Alternative strategies for lignocellulose fermentation through lactic acid bacteria: the state of the art and perspectives

Alternative strategies for lignocellulose fermentation through lactic acid bacteria: state-of-the-art and perspectives

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Abstract

Lactic acid bacteria (LAB) have a long history in industrial processes as food starters and biocontrol agents, but also as producers of high-value compounds. Lactic acid, their main product, is among the most requested chemicals owing to its multiple applications including synthesis of biodegradable plastic polymers. Moreover, LAB are attracting candidates for production of ethanol, polyhydroalkanoates, sweeteners, exopolysaccharides, etc.. LAB generally have complex nutritional requirements. Moreover, they cannot directly ferment inexpensive feedstocks such as lignocellulose. This significantly increases the cost of LAB fermentation and hinders its application to produce high-volume low-cost chemicals. Different strategies have been explored to extend LAB fermentation to lignocellulosic biomass. Fermentation of lignocellulose hydrolysates by LAB has most often been reported and is the most mature technology. However, current economic constraints of this strategy have driven research for other alternative approaches. Co-cultivation of LAB with native cellulolytic microorganisms may allow to reduce the high cost of exogenous-cellulase supplementation. Special attention will be given here to construction of recombinant cellulolytic LAB by metabolic engineering which may generate strains able to directly ferment plant biomass. The present review will illustrate the state-of-the-art of these strategies and perspectives towards their application to industrial 2nd generation biorefinery processes.

Key words: Lactobacillus, lactococcus, cellulase, recombinant cellulolytic strategy, metabolic engineering, cellulosome

Introduction

Lactic acid bacteria (LAB) have extensive industrial application, mainly in food fermentation and as probiotics (Mazzoli et al. 2014). Relevant industrial processes involving LAB also include fermentative production of lactic acid (LA). LA is among the most requested chemicals because of its several applications in food (e.g. acidifier and flavour-enhancing agent), cosmetic (emulsifying and moisturing agent) and pharmaceutical (intermediate) industries and as building block for the synthesis of biodegradable plastic polymers (e.g. polylactides, PLAs) (Abdel-Rahman *et al.* 2013). It has been estimated that about 90% of the worldwide LA is produced through LAB fermentation (Sauer *et al.* 2008). LA can be produced also by chemical synthesis, but this gives rise to a racemic mixture of D- and L-LA which is not suitable for PLA production (Abdel-Rahman et al., 2016). Furthermore, D-LA can cause metabolic problems to humans and therefore cannot be used in the food, drink, and pharmaceutical industries (Jem *et al.* 2010). Depending on specific LAB strain genome, i.e. the presence of gene(s) encoding D- or L- lactate dehydrogenase and/or racemase, D- or L-LA or their mixtures can be produced. In addition, LAB have been considered as candidates for production of other high-value compounds such as ethanol, polyhydroalkanoates, polyols, and exopolysaccharides (Mazzoli *et al.* 2014). However, most LAB are auxotrophic for several amino acids, nucleotides and vitamins (that should be supplemented to their growth media). Furthermore, LAB, with few exceptions, cannot ferment abundant inexpensive biomass, such as starchy or lignocellulosic feedstocks. These are significant limits for LAB to be applied to economically viable biorefinery processes, especially those aimed at high-volume low-value molecules (e.g. ethanol). Nowadays, most LA is produced by bioconversion of dedicated crops (mainly corn) by industries such as Corbion-Purac (The Netherlands), Galactic (Belgium), NatureWorks LLC-Cargill (USA) (Abdel-Rahman *et al.* 2013; de Oliveira *et al.* 2018). As the global demand for LA is rapidly increasing (16.2 % annual growth) (de Oliveira *et al.* 2018), such a process represents a threat for these food crops. Development of fermentation processes based on 2^{nd} generation (i.e. lignocellulosic) feedstocks appears as a priority for extensive application of LAB in biorefinery.

So far, no native cellulolytic and/or hemicellulolytic LAB has been identified. However, a number of LAB strains have been isolated from "plant environments", e.g. from fermented vegetables or the gastrointestinal tract of herbivores where plant biomass is the main carbon source. These LAB developed the ability to ferment a variety of soluble sugars derived from plant polysaccharide hydrolysis (see next section). Supplementation of cellulases in the growth medium (Adsul *et al*. 2007;° Wee and Ryu 2009; Shi *et al*. 2015; Bai *et al.* 2016; Hu *et al*. 2016; Overbeck

et al. 2016; Wang *et al*. 2017; Grewal and Khare 2018) or co-cultivation with cellulolytic microorganisms (Shahab *et al.* 2018) have therefore been used as efficient strategies to allow plant biomass fermentation by LAB. Alternatively, the development of recombinant LAB equipped with heterologous cellulase systems has been pursued so as to obtain strains that can directly ferment lignocellulosic feedstocks (i.e. consolidated bioprocessing, CBP) (Mazzoli et al., 2014). The state-of-the-art of these strategies and future research directions towards their application in industrial processes will be described in the next sections.

LAB ability to ferment soluble mono-/oligo-saccharides from lignocellulosic biomass

LAB can metabolize several monosaccharides, including both hexoses (e.g. fructose, glucose, galactose) and pentoses (e.g. xylose) (Kandler 1983), which are common components of lignocellulosic materials. Based on their metabolism, LAB are classified as homo-, hetero- and mixed acid-fermenters (Kandler 1983). In homofermentative metabolism, sugars are catabolized through the Embden-Meyerhof-Parnas pathway and converted to pyruvate which is finally reduced to LA. Heterofermentative metabolism involves sugar conversion through the phosphoketolase pathway giving rise to equimolar mixtures of LA and ethanol/or acetic acid (Kandler 1983). Finally, in mixed acid fermenters, glycolysis-derived pyruvate is metabolized through multiple pathways resulting in production of LA and ethanol and/or acetic and/or formic acid mixtures (Kandler 1983). Efficient metabolism of pentose sugars is particularly important when hemicellulose fermentation is addressed (Jordan *et al.* 2012). Some LAB such as *Lactobacillus* (*Lb.*) *pentosus*, *Lb. brevis*, *Lb. plantarum* and *Leuconostoc (Leu.) lactis* