol Lett 1998;159:293–7.

Bader J, Mast-Gerlach E, Popovic MK, Bajpai R, Stahl U. Relevance of microbial co-culture fermentations in biotechnology. J Appl Microbiol 2010;109:371–87.

Balusu R, Paduru RM, Seenayya G, Reddy G. Production of ethanol from cellulosic biomass by *Clostridium thermocellum* SS19 in submerged fermentation: screening of nutrients using Plackett-Burman design. Appl Biochem Biotechnol 2004;117:133–41.

Bates EEM, Gilbert HJ, Hazlewood GP, Huckle J, Laurie JI, Mann SP. Expression of a *Clostridium thermocellum* endoglucanase gene in *Lactobacillus plantarum*. Appl Environ Microbiol 1989;55:2095–7.

Bayer EA, Chanzy H, Lamed R, Shoham Y. Cellulose, cellulases and cellulosomes. Curr Opin Struct Biol 1998;8:548-557.

Bayer EA, Lamed R, Himmel ME. The potential of cellulases and cellulosomes for cellulosic waste management. Curr Opin Biotechnol 2007;18:237–45.

Bayer EA, Morag E, Lamed R. The cellulosome — A treasure-trove for biotechnology. Trends Biotechnol 1994;12:379-386.

Bayer EA, Shoham Y, Lamed R: Lignocellulose-decomposing bacteria and their enzyme systems. In: Rosenberg E, DeLong F, Lory S, Stackebrandt E, Thompson F, editors. The Prokaryotes. Springer Berlin Heidelberg; 2013. p. 216-266.

Bayer EA, Lamed R, White BA, Flint HJ. From cellulosomes to cellulosomics. Chem Rec 2008;8:364–77.

Berezina OV, Zakharova NV, Brandt A, Yarotsky SV, Schwarz WH, Zverlov VV. Reconstructing the clostridial n-butanol metabolic pathway in *Lactobacillus brevis*. Appl Microbiol Biotechnol 2010;87:635–46.

Berlec A, Strukelj B. Novel applications of recombinant lactic acid bacteria in therapy and in metabolic engineering. Recent Pat Biotechnol 2009;3:77–87.

Blencowe DK, Morby AP. Zn(II) metabolism in prokaryotes. FEMS Microbiol Rev 2003;27:291–311.

Boels IC, Ramos A, Kreerebezem M, de Vos WM. Functional analysis of the *Lactococcus lactis galU* and *galIE* genes and their impact on sugar nucleotide and exopolysaccharide biosynthesis. Appl Environ Microbiol 2001a;67:3033–40.

Boels IC, van Kranenburg R, Hugenholtz J, Kleerebezem M, de Vos WM. Sugar catabolism and its impact on the biosynthesis and engineering of exopolysaccharide production in lactic acid bacteria. Int Dairy J 2001b;11:723–32.

Bron PA, Wels M, Bongers RS, van Bokhorst-van de Veen H, Wiersma A, Overmars L, et al. Transcriptomes reveal genetic signatures underlying physiological variations imposed by different fermentation conditions in *Lactobacillus plantarum*. PLoS One 2012;7:e38720.

Burgess C, O'Connell-Motherway M, Sybesma W, Hugenholtz J, van Sinderen D. Riboflavin production in *Lactococcus lactis*: potential for in situ production of vitamin-enriched foods. Appl Environ Microbiol 2004;70:5769–77.

Buschke N, Schafer R, Becker J, Wittmann C. Metabolic engineering of industrial platform microorganisms for biorefinery applications-optimization of substrate spectrum and process robustness by rational and evolutive strategies. Bioresour Technol 2013;135:544–54.

Bustos G, Moldes AB, Cruz JM, Domínguez JM. Influence of the metabolism pathway on lactic acid production from hemicellulosic trimming vine shoots hydrolyzates using *Lactobacillus pentosus.* Biotechnol Prog 2005;21:793–8.

Calabia BP, Tokiwa Y. Production of D-lactic acid from sugarcane molasses, sugarcane juice and sugar beet juice by *Lactobacillus delbrueckii*. Biotechnol Lett 2007;29:1329–32.

Calomme M, Hu J, Van Den Branden K, Van den Berghe D. Seleno-*lactobacillus* – an organic selenium source. Biol Trace Elem Res 1995;47:379–83.

Carvalho AL, Turner DL, Fonseca LL, Solopova A, Catarino T, Kuipers OP, et al. Metabolic and transcriptional analysis of acid stress in *Lactococcus lactis*, with a focus on the kinetics of lactic acid pools. PLoS One 2013;8:e68470.

Chaillou S, Bor YC, Batt CA, Postma PW, Pouwels PH. Molecular cloning and functional expression in *Lactobacillus plantarum* 80 of *xylT*, encoding the D-xylose-H_ symporter of *Lactobacillus brevis.* Appl Environ Microbiol 1998;64:4720–8.

Chandel AK, Chandrasekhar G, Silva MB, Silvério da Silva S. The realm of cellulases in biorefinery development. Crit Rev Biotechnol 2012;32:187–202.

Chapot-Chartier MP, Monnet V, De Vuyst L. Cell-wall and exopolysaccharides of lactic acid bacteria. In: Ledeboer A, Hugenholtz J, Kok J, Konings W, Wouters J, editors. Thirty years of research on lactic acid bacteria. Rotterdam, The Netherlands: Media Labs; 2011. p. 113–132.

Charalampopoulos D, Pandiella SS, Webb C. Growth studies of potentially probiotic lactic acid bacteria in cereal-based substrates. J Appl Microbiol 2002;92:851–9.

Chen J, Zhang W, Tan L, Wang Y, He G. Optimization of metabolic pathways for bioconversion of lignocellulose to ethanol through genetic engineering. Biotechnol Adv 2009;27:593–8.

Cheng Y, Deng S, Chen P, Ruan R. Polylactic acid (PLA) synthesis and modi*f*ications: a review. Front. Chem. China 2009;4: 259–64.

Cho JS, Choi YJ, Chung DK. Expression of *Clostridium thermocellum* endoglucanase gene in *Lactobacillus gasseri* and *Lactobacillus johnsonii* and characterization of the genetically modified probiotic Lactobacilli. Current Microbiology 2000;40:257–63.

Cho YR, Chang JY, Chang HC. Production of gamma-aminobutyric acid (GABA) by *Lactobacillus buchneri* isolated from kimchi and its neuroprotective effect on neuronal cells. J Microbiol Biotechnol 2007;17:104–9.

Coakley M, Ross RP, Nordgren M, Fitzgerald G, Devery R, Stanton C. CLA biosynthesis by human-derived *Bifidobacterium* species. J Appl Microbiol 2003;94:138–45.

Coelho LF, de Lima CJ, Bernardo MP, Contiero J. D(-)-lactic acid production by *Leuconostoc mesenteroides* B512 using different carbon and nitrogen sources. Appl Biochem Biotechnol 2011;164:1160–71.

Cotter PD, Hill C, Ross RP. Bacteriocins: developing innate immunity for food. Nat Rev Microbiol 2005;3:777–88.

Cotter PD, Ross RP, Hill C. Bacteriocins: a viable alternative to antibiotics ? Nat Rev Microbiol 2013;11:95–105.

Daguer JP, Chambert R, Petit-Glatron MF. Increasing the stability of *sacB* transcript improves levansucrase production in *Bacillus subtilis*. Lett Appl Microbiol 2005;41:221–6.

Datta R, Henry M. Lactic acid: recent advances in products, processes and technologies - a review. J Chem Technol Biotechnol 2006;81:1119–29.

de Vos WM, Hugenholtz J. Engineering metabolic highways in Lactococci and other lactic acid bacteria. Trends Biotechnol 2004;22:72–9.

de Vos WM. Gene expression systems for lactic acid bacteria. Curr Opin Microbiol 1999;2:289–95.

de Vos WM, Kleerebezem, MK, Oscar P. Expression systems for industrial Gram-positive bacteria with low guanine and cytosine content. Curr Opin Biotechnol 1997;8:547–53.

Defoirdt T, Boon N, Sorgeloos P, Verstraete W, Bossier P. Short-chain fatty acids and poly-β-hydroxyalkanoates: (New) Biocontrol agents for a sustainable animal production. Biotechnol Adv 2009;27:680–5.

Dias JM, Lemos PC, Serafim LS, Oliveira C, Eiroa M, Albuquerque MG, et al. Recent advances in polyhydroxyalkanoate production by mixed aerobic cultures: from the substrate to the final product. Macromol Biosci 2006;6:885–906.

Domagk GF, Horecker BL. Pentose fermentation by *Lactobacillus plantarum*. V. Fermentation of 2-deoxy-D-ribose. J Biol Chem 1958;233:283–6.

Doran-Peterson J, Cook DM, Brandon S. Microbial conversion of sugars from plant biomass to lactic acid or ethanol. Plant J 2008;54:582–92.

Douglas GL, Jun Goh Y, Klaenhammer TR. Integrative Food Grade Expression System for Lactic Acid Bacteria. In: Williams JA, editor. Strain Engineering: Methods and Protocols, Methods in Molecular Biology, vol. 765, DOI 10.1007/978-1-61779-197-0_22, © Springer Science+Business Media, LLC 2011.

Draper LA, Grainger K, Deegan LH, Cotter PD, Hill C, Ross RP. Cross-immunity and immune mimicry as mechanisms of resistance to the lantibiotic lacticin (3147). Mol Microbiol 2009;71:1043–54.

Ewaschuk JB, Walker JW, Diaz H, Madsen KL. Bioproduction of conjugated linoleic acid by probiotic bacteria occurs in vitro and in vivo in mice. J Nutr 2006;136:1483–7.

Fernandez L, Delgado S, Herrero H, Maldonado A, Rodriguez JM. The bacteriocin nisin, an effective agent for the treatment of staphylococcal mastitis during lactation. J Hum Lact 2008;24:311–6.

Field D, Begley M, O'Connor P, Daly KM, Hugenholtz F, Cotter PD, et al. Bioengineered nisin A derivatives with enhanced activity against both Gram positive and Gram negative pathogens. PLoS One 2012;7:e 46884.

Fimland G, Johnsen L, Dalhus B, Nissen-Meyer J. Pediocin-like antimicrobial peptides (class IIa bacteriocins) and their immunity proteins: biosynthesis, structure, and mode of action. J Pept Sci 2005;11:688–96.

Fontes CM, Gilbert HJ. Cellulosomes: highly efficient nanomachines designed to deconstruct plant cell wall complex carbohydrates. Annu Rev Biochem 2010;79:655–81.

Galano E, Mangiapane E, Bianga J, Palmese A, Pessione E, Szpunar J, et al. Privileged incorporation of selenium as selenocysteine in *Lactobacillus reuteri* proteins demonstrated by selenium-specific imaging and proteomics**.** Mol Cell Proteomics 2013;12:2196–204.

Gänzle MG, Vermeulen N, Vogel RF. Carbohydrate, peptide and lipid metabolism of lactic acid bacteria in sourdough. Food Microbiol 2007;24:128–138.

Gardiner GE, Rea MC, O'Riordan B, O'Connor P, Morgan SM, Lawlor PG, et al. Fate of the two-component lantibiotic lacticin 3147 in the gastrointestinal tract. Appl Environ Microbiol 2007;73:7103–9.

Gaspar P, Carvalho AL, Vinga S, Santos H, Neves AR. From physiology to systems metabolic engineering for the production of biochemicals by lactic acid bacteria. Biotechnol Adv 2013;31:764–88.

Gibbons WR, Hughes SR. Integrated biorefineries with engineered microbes and high-value co-products for profitable biofuels production. In Vitro Cell Dev Biol-Plant 2009;45:218–28.

Gillor O, Vriezen JA, Riley MA. The role of SOS boxes in enteric bacteriocin regulation. Microbiology 2008;154:1783–92.

Giraud E, Champailler A, Raimbault M. Degradation of raw starch by a wild amylolytic strain of *Lactobacillus plantarum*. Appl Environ Microbiol 1994;60:4319–23.

Goffin P, Deghorain M, Mainardi JL, Tytgat I, Champomier-Vergès MC, Kleerebezem M, et al. Lactate racemization as a rescue pathway for supplying D-lactate to the cell wall biosynthesis machinery in *Lactobacillus plantarum*. J Bacteriol 2005;187:6750–61.

Göksungur Y, Güvenç U. Production of lactic acid from beet molasses by calcium alginate immobilized *Lactobacillus delbrueckii* IFO 3202. J Chem Technol Biotechnol 1999;74:131–6.

Gold RS, Meagher MM, Tong S, Hutkins RW, Conway T. Cloning and expression ofthe *Zymomonas mobilis* "production of ethanol" genes in *Lactobacillus casei*. Curr Microbiol. 1996;33:256–60.

Guo T, Kong J, Zhang L, Zhang C, Hu S. Fine tuning of the lactate and diacetyl production through promoter engineering in *Lactococcus lactis*. PLoS ONE 2012;7:e36296.

Guo W, Jia W, Li Y, Chen S. Performances of *Lactobacillus brevis* for producing lactic acid from hydrolysate of lignocellulosics. Appl Biochem Biotechnol 2010;161:124–36.

Guyot JP, Calderon M, Morlon-Guyot J. Effect of pH control on lactic acid fermentation of starch by *Lactobacillus manihotivorans* LMG 18010T. J Appl Microbiol 2000;88:176–82.

Hammami R, Fernandez B, Lacroix C, Fliss I. Anti-infective properties ofbacteriocins: an update. Cell Mol Life Sci 2013;70:2947–67.

Harris LJ, Flemming HP, Klaenhammer T. Sensitivity and resistance of *Listeria monocytogenes* ATCC 19115, Scott A, and UAL500 to nisin. J Food Prot 1991;54:836–840.

Hassan M, Kjos M, Nes IF, Diep DB, Lotfipour F. Natural antimicrobial peptides from bacteria: characteristics and potential applications to fight against antibiotic resistance. J Appl Microbiol 2012;113:723–36.

Helando M, Aarnikunnas J, von Weymarn N, Airaksinen U, Palva A, Leisola M. Improved mannitol production by a random mutant of *Leuconostoc pseudomesenteroides*. J Biotechnol 2005;116:283–94.

Higashide W, Li Y, Yang Y, Liao JC. Metabolic engineering of *Clostridium cellulolyticum* for production of isobutanol from cellulose. Appl Environ Microbiol 2011;77:2727–33.

Himmel ME, Xu Q, Luo Y, Ding SY, Lamed R, Bayer EA. Microbial enzyme systems for biomass conversion: Emerging paradigms. Biofuels 2010;1:323–41.

Hoefnagel MHN, Starrenburg MJC, Martens DE, Hugenholtz J, Kleerebezem M, van Swam II, et al. Metabolic engineering of lactic acid bacteria, the combined approach: kinetic modeling, metabolic control and experimental analysis. Microbiology 2002;148:1003–13.

Hofvendahl K, Hahn-Hägerdal B. Factors affecting the fermentative lactic acid production from renewable resources. Enzyme Microb Technol 2000;26:87–107.

Hols P, Kleerebezem M, Schanck AN, Ferain T, Hugenholtz J, Delcour J, et al. Conversion of *Lactococcus lactis* from homolactic to homoalanine fermentation through metabolic engineering. Nat Biotechnol 1999;17:588–92.

Hugenholtz J, Hols P, Snoep J. Metabolic engineering of lactic acid bacteria: 30 years of research and discoveries. In: Ledeboer A, Hugenholtz J, Kok J, Konings W, Wouters J, editors. Thirty years of research on lactic acid bacteria. Rotterdam, The Netherlands: Media Labs; 2011. p. 113–132.

Hugenholtz J, Sybesma W, Groot MN, Wisselink W, Ladero V, Burgess K, et al. Metabolic engineering of lactic acid bacteria for the production of nutraceuticals. Antonie Van Leeuwenhoek. 2002;82:217–35.

Jarboe LR, Grabar TB, Yomano LP, Shanmugan KT, Ingram LO. Development of ethanologenic bacteria. Adv Biochem Eng Biotechnol 2007;108:237–61.

Jin, T.; Zhang, H.; Boyd, G. Incorporation of preservatives in polylactic acid films for inactivating *Escherichia coli* O157:H7 and extending microbiological shelf life of strawberry puree. J. Food Prot. 2010, 73, 812–818.

John RP, Nampoothiri KM, Pandey A. Simultaneous saccharification and L-(+)-lactic acid fermentation of protease-treated wheat bran using mixed culture of lactobacilli. Biotechnol Lett 2006;28:1823–26.

John RP, Nampoothiri KM, Pandey A. Fermentative production of lactic acid from biomass: an overview on process developments and future perspectives. Appl Microbiol Biotechnol 2007;74:524–34.

Jojima T, Omumasaba CA, Inui M, Yukawa H. Sugar transporters in efficient utilization of mixed sugar substrates: current knowledge and outlook. Appl Microbiol Biotechnol 2010;85:471–80.

Jordan DB, Bowman MJ, Braker JD, Dien BS, Hector RE, Lee CC, et al. Plant cell walls to ethanol. Biochem J 2012;442:241–52.

Joshi DS, Singhvi MS, Khire JM, Gokhale DV. Strain improvement of *Lactobacillus lactis* for D-lactic acid production. Biotechnol Lett 2010;32:517–20.

Kandler O. Carbohydrate metabolism in lactic acid bacteria. Antonie Van Leeuwenhoek 1983;49:209–24.

Keshavarz T, Roy I. Polyhydroxyalkanoates: bioplastics with a green agenda. Curr Opin Microbiol 2010;13:321–6.

Keskin T, Abo-Hashesh M, Hallenbeck PC. Photofermentative hydrogen production from wastes. Bioresour Technol 2011;102: 8557–68.

Kim JY, Lee MY, Ji GE, Lee YS, Hwang KT. Production of gamma-aminobutyric acid in black raspberry juice during fermentation by *Lactobacillus brevis* GABA100. Int J Food Microbiol 2009;130:12–6.

Kim JH, Block DE, Shoemaker SP, Mills DA. Atypical ethanol production bycarbon catabolite derepressed lactobacilli. Bioresour Technol 2010;101:8790–7.

Kishino S, Ogawa J, Ando A, Iwashita T, Fujita T, Kawashima H, et al. Structural analysis of conjugated linoleic acid produced by *Lactobacillus plantarum* and factors affecting isomers production. Biosci Biotechnol Biochem 2003;67:179–82.

Kleerebezem M, Boekhorst J, van Kranenburg R, Molenaar D, Kuipers OP, Leer R, et al. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc Natl Acad Sci USA. 2003;100:1990–5.

Kleerebezem M, Hugenholtz J. Metabolic pathway engineering in lactic acid bacteria. Curr Opin Biotechnol. 2003;14:232–7.

Knoshaug EP, Zhang M. Butanol Tolerance in a Selection of Microorganisms. Appl Biochem Biotechnol 2009;153:13–20.

Koller M, Hesse P, Bona R, Kutschera C, Atlic A, Braunegg G. Potential of various Archae- and Eubacterial strains as industrial polyhydroxyalcanoate producers from whey. Macromol Biosci 2007;7:218–26.

Komarova AV, Tchufistova LS, Dreyfus M, Boni IV. AU-rich sequences within 5' untranslated leaders enhance translation and stabilize mRNA in *Escherichia coli*. J Bacteriol 2005;187:1344–9.

Konings WN. Microbial transport: adaptations to natural environments. Antonie Van Leeuwenhoek. 2006;90:325–42.

Kotzanmanidis C, Roukas T, Skaracis G. Optimization of lactic acid production from beet molasses *Lactobacillus delbrueckii* NCIMB 8130. World J Microbiol Biotechnol 2002;18:442–8.

Kowalczyk M, Cocaign-Bousquet M, Loubiere P, Bardowski J. Identification and functional characterisation of cellobiose and lactose transport systems in *Lactococcus lactis* IL1403. Arch Microbiol 2008;189:187–96.

Krauss J, Zverlov VV, Schwarz WH. In vitro reconstitution of the complete *Clostridium thermocellum* cellulosome and synergistic activity on crystalline cellulose. Appl Environ Microbiol 2012;78:4301–7.

Lacourciere GM, Stadtman TC. The NIFS protein can function as a selenide delivery protein in the biosynthesis of selenophosphate. J Biol Chem 1998;273:30921–6.

Ladero V, Ramos A, Wiersma A, Goffin P, Schanck A, Kreerebezem M, et al. High-level production of the low-calorie sugar sorbitol by *Lactobacillus plantarum* through metabolic engineering. Appl Environ Microbiol 2007;73:1864–72.

Lambert JM, Bongers RS, Kleerebezem M. Cre–lox-based system for multiple gene deletions and selectable-marker removal in *Lactobacillus plantarum*. Appl Environ Microbiol 2007;73:1126–35.

Lamberti C, Genovese F, Coisson JD, LoBianco G., Cocolin LS, Pessione E. Anti-*S. aureus* and anti-*L. monocytogenes* molecules produced by cheese-isolated LAB. Czec J Food Sci 2014;32:54–60.

Lamberti C, Mangiapane E, Pessione A, Mazzoli R, Giunta C, Pessione E. Proteomic characterization of a selenium-metabolizing probiotic *Lactobacillus reuteri* Lb2 BM for nutraceutical applications. Proteomics 2011;11:2212–21.

Laopaiboon P, Thani A, Leelavatcharamas V, Laopaiboon L. Acid hydrolysis of sugarcane bagasse for lactic acid production. Bioresour Technol 2010;101:1036–43.

Leroy F, De Vuyst L. Bacteriocin production by *Enterococcus faecium* RZS C5 is cell density limited and occurs in the very early growth phase. Int J Food Microbiol 2002;72:155–64.

Levander F, Svensson M, Radstrom P. Enhanced expopolysacharide production by metabolic engineering of *Streptococcus thermophillus*. Appl Environ Microbiol 2002;68:784–90.

Li H, Cao Y. Lactic acid bacterial cell factories for gamma-aminobutyric acid. Amino Acids 2010;39:1107–16.

Li H, Qiu T, Huang G, Cao Y. Production of gamma-aminobutyric acid by *Lactobacillus brevis* NCL912 using fed-batch fermentation. Microb Cell Fact 2010a;9:85.

Li Z, Hana L, Ji Y, Wanga X, Tana T.Fermentative production of l-lactic acid from hydrolysate of wheat bran by *Lactobacillus rhamnosus*. Biochem Eng J 2010b:49:138–142

Limayem A, Hanning IB, Muthaiyan A, Illeghems K, Kim JW, Crandall PG, et al. Alternative antimicrobial compounds to control potential *Lactobacillus* contamination in bioethanol fermentations. J Environ Sci Health B 2011;46:709–14.

Liu S, Nichols NN, Dien BS, Cotta MA. Metabolic engineering of a *Lactobacillus plantarum* double *ldh* knockout strain for enhanced ethanol production. J Ind Microbiol Biotechnol 2006;33:1–7.

London J. Uncommon pathways of metabolism among lactic acid bacteria. FEMS Microbiol Rev 1990;7:103–11.

Lu J, Tappel RC, Nomura CT. Mini-Review: biosynthesis of poly(hydroxyalkanoates), Polym Rev 2009;49:226–48

Lucena BT, dos Santos BM, Moreira JL, Moreira AP, Nunes AC, Azevedo V, et al. Diversity of lactic acid bacteria of the bioethanol process. BMC Microbiol 2010;10:298.

Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 2002;66:506–77.

Madhavan Nampoothiri K, Nair NR, John RP. An overview of the recent developments in polylactide (PLA) research. Bioresour Technol 2010;101:8493–501.

Marques S, Santos JAL, Gırio FM, Roseiroa JC. Lactic acid production from recycled paper sludge by simultaneous saccharification and fermentation. Biochem Eng J 2008; 41:210–6.

Mathiesen G, Sveen A, Piard JC, Axelsson L, Eijsink VG. Heterologous protein secretion by *Lactobacillus plantarum* using homologous signal peptides. J Appl Microbiol 2008;105:215–26.

Mazzoli R, Lamberti C, Pessione E. Engineering new metabolic capabilities in bacteria: lessons from recombinant cellulolytic strategies. Trends Biotechnol 2012; 30:111–9.

Mazzoli R, Pessione E, Dufour M, Laroute V, Giuffrida MG, Giunta C, et al. Glutamate-induced metabolic changes in *Lactococcus lactis* NCDO 2118 during GABA production: combined transcriptomic and proteomic analysis. Amino Acids 2010;39:727–37.

Mazzoli R. Development of microorganisms for cellulose-biofuel consolidated bioprocessings: metabolic engineers' tricks. Comput Struct Biotechnol J 2012;3:e201210007. doi: http://dx.doi.org/10.5936/csbj.201210007

Mazzoli R. Neuro-active compounds produced by probiotics. Towards a microbiota-(gut-) brain axis control? In: Pessione E, editor. Interactive probiotics. Boca Raton, FL 33487, U.S.A.: CRC Press, Taylor & Francis Group, LLC; 2014. p. 148–76.

Melchiorsen CR, Jokumsen KV, Villadsen J, Israelsen H, Arnau J. The level of pyruvate-formate lyase controls the shift from homolactic to mixed-acid product formation in *Lactococcus lactis*. Appl Microbiol Biotechnol 2002;58: 338–44.

Mingardon F, Chanal A, Tardif C, Fierobe HP. The issue of secretion in heterologous expression of *Clostridium cellulolyticum* cellulase-encoding genes in *Clostridium acetobutylicum* ATCC 824. Appl Environ Microbiol. 2011;77:2831–8.

Mizrahi I. Rumen Symbioses. In: Rosenberg E, DeLong F, Lory S, Stackebrandt E, Thompson F, editors. The Prokaryotes. Springer Berlin Heidelberg; 2013.p. 533–44.

Moldes AB, Torrado A, Converti A, Domínguez JM. Complete bioconversion of hemicellulosic sugars from agricultural residues into lactic acid by *Lactobacillus pentosus*. Appl Biochem Biotechnol 2006;135:219–28.

Montalbán-López M, Sánchez-Hidalgo M, ValdiviaE, Martínez-Bueno M, Maqueda M. Are bacteriocins underexploited? Novel applications for old antimicrobials. Curr Pharm Biotechnol2011;12:1205–20.

Monteagudo JM, Rodríguez L, Rincón J, Fuertes J. Kinetics of lactic acid fermentation by *Lactobacillus delbrueckii* grown on beet molasses. J Chem Technol Biotechnol 1997;68:271–6.

Morais S, Shterzer N, Grinberg IR, Mathiesen G, Eijsink VG, Axelsson L, et al. Establishment of a simple *Lactobacillus plantarum* cell consortium for cellulase-xylanase synergistic interactions. Appl Environ Microbiol. 2013;79:5242–9.

Morello E, Bermúdez-Humarán LG, Llull D, Solé V, Miraglio N, Langella P, et al. *Lactococcus lactis*, an efficient cell factory for recombinant protein production and secretion. J. Mol. Microbiol. Biotechnol 2008;14:48–58.

Mota-Meira M, Morency H, Lavoie MC. In vivo activity of mutacin B-Ny266. J Antimicrob Chemother 2005;56: 869–71.

Narita J, Ishida S, Okano K, Kimura S, Fukuda H, Kondo A. Improvement of protein production in lactic acid bacteria using 5'-untranslated leader sequence of *slpA* from *Lactobacillus acidophilus*. Improvement in protein production using UTLS. Appl Microbiol Biotechnol 2006;73:366–73.

Narita J, Nakahara S, Fukuda H, Kondo A. Efficient production of L-(+)-lactic acid from raw starch by *Streptococcus bovis* 148. J Biosci Bioeng 2004;97:423–5.

Naveena BJ, Altaf M, Bhadrayya K, Madhavendra SS, Reddy G. Direct fermentation of starch to l(+) lactic acid in SSF by *Lactobacillus amylophilus* GV6 using wheat bran as support and substrate:medium optimization using RSM. Process Biochem 2005a;40:681–90.

Naveena BJ, Altaf M, Bhadriah K, Reddy G. Selection of medium components by Plackett–Burman design for production of L(+) lactic acid by *Lactobacillus amylophilus* GV6 in SSF using wheat bran. Bioresour Technol 2005b;96:485–90

Neysens P, De Vuyst L. Carbon dioxide stimulates the production of amylovorin L by *Lactobacillus amylovorus* DCE 471, while enhanced aeration causes biphasic kinetics of growth and bacteriocin production. Int J Food Microbiol 2005;105:191–202.

Ng TK, Ben-Bassat A, Zeikus JG. Ethanol production by thermophilic bacteria: fermentation of cellulosic substrates by cocultures of *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*. Appl Environ Microbiol 1981;41:1337–43.

Nichols NN, Dien BS, Bothast RJ. Engineering lactic acid bacteria with pyruvate decarboxylase and alcohol dehydrogenase genes for ethanol production from *Zymomonas mobilis*. J Ind Microbiol Biotechnol 2003;30:315–21.

Nicolaou SA, Gaida SM, Papoutsakis ET. A comparative view of metabolite and substrate stress and tolerance in microbial bioprocessing: From biofuels and chemicals, to biocatalysis and bioremediation. Metab Eng 2010;12:307–31.

Nishie M, Nagao J, Sonomoto K. Antibacterial peptides "bacteriocins": an overview of their diverse characteristics and applications. Biocontrol Sci 2012;17:1–16.

Nyyssölä A, Pihlajaniemi A, Palva A, von Weymarn N, Leisola M. Production of xylitol from D-xylose by recombinant *Lactococcus lactis*. J Biotechnol 2005;118:55–66.

Ogawa J, Kishino S, Ando A, Sugimoto S, Mihara K, Shimizu S. Production of conjugated fatty acids by lactic acid bacteria. J Biosci Bioeng 2005;100:355–64.

O'Shea EF, Cotter PD, Ross RP, Hill C. Strategies to improve the bacteriocin protection provided by lactic acid bacteria. Curr Opin Biotechnol 2013;24:130–4.

O'Shea EF, O'Connor PM, Cotter PD, Ross RP, Hill C. Synthesis of trypsin resistant variants of the Listeria-active bacteriocin salivaricin P. Appl Environ Microbiol 2010;76:5356–62.

Oh H, Wee YJ, Yun JS, Han SH, Jung S, Ryu HW. Lactic acid production from agricultural resources as cheap raw materials. Bioresour Technol 2005;96:1492–8.

Oh YK, Raj SM, Jung GY, Park S. Current status of the metabolic engineering of microorganisms for biohydrogen production. Bioresour Technol 2011;102:8357–67.

Ohara H, Owaki M, Sonomoto K. Xylooligosaccharide fermentation with *Leuconostoc lactis*. J Biosci Bioeng 2006;101:415–20.

Ohara H. Biorefinery. Appl Microbiol Biotechnol 2003;62:474–7.

Ohashi R, Yamamoto T, Suzuki T. Continuous production of lactic acid from molasses by perfusion culture of *Lactococcus lactis* using a stirred ceramic membrane reactor. J Biosci Bioeng 1999;87:647–54.

Okano K, Kimura S, Narita J, Fukuda H, Kondo A. Improvement in lactic acid production from starch using alpha-amylase-secreting *Lactococcus lactis* cells adapted to maltose or starch. Appl Microbiol Biotechnol 2007;75:1007–13.

Okano K, Tanaka T, Ogino C, Fukuda H, Kondo A. Biotechnological production of enantiomeric pure lactic acid from renewable resources: recent achievements, perspectives, and limits. Appl Microbiol Biotechnol 2010a;85:413–23.

Okano K, Yoshida S, Tanaka T, Ogino C, Fukuda H, Kondo A. Homo-D-lactic acid fermentation from arabinose by redirection of the phosphoketolase pathway to the pentose phosphate pathway in L-LDH gene-deficient *Lactobacillus plantarum*. Appl Environ Microbiol 2009a;75:5175–8.

Okano K, Yoshida S, Yamada R, Tanaka T, Ogino C, Fukuda H, et al. Improved production of homo-D-lactic acid via xylose fermentation by introduction of xylose assimilation genes and redirection of the phosphoketolase pathway to the pentose phosphate pathway in L-lactate dehydrogenase gene-deficient *Lactobacillus plantarum*. Appl Environ Microbiol 2009b;75:7858–61.

Okano K, Zhang Q, Kimura S, Narita J, Tanaka T, Fukuda H, et al. System using tandem repeats of the cA peptidoglycan-binding domain from *Lactococcus lactis* for display of both N- and C-terminal fusions on cell surfaces of lactic acid bacteria. Appl Environ Microbiol 2008;74:1117–23.

Okano K, Zhang Q, Shinkawa S, Yoshida S, Tanaka T, Fukuda H, Kondo A. Efficient production of optically pure D-lactic acid from raw corn starch by using a genetically modified L-lactate dehydrogenase gene-deficient and alpha-amylase-secreting *Lactobacillus plantarum* strain. Appl Environ Microbiol 2009c;75:462–7.

Okano K, Zhang Q, Yoshida S, Tanaka T, Ogino C, Fukuda H, et al. D-lactic acid production from cellooligosaccharides and beta-glucan using L-LDH gene-deficient and endoglucanase-secreting *Lactobacillus plantarum*. Appl Microbiol Biotechnol 2010b;85:643–50.

Olofsson K, Bertilsson M, Lidén G. A short review on SSF - an interesting process option for ethanol production from lignocellulosic feedstocks. Biotechnol Biofuels 2008;1:7.

Olson DG, McBride JE, Shaw AJ, Lynd LR. Recent progress in consolidated bioprocessing. Curr Opin Biotechnol 2012;23:396–405.

Ozkose E, Akyol I, Kar B, Comlekcioglu U, Ekinci MS. Expression of fungal cellulase gene in *Lactococcus lactis* to construct novel recombinant silage inoculants. Folia Microbiol. (Praha) 2009;54:335–42.

Panesar PS, Kennedy JF, Gandhi DN, Bunko K. Bioutilisation of whey for lactic acid production Food Chemistry 2007a;105:1–14

Panesar PS, Kennedy JF, Knill CJ, Kosseva MR. Applicability of pectate-entrapped *Lactobacillus casei* cells for L(+) lactic acid production from whey. Appl Microbiol Biotechnol 2007b;74:35–42.

Papadimitriou K, Alexandraki V and Tsakalidou E. Fighting off human infections: a new role for bacteriocin molecules. In: Pessione E, editor. Interactive probiotics. Boca Raton, FL 33487, U.S.A.: CRC Press, Taylor & Francis Group, LLC; 2014. p. 148–176.

Papagianni M. Metabolic engineering of lactic acid bacteria for the production of industrially important compounds. Computat Struct Biotechnol J 2012;3:e201210003.

Park KB, Oh SH. Production of yogurt with enhanced levels of gamma-aminobutyric acid and valuable nutrients using lactic acid bacteria and germinated soybean extract. Bioresour Technol 2007;98:1675–9.

Park SH, Itoh K, Kikuchi E, Niwa H, Fujisawa T. Identification and characteristics of nisin Z-producing *Lactococcus lactis* subsp. *lactis* isolated from Kimchi. Curr Microbiol 2003;46:385–8.

Passoth V, Blomqvist J, Schnürer J. *Dekkera bruxellensis* and *Lactobacillus vini* form a stable ethanol-producing consortium in a commercial alcohol production process. Appl Environ Microbiol 2007;73:4354–6.

Patnaik PR. Cognitive optimization of microbial PHB production in an optimally dispersed bioreactor by single and mixed cultures. Bioprocess Biosyst Eng 2009;32:557–68.

Patnaik PR. Perspectives in the modelling and optimization of PHB production by pure and mixed cultures. Crit Rev Biotechnol 2005;25:153–71.

Pessione A. The Double Face of Bacteria and the Ambiguous Role of Some Probiotics. In: Pessione E, editor. Interactive probiotics. Boca Raton, FL 33487, U.S.A.: CRC Press, Taylor & Francis Group, LLC; 2014. p. 196–214.

Pessione A, Zapponi M, Mandili G, Fattori P, Mangiapane E, Mazzoli R, et al. Enantioselective lactic acid production by an *Enterococcus faecium* strain showing potential in agro-industrial waste bioconversion: physiological and proteomic studies. J Biotechnol 2014;173:31-40.

Pessione E, Mazzoli R, Giuffrida MG, Lamberti C, Garcia-Moruno E, Barello C, et al. A proteomic approach to studying biogenic amine producing lactic acid bacteria. Proteomics. 2005;5: 687–98.

Pessione E, Pessione A, Lamberti C, Coïsson DJ, Riedel K, Mazzoli R, et al. First evidence of a membrane-bound, tyramine and beta-phenylethylamine producing, tyrosine decarboxylase in *Enterococcus faecalis*: a two-dimensional electrophoresis proteomic study. Proteomics 2009;9:2695–710.

Pessione E. Lactic acid bacteria contribution to gut microbiota complexity: lights and shadows. Front Cell Infect Microbiol 2012;2:86.

Pinto JP, Zeyniyev A, Karsens H, Trip H, Lolkema JS, Kuipers OP, Kok J. pSEUDO, a genetic integration standard for *Lactococcus lactis*. Appl Environ Microbiol 2011;77:6687–90.

Piper C, Hill C, Cotter PD, Ross RP. Bioengineering of a Nisin A-producing *Lactococcus lactis* to create isogenic strains producing the natural variants Nisin F, Q and Z. Microb Biotechnol 2011;4:375–82.

Racine FM, Saha BC. Production of mannitol by *Lactobacillus intermedius* NRRL B-3693 in fed-batch and continuous cell-recycle fermentations. Process Biochem 2007;42:1609–13.

Raha AR, Chang LY, Sipat A, Yusoff K, Haryanti T. Expression of a thermostable xylanase gene from *Bacillus coagulans* ST-6 in *Lactococcus lactis*. Lett Appl Microbiol 2006;42:210–4.

Reddy G, Altaf Md, Naveena BJ, Venkateshwar M, Kumar EV. Amylolytic bacterial lactic acid fermentation - A review. Biotechnol Adv 2008;26:22–34.

Richter H, Vlad D, Unden G. Significance of pantothenate for glucose fermentation by *Oenococcus oeni* and for suppression of the erythritol and acetate production. Arch Microbiol 2001;175: 26–31.

Roach DR, Khatibi PA, Bischoff KM, Hughes SR, Donovan DM. Bacteriophage-encoded lytic enzymes control growth of contaminating *Lactobacillus* found in fuel ethanol fermentations. Biotechnol Biofuels 2013;6:20.

Rossi F, Rudella A, Marzotto M, Dellaglio F. Vector-free cloning of a bacterial endo-1,4-β-glucanase in *Lactobacillus plantarum* and its effect on the acidifying activity in silage: Use of recombinant cellulolytic *Lactobacillus plantarum* as silage inoculants. Antonie van Leeuwenhoek 2001;80:139–47.

Rouse S, Field D, Daly KM, O'Connor P, Cotter PD, Hill C, et al. Bioengineered nisin derivatives with enhanced activity in complex matrices. Microb Biotechnol 2012;5:501–8.

Roy D, Goulet J, LeDuy A. Batch fermentation of whey ultrafiltrate by *Lactobacillus helveticus* for lactic acid production. Applied Microbiol Biotechnol 1986;24:206–13.

Roy D, Goulet J, LeDuy A. Continuous production of lactic acid from whey permeate by free and calcium alginate entrapped *Lactobacillus bulgaricus*. J Dairy Sci 1987;70:506–13.

Salvatore S, Hauser B, Devreker T, Vieira MC, Luini C, Arrigo S, et al. Probiotics and zinc in acute infectious gastroenteritis inchildren: are they effective? Nutrition 2007;23:498–506.

Sang Y, Blecha F. Antimicrobial peptides and bacteriocins: alternatives to traditional antibiotics. Anim Health Res Rev 2008;9:227–35.

Sauer M, Porro D, Mattanovich D, Branduardi P. Microbial production of organic acids: expanding the markets. Trends Biotechnol 2008;26:100–8.

Scheirlinck T, Mahillon J, Joos K, Dhaese P, Michiels F. Integration and expression of α-amylase and endoglucanase genes in the *Lactobacillus plantarum* chromosome. Appl Environ Microbiol 1989;55:2130–7.

Schepers AW, Thibault J, Lacroix C. Continuous lactic acid production in whey permeate/yeast extract medium with immobilized *Lactobacillus helveticus* in a two-stage process: Model and experiments. Enzyme Microb Technol 2006;38:324–37

Settanni L, Corsetti A. Application of bacteriocins in vegetable food biopreservation. Int J Food Microbiol 2008;121:123–38.

Settanni L, Moschetti G. Non-starter lactic acid bacteria used to improve cheese quality and provide health benefits. Food Microbiol 2010;27:691–7.

Shaw AJ, Podkaminer KK, Desai SG, Bardsley JS, Rogers SR, Thorne PG, et al. Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield. Proc Natl Acad Sci U S A 2008;105:13769–74.

Shen CR, Lan EI, Dekishima Y, Baez A, Cho KM, Liao JC. Driving forces enable high-titer anaerobic 1-butanol synthesis in *Escherichia coli*. Appl Environ Microbiol 2011;77:2905–15.

Siegel RE. Emerging gram-negative antibiotic resistance: daunting challenges, declining sensitivities, and dire consequences. Respir Care 2008;53:471–9.

Silveira MM, Jonas R. The biotechnological production of sorbitol. Appl Microbiol Biotechnol 2002;59:400–8.

Singh SK, Ahmed SU, Pandey A. Metabolic engineering approaches for lactic acid production. Process Biochemistry 2006;41:991–1000.

Singh S, Ray SS. Polylactide based nanostructured biomaterials and their applications. J Nanosci Nanotechnol 2007;7:2596–615.

Siragusa S, De Angelis M, Di Cagno R, Rizzello CG, Coda R, Gobbetti M. Synthesis of gamma-aminobutyric acid by lactic acid bacteria isolated from a variety of Italian cheeses. Appl Environ Microbiol 2007;73:7283–90.

Solem C, Dehli T, Jensen PR. Rewiring *Lactococcus lactis* for ethanol production. Appl Environ Microbiol 2013;79:2512–8.

Sørvig E, Mathiesen G, Naterstad K, Eijsink VG, Axelsson L. High-level,inducible gene expression in *Lactobacillus sakei* and *Lactobacillus plantarum* using versatile expression vectors. Microbiology 2005;151:2439–49.

Sosunov VV, Mischenko B, Eruslanov E, Svetoch Y, Shakina N, Stern K, et al. Antimycobacterial activity of bacteriocins and their complexes with liposomes. J Antimicrob Chemother 2007;59:919–25.

Sun Y, Olivier JD. Antimicrobial action of some GRAS compounds against *Vibrio vulnificus*. Food Addit Contam 1994;11:549–58.

Sybesma W, Burgess C, Starrenburg M, van Sinderen D, Hugenholtz J. Multivitamin production in *Lactococcus lactis* using metabolic engineering. Metab Eng 2004;6:109–115.

Tamura T, Noda M, Ozaki M, Maruyama M, Matoba Y, Kumagai T, et al. Establishment of an efficient fermentation system of gamma-aminobutyric acid by a lactic acid bacterium, *Enterococcus avium* G-15, isolated from carrot leaves. Biol Pharm Bull 2010;33:1673–9.

Tango MSA, Ghali AE. Effect of temperature on lactic acid production from cheese whey using *Lactobacillus helveticus* under batch conditions. Biomass Bioenergy 1999;16:61–78.

Tanaka K, Katamune K, Ishizaki A. Fermentative production of poly-(β-hydroxybutyric acid) from xylose via L-lactate by a two stage culture method employing *Lactococcus lactis* IO-1 and *Alcaligenes eutrophus*. Can J Microbiol 1995;41:257–61.

Tanaka K, Komiyama A, Sonomoto K, Ishizaki A, Hall SJ, Stanbury PF. Two different pathways for D-xylose metabolism and the effect of xylose concentration on the yield coefficient of L-lactate in mixed-acid fermentation by the lactic acid bacterium *Lactococcus lactis* IO-1. Appl Microbiol Biotechnol 2002;60:160–7.

Terpstra AHM. Effet of conjugated linoleic acid on body composition and plasma lipids in humans: an overview of the literature. Am J Clin Nutrit 2004;79:352–61.

Thomsen MH, Guyot JP, Kiel P. Batch fermentations on synthetic mixed sugar and starch medium with amylolytic lactic acid bacteria. Appl Microbiol Biotechnol 2007;74:540–6.

Tohyama M, Patarinska T, Qiang Z, Shimizu K. Modeling of the mixed culture and periodic control for PHB production. J Biochem Eng 2002;10:157–73.

Tohyama M, Shimizu K. Control of a mixed culture of *Lactobacillus delbrueckii* and *Ralstonia eutropha* for the production of PHB from glucose via lactate. J Biochem Eng 1999;4:45–53.

Tohyama M, Takagi S, Shimizu K. Effect of controlling lactate concentration and periodic change in DO concentration on fermentation characteristics of a mixed culture of *Lactobacillus delbrueckii* and *Ralstonia eutropha* for PHB production. J Biosci Bioeng 2000;89:323–8.

Tomas CA, Welker NE, Papoutsakis ET. Overexpression of groESL in *Clostridium acetobutylicum* results in increased solvent production and tolerance, prolonged metabolism, and changes in the cell's transcriptional program. Appl Environ Microbiol 2003;69:4951–65.

Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 2006;444:1027–31.

van Asseldonk M, de Vos WM, Simons G. Functional analysis of the *Lactococcus lactis* usp45 secretion signal in the secretion of a homologous proteinase and aheterologous alpha-amylase. Mol Gen Genet 1993;240:428–34.

van Pijkeren JP, Britton RA. High efficiency recombineering in lactic acid bacteria. Nucleic Acids Res 2012;40:e76.

van Staden AD, Brand AM, Dicks LM. Nisin F-loaded brushite bone cement prevented the growth of *Staphylococcus aureus in vivo*. J Appl Microbiol 2012;112:831–40.

Vasala A, Panula J, Neubauer P. Efficient lactic acid production from high salt containing dairy by-products by *Lactobacillus salivarius* ssp *salicinius* with pre-treatment by proteolytic microorganisms. J Biotechnol 2005;117:421–31.

Vishnu C, Seenayya G, Reddy G. Direct fermentation of starch to L(+) lactic acid by amylase producing *Lactobacillus amylophilus* GV6. Bioprocess Eng 2000;23:155–8.

Vishnu C, Seenayya G, Reddy G. Direct fermentation of various pure and crude starchy substrates to L(+) lactic acid using *Lactobacillus amylophilus* GV6. World J Microbiol Biotechnol 2002;18:429–33.

Vos P, Garrity G, Jones D, Krieg NR, Ludwig W, Rainey FA, et al. Bergey's Manual of Systematic Bacteriology: Volume 3: The Firmicutes. Springer; 2011.

Wahle KWJ, Heys SD, Rotondo D. CLA: are they beneficial or detrimental to health? Progr Lip Res 2004;43:553–87.

Wee YJ, Kim JN, Yun JS, Ryu HW. Utilization of sugar molasses for economical L(+)-lactic acid production by batch fermentation of *Enterococcus faecalis*. Enzyme Microb Technol 2004;35:568–73.

Wee YJ, Kim JN, Ryu HW. Biotechnological production of lactic acid and its recent applications. Food Technol Biotechnol 2006;44:163–72.

Wee YJ, Ryu HW. Lactic acid production by *Lactobacillus sp* RKY2 in a cell-recycle continuous fermentation using lignocellulosic hydrolyzates as inexpensive raw materials. Bioresour Technol 2009;100:4262–70.

Welman AD, Maddox IS. Exopolysaccharides from lactic acid bacteria: perspectives and challenges. Trends Biotechnol 2003;21:269–74.

Wieczorek AS, Martin VJ. Effects of synthetic cohesin-containing scaffold protein architecture on binding dockerin-enzyme fusions on the surface of *Lactococcus lactis*. Microb Cell Fact 2012;11:160.

Wieczorek AS, Martin VJ. Engineering the cell surface display of cohesins for assembly of cellulosome-inspired enzyme complexes on *Lactococcus lactis*. Microb Cell Fact. 2010;9:69.

Willey JM, van der Donk WA. Lantibiotics: peptides of diverse structure and function. Annu Rev Microbiol 2007;61:477–501.

Wilson DB. Microbial diversity of cellulose hydrolysis. Curr Opin Microbiol 2011;14:259–63.

Wisselink H, Moers AP, Mars AE, Hoefnagel MHN, de Vos WM, Hugenholtz J. Overproduction of heterologous mannitol1-phosphatase: a key factor for engineering mannitol production by *Lactococcus lactis*. Appl Environ Microbiol 2005;71:1507–14.

Wisselink HW, Weusthuis RA, Eggink G, Hugenholtz J, Grobben GJ. Mannitol production by lactic acid bacteria: a review. Int Dairy J 2002;12:151–61.

Wong DWS, Camirand WM, Pavlath AE, Parris N, Friedman M. Structures and functionalities of milk proteins. Crit Rev Food Sci 1996;36:807–44.

Xiaodong W, Xuan G, Rakshit SK. Direct fermentation of lactic acid from cassava or other starch substrates. Biotechnol Lett 1997;9: 841–3.

Yamada R, Hasunuma T, Kondo A. Endowing non-cellulolytic microorganisms with cellulolytic activity aiming for consolidated bioprocessing. Biotechnol Adv 2013;31:754–63.

Yilmaz M, Soran H, Beyatli Y. Determination of poly-beta-hydroxybutyrate (PHB) production by some *Bacillus spp.* World J Microbiol Biotechnol 2005;21:565–6.

You C, Zhang XZ, Sathitsuksanoh N, Lynd LR, Zhang YH. Enhanced microbial utilization of recalcitrant cellulose by an *ex vivo* cellulosome-microbe complex. Appl Environ Microbiol 2012;78:1437–44.

Yu RU, Hang YD. Kinetics of direct fermentation of agricultural commodities to L(+) lactic acid by *Rhizopus oryzae*. Biotechnol Lett 1989;11:597–600.

Zaunmüller T, Eichert M, Richter H, Unden G. Variations in the energy metabolism of biotechnologically relevant heterofermentative lactic acid bacteria during growth on sugars and organic acids. Appl Microbiol Biotechnol 2006;72:421–9.

Figure captions

Fig. 1. Schematic overview of the metabolic pathways enabling LAB to produce some of the most industrially desired molecules by sugar fermentation. Thin arrows stand for single enzymatic reactions, while thick arrows stands for multiple reaction pathways. AlaDH, alanine dehydrogenase; ALS, α-acetolactate synthase; EPS, exopolysaccharides; GAP, glyceraldehyde-3-phosphate; LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase; PFL, pyruvate-formiate lyase; PHB, polyhydroxybutyrate.

Fig. 2. Diacetyl biosynthetic pathway in *Lc. lactis*. ALDB, α-acetolactate decarboxylase; ALS, α-acetolactate synthase; ILVNB, acetohydroxy acid synthase.

Fig. 3. Examples of effective strategies for improving the production of sweeteners: 1) sorbitol, 2) mannitol, and 3) L-alanine, from sugars by means of LAB (adapted from Hugenholtz et al., 2011). Inactivation of LDH (ΔLDH) is a common feature to improve intracellular NADH concentration. AlaDH, alanine dehydrogenase; Mth1PDH, mannitol-1-phosphate dehydrogenase; Mtl1Pase, mannitol-1-phosphate phosphatase; Stl6PDH, sorbitol-6-phosphate dehydrogenase; Stl6Pase, sorbitol-6-phosphate phosphatase.

Fig. 4. Scheme representing: A) general routes for homo-EPS (i.e., glucans and fructans) biosynthesis from sucrose; B) model of hetero-EPS biosynthesis in Lc. lactis NIZO (adapted from Welman and Maddox, 2003). GlysucA, glycansucraseA; GlysucB, glycansucrase B, EpsA, B, D, E, F, G, H, I, J, and K, gene products involved in EPS biosynthesis; Frt, fructose; Glc, glucose; Gal, galactose; Rha, rhamnose.

Fig. 5. Prospects and broad applications of a future LAB-based biorefinery. EPS, exopolysaccharides; GABA, γ-amino butyric acid; PHA, polyhydroxyalkanoates; PLA, polylactide.

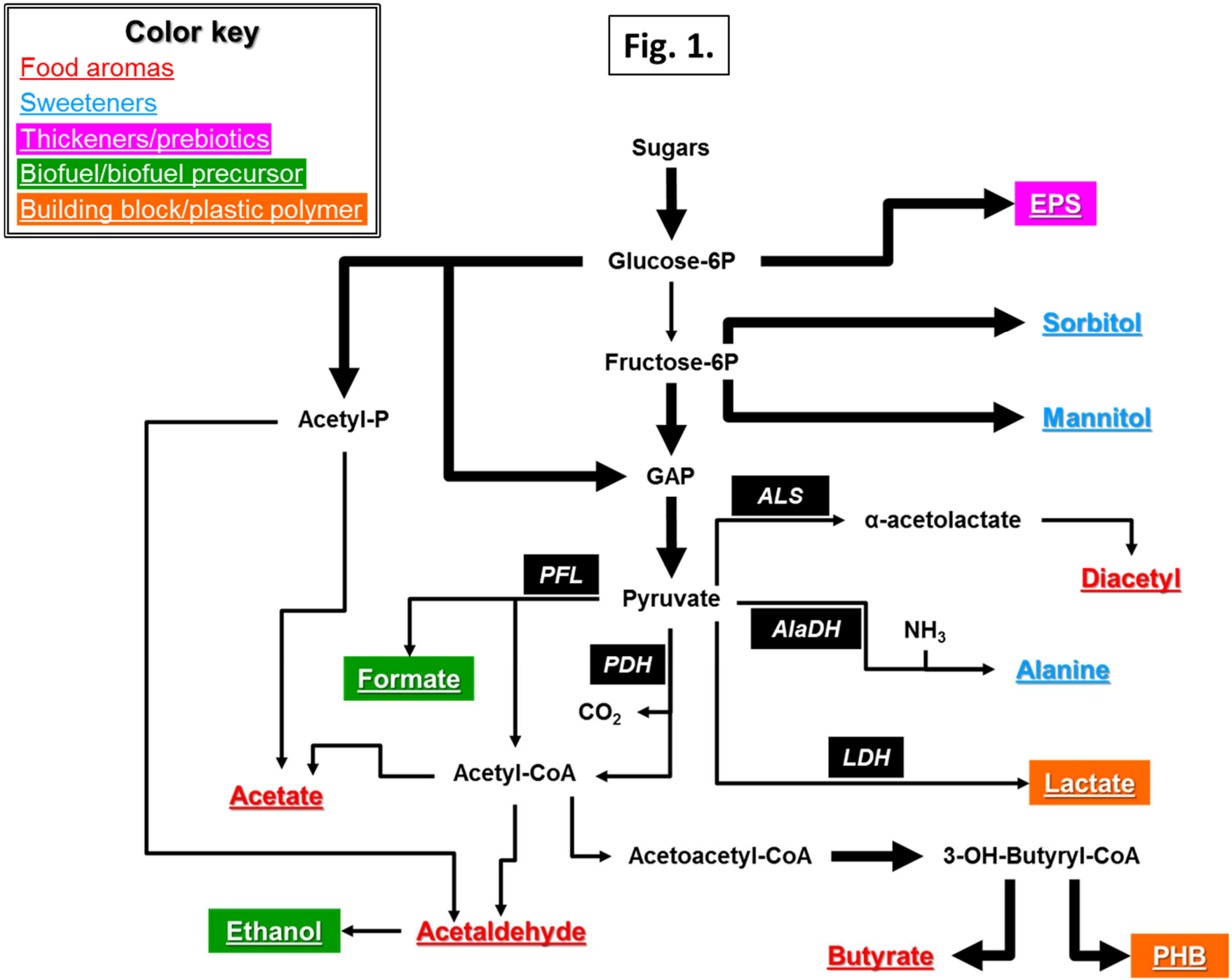

Color key
Food aromas
Sweeteners
Thickeners/prebiotics
Biofuel/biofuel precursor
Building block/plastic polymer
Fig. 1.
Sugars
Glucose-6P
EPS
Fructose-6P
Sorbitol
Mannitol
Acetyl-P
GAP
ALS
α-acetolactate
Diacetyl
PFL
Pyruvate
AlaDH
NH3
Alanine
Formate
PDH
CO2
LDH
Lactate
Acetyl-CoA
Acetate
Acetoacetyl-CoA
3-OH-Butyryl-CoA
Ethanol
Acetaldehyde
Butyrate
PHB

Figure 2

Fig. 2.

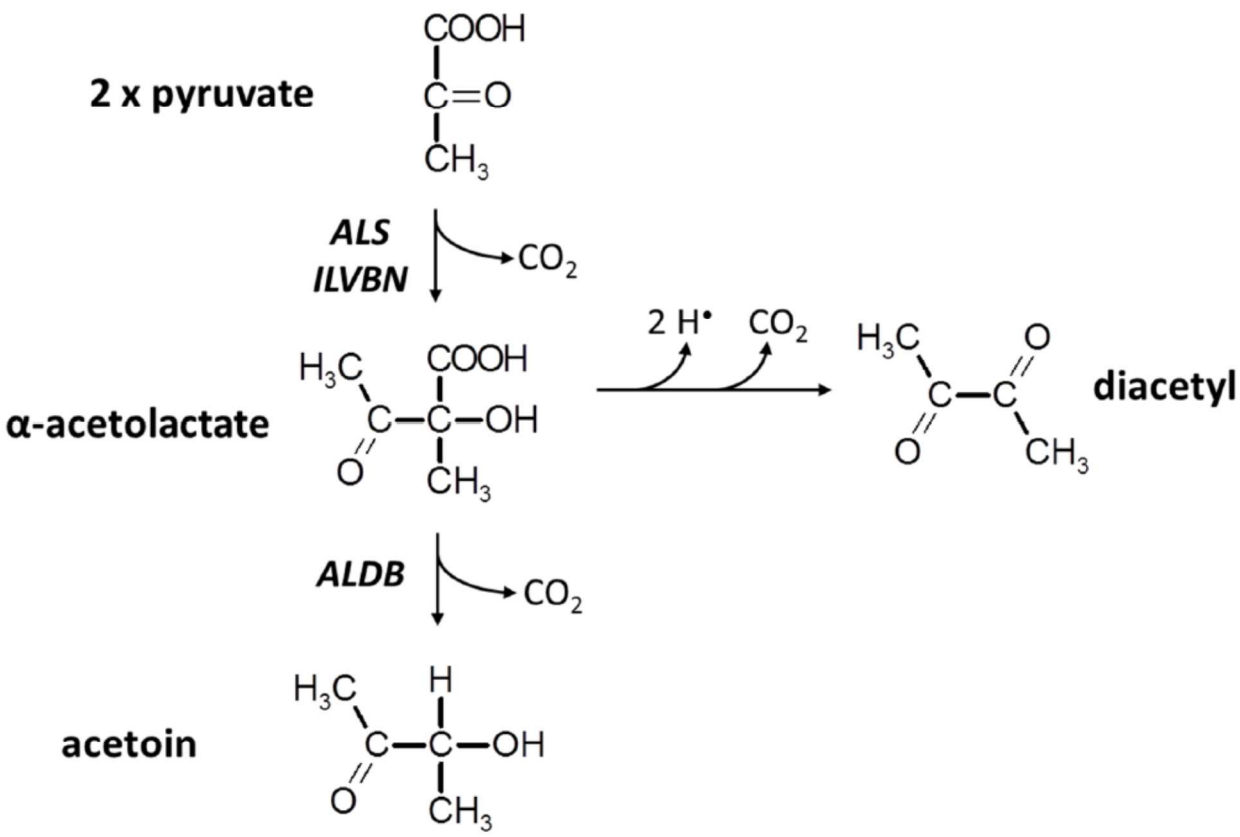


Figure 3

Fig. 3.

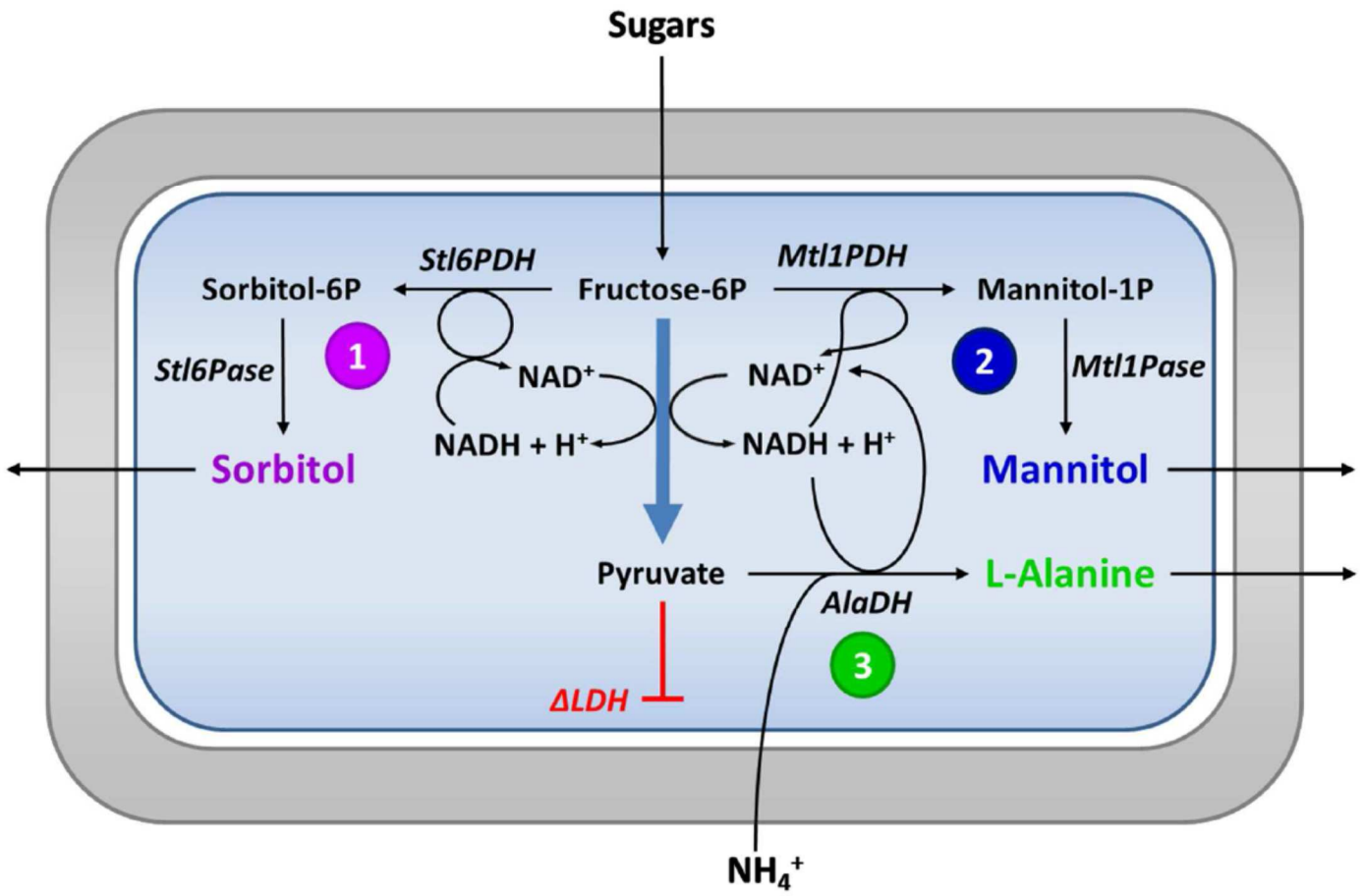
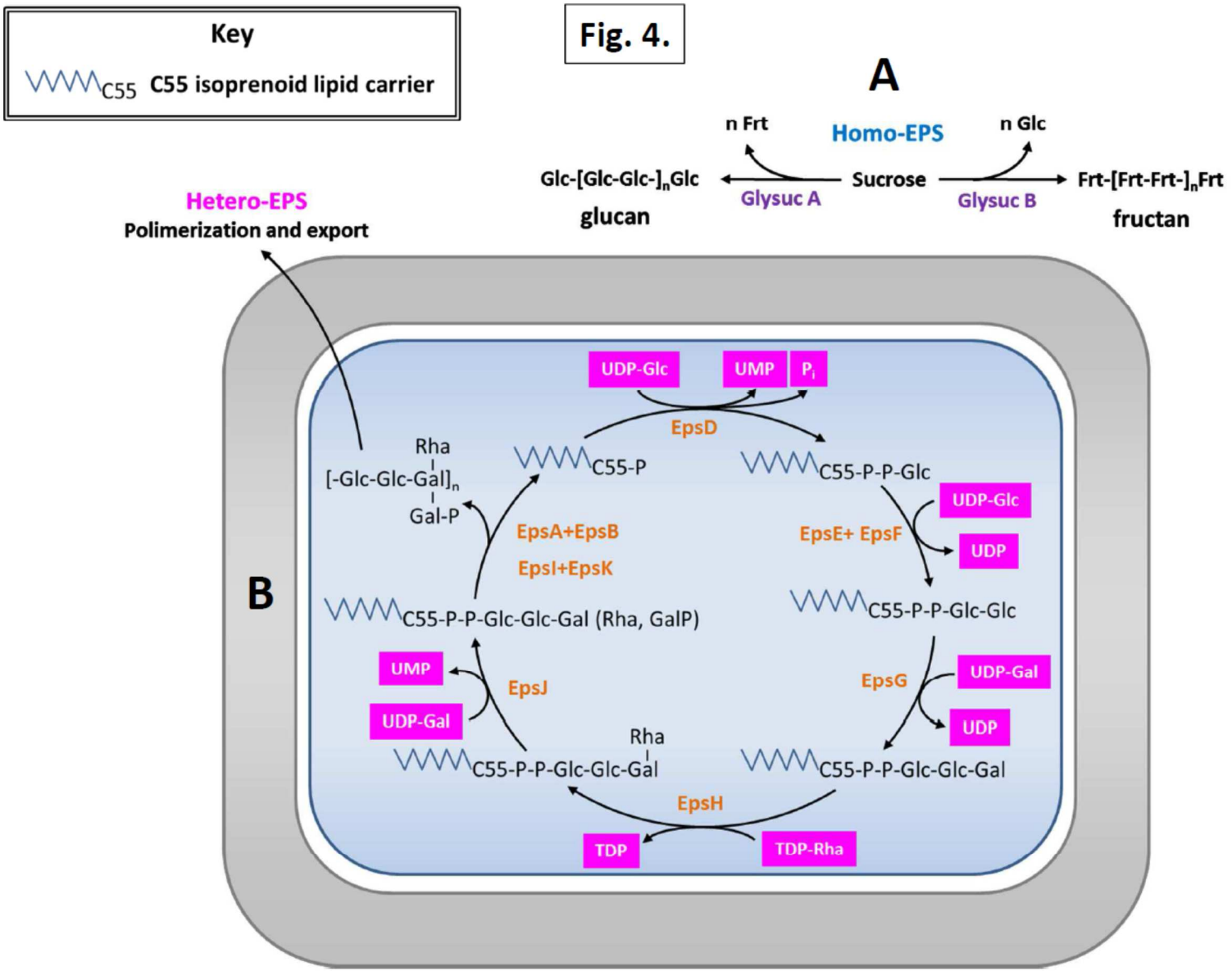


Figure 4


Key
C55 C55 isoprenoid lipid carrier
Fig. 4.
A
n Frt
Homo-EPS
n Glc
Glc-[Glc-Glc-]nGlc
Sucrose
Frt-[Frt-Frt-]nFrt
Glysuc A
Glysuc B
glucan
fructan
Hetero-EPS
Polimerization and export
B
UDP-Glc
UMP
Pi
EpsD
C55-P
C55-P-P-Glc
Rha
[-Glc-Glc-Gal]n
Gal-P
EpsA+EpsB
EpsI+EpsK
EpsE+ EpsF
UDP-Glc
UDP
C55-P-P-Glc-Glc-Gal (Rha, GalP)
C55-P-P-Glc-Glc
UMP
UDP-Gal
EpsJ
EpsG
UDP-Gal
UDP
Rha
C55-P-P-Glc-Glc-Gal
C55-P-P-Glc-Glc-Gal
EpsH
TDP
TDP-Rha

new Table 1

Strains	Heterologous protein(s) expressed	Heterologous protein expression/secretion level[a]	Improved phenotypic properties of the strain	References
Strains with improved amylolytic properties				
Lb. casei BLSJ 03135	α-amylase (AmyA) from *Str. bovis* 148	≈ 900 U/L (N3-G5-β-CNP)[b]	Not described	Narita et al., 2006
Lb. plantarum NCIMB 8826 (Δ*ldhL1*)	α-amylase (AmyA) from *Str. bovis* 148	714 U/L (N3-G5-β-CNP)[b]	Growth on raw corn starch	Okano et al., 2009c
Lc. lactis IL 1403	α-amylase (AmyA) from *Str. bovis* 148	≈ 500 U/L (N3-G5-β-CNP)[b]	Growth on soluble starch	Okano et al. 2007
Lc. lactis MG1363	α-amylase (AmyS) from *Bacillus stearothermophilus*	600 U/L (amylose azure)[b]	Hydrolysis of starch	Van Asseldonk et al., 1993
Strains with improved cellulolytic properties				
Lb. gasseri ATCC 33323	Cel8A endoglucanase from *C. thermocellum*	722 U/L (CMC)[b]	Hydrolysis of CMC	Cho et al., 2000
Lb. jonhsonii NCK 88	Cel8A endoglucanase from *C. thermocellum*	759 U/L (CMC)[b]	Hydrolysis of CMC	Cho et al., 2000
Lb. plantarum strains B41 and Lp80	Cel8A cellulase from *Bacillus sp.* N-4	34.24/43.61 U/L (CMC)[b]	Increased silage acidification	Rossi et al., 2001
Lb. plantarum Lp80	Cel8A endoglucanase from *C. thermocellum*	≈ 90 U/L (CMC)[b]	Hydrolysis of CMC	Scheirlinck et al., 1989
Lb. plantarum NCDO 1193	Cel5E endoglucanase from *C. thermocellum*	1996 U/L (CMC)[b]	Hydrolysis of CMC	Bates et al., 1989
Lb. plantarum NCIMB 8826 (Δ*ldh1*)	Cel8A endoglucanase from *C. thermocellum*	6.03 U/L (barley β-glucan)[b]	Growth on cellohexaose	Okano et al., 2010b
Lb. plantarum WCFS1	Cel6A endoglucanase from *Thermobifida fusca*	280 U/L (PASC)[b]	Hydrolysis of sodium hypochlorite-pretreated wheat straw	Morais et al., 2013
Lc. lactis strains IL1403 and MG1363	Cellulase from *Neocallimastix sp.*	5.9 U (CMC)[b, c]	Hydrolysis of CMC	Ozkose et al., 2009
Lc. lactis HtrA NZ9000	Fragments of CipA scaffoldin from *C. thermocellum*	9×10^3 scaffolds/cell[d]	Scaffoldins displayed on the cell surface	Wieckzoreck and Martin, 2010
Strains with improved hemicellulose-metabolizing properties				
Lb. plantarum NCIMB 8826 (Δ*ldh1-xpk1*)	Transketolase (Tkt) from *Lc. lactis* IL1403 (replacing endogenous phosphoketolase Xpk1)	Not determined	Almost homolactic fermentation of arabinose	Okano et al. 2009a
Lb. plantarum NCIMB	Transketolase (Tkt) from *Lc. lactis* IL1403	Not determined	Almost homolactic	Okano et al. 2009b

8826 (*Δldh1-xpk1-xpk2*)	(replacing endogenous phosphoketolase Xpk1 and Xpk2); Xylose isomerase (XylA) and xylulose kinase (XylB) from *Lb. pentosus* NRIC 1069		fermentation of xylose	
Lb. plantarum WCFS1	Xyn11A endoxylanase from *Thermobifida fusca*	3360 U/L (oat spelt xylan)[b]	Hydrolysis of sodium hypochlorite-pretreated wheat straw	Morais et al., 2013
Lc. lactis MG1316	Xylanase from *Bacillus coagulans* ST-6	≈87 U/L (xylan)[c]	Hydrolysis of RBB-xylan	Raha et al., 2006

[a]Maximum values reported in each study. Substrates used for determining enzyme activity are indicated in parentheses. CMC, carboxy methyl cellulose; N3-G5-β-CNP, 2-chloro-4-nitrophenyl-6^5-azido-6^5-deoxy-β-maltopentaoside; PASC, phosphoric acid-swollen cellulose

[b]Enzyme activity/protein quantification measured in extracellular fraction

[c]The volume of extracellular extract used in this study was not reported

[d]Proteins displayed on the cell surface

Table 1. Recombinant LAB showing improved amylolytic, cellulolytic or hemicellulolytic properties described in this study.

1

new Table 2

Microorganisms	Carbon source	T and pH	Fermentation mode	LA (g/L)	Yield $Y_{P/S}$ (g/g)	Productivity (g/L/h)	References
Strains able to ferment whey							
Lb. casei NBIMCC 1013	Whey permeate (+YE)	37°C, pH 6.5	Ca-pectate immobilized cells	≈33	≈0.87	-	Panesar et al., 2007b
Lb. helveticus ATCC 15009	Cheese whey	42°C, pH 5.8	Batch	9.3	0.36	-	Tango and Ghaly, 1999
Lb. helveticus milano	Whey permeate (+ CSL)	42°C, pH 5.9	Batch	35	≈0.5	2.7	Roy et al., 1986
Lb. helveticus milano	Whey permeate (+ YE)	42°C, pH 5.9	Continuous fermentation (dilution rate 0.35 h^{-1}), Ca-alginate entrapped cells	-	-	9.7	Roy et al., 1987
Lb. helveticus milano	Whey permeate powder (+ YE)	42°C pH 5.9	Batch	-	-	5.4	Amrane and Prigent, 1998
Lb. helveticus R211	Whey permeate (+YE)	42°C, pH 5.5	Continuous fermentation, K-carrageenan/locust bean gum immobilized cells	-	-	19-22	Schepers et al., 2006
Lb. salivarum ssp. *salicinius*	Whey	30°C, pH 6.5	Batch	≈10	-	0.2	Vasala et al., 2005
	Whey (+ protease enzymes)			≈50	-	0.9	
	Whey (+ proteolytic *Bacillus megaterium*)			≈50	-	0.8	
Strains able to ferment molasses							
Ent. faecalis RKY1	Molasses (+ YE)	38°C, pH 7.0	Batch	95.7	0.95	4.0	Wee et al., 2004
Lb. delbrueckii C.E.C.T. 286	Beet molasses	49°C, pH 5.9	Batch	-	0.91	-	Monteagudo et al., 1997
Lb. delbrueckii IFO3202	Beet molasses	45°C, pH 6.0	Batch	61	0.96	-	Göksungur and Güvenç, 1999
			Batch, Ca-alginate immobilized cells	59	0.90	-	
Lb. delbrueckii JCM 1148	Sugarcane molasses	40°C, pH 6	Batch (fermenter)	107	0.9	1.48	Calabia and Tokiwa, 2007
Lb. delbrueckii NCIMB 8130	Sucrose (+ YE + $CaCO_3$)	45°C	Batch	88.4	0.97	-	Kotzanmanidis et al., 2002
Lb. lactis NCIM 2368 RM2-24	Hydrolyzed cane sugar	42°C, pH 7	Batch	81	-	1.68	Joshi et al., 2010
	Molasses			70	-	1.45	

Lc. lactis ssp. *cremoris* IFO3427	Molasses	37°C, pH 6.8	Stirred ceramic membrane reactor perfusion	46	-	15.8	Ohashi et al., 1999
Strains able to ferment starchy substrates							
Lb. amylophilus GV6	Soluble starch	37°C, pH 6.5	Batch	-	0.90	-	Vishnu et al., 2000
	Corn starch			-	0.82	-	
Lb. amylophilus GV6	Soluble starch	37°C, pH 6.5	Batch	9.6	0.96	-	Vishnu et al., 2002
	Corn starch			8.8	0.94	-	
	Potato starch			8.7	0.92	-	
	Sorghum starch grain			11.6	0.92	-	
	Cassava starch grain			14	0.92	-	
	Barley starch grain			11.3	0.90	-	
	Rice starch grain			13	0.91	-	
	Wheat starch grain			11.7	0.93	-	
Lb. amylophilus GV6	Starch (+ red lentil and baker's yeast)	37°C, pH 6.5	Batch	12.2	0.92	-	Altaf et al., 2005
Lb. manihotivorans LMG 18010T	Starch	35°C, pH 6.0	Batch (fermenter)	12.6	0.67	0.5	Guyot et al., 2000
Lb. plantarum A6	Cassava raw starch	30°C, pH 6.0	Batch (fermenter)	41	0.9	-	Giraud et al., 1994
Lb. plantarum A6	Synthetic brown juice	40°C	Batch (fermenter)l	14.25	-	≈0.59	Thomsen et al., 2007
Str. bovis 148	Raw starch	37°C, pH 6.0	Batch (fermenter)	14.7	0.88	-	Narita et al., 2004
Strains able to ferment lignocellulosic substrates							
Ent. faecalis RKY1	Barley	38°C, pH 7.0	Batch (fermenter)	-	0.94	0.88	Oh et al., 2005
	Wheat			-	0.93	0.81	
	Corn			-	0.94	0.51	
Lactobacillus sp. RKY2	Oak wood chip hydrolyzate (+ CSL)	36°C, pH 6	Continuous cell recycle (dilution rate 0.16 h^{-1})	42	0.95	6.7	Wee and Ryu, 2009
Lb. amylophilus GV6	Wheat bran	37°C	Solid state fermentation	-	0.42	-	Naveena et al., 2005b
Lb. brevis S3F4	Corn stover hydrolysate	30°C	Batch	18.2	0.74	0.76	Guo et al., 2010
	Corncob hydrolysate			39.1	0.69	0.81	
Lb. casei + *Lb. delbrueckii*	Protease-treated wheat bran	37°C	Batch	123	0.95	2.3	John et al., 2006

Lb. delbrueckii mutant Uc-3	Sugarcane bagasse cellulose	42°C, pH 6	Batch (Simultaneous saccharification and fermentation)	67	0.83	0.93	Adsul et al., 2007a
Lb. delbrueckii mutant Uc-3	Cellobiose	42°C, pH 6.5	Batch	90	0.9	2.25	Adsul et al., 2007b
	Cellotriose			1.7	0.85	-	
Lb. pentosus CECT-4023T	trimming vine shoots hydrolyzate	31°C, pH 6.5	Batch	46	0.78	0.933	Bustos et al., 2005
Lb. pentosus CECT-4023T	Barley bran hydrolysate	31°C, pH 6.0	Batch (fermenter)	33	0.57	0.60	Moldes et al., 2006
	Corncob hydrolysate			26	0.53	0.34	
	Trimming vine shoot hydrolysate			24	0.76	0.51	
	Detoxified *Eucalyptus globulus* hydrolysate			14.5	0.70	0.28	
Lb. rhamnosus ATCC 7469	Recycled paper sludge	37°C, pH 5.5	Batch (Simultaneous saccharification and fermentation)	73	0.97	2.9	Marques et al, 2008
Lb. rhamnosus LA-04-1	Wheat bran hydrolysate (+ CSL)	42°C, pH 6.25	Batch (fermenter)	-	0.87	1.68	Li et al., 2010b
Lc. lactis I0-1 JCM 7638	Xylose	-	-	33	0.60	-	Doran-Peterson et al., 2008
Lc. lactis IO-1 JCM 7638	Sugarcane bagasse	37°C	Batch	10.9	-	≈ 0.17	Laopaiboon et al., 2010
Leu. lactis	Hydrolyzed xylan	37°C	Batch	2.3	-	-	Ohara et al., 2006

Table 2. Natural and mutant (i.e., not-engineered) LAB used to ferment lignocellulosic, molasses, starchy and whey substrates described in this study. CSL, corn steep liquor; YE, yeast extract

1

new Table 3

	Carbon source	*Lb. brevis*	*Lb. hilgardii*	*Lb. kunkeei*	*Lb. plantarum*	*Ped. damnosus*	*Ped. parvulus*	*Ped. pentosaceus*	*Saccharomyces cerevisiae*	*Scheffersomyces stipitis* (*Pichia stipitis*)	*Candida shahatae*	*Kluyveromyces marxianus*	*Escherichia coli* (FBR2)	*Zymomonas mobilis* (Zm4)
Natural carbon utilization	Arabinose	+	–	–	**v**	–	–	+	–	–	+	+	+	+
	Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+
	Lactose	**v**	**v**	–	+	–	–	**v**	–	–	–	–	–	+
	Mannitol	–	–	+	+	–	–	–	–	–	–	–	–	–
	Maltose	+	+	–	+	**v**	+	+	+	+	+	+	+	+
	Melezitose	–	**v**	–	+	**v**	–	–	–	–	–	–	–	–
	Ribose	+	+	–	+	–	–	+	–	–	–	–	–	–
	Sucrose	**v**	**v**	+	+	**v**	–	–	+	+	+	+	+	+
	Trehalose	–	–	–	+	+	**v**	+	+	+	+	–	–	–
	Xylose	**v**	+	–	**v**	–	–	**v**	-	+	+	+	+	+
Growth range	Temperature	26–45°C	20–40°C	26–40°C	26–40°C	20–35°C	26–39°C	26–40°C	<44°C	26–35°C	10–40°C	<40°C	<49°C	27–37.5°C
	pH	3-4	4-5	4-5	4-6	4-8	4-7	4-8	3.0-8.0	4.0-7.5	3.0-7.5	4.8-6.3	4.8-6.3	5.5-6.8

Table 3. Comparison of potential biorefinery organisms and several LAB species from the genera *Lactobacillus* and *Pediococcus* for their growth conditions and carbon source utilization (Buschke et al., 2013, Charalampopoulos et al., 2002, Gaspar et al., 2013, Gibbons and Hughes, 2009, Vos et al., 2011). v, varies among strains.

1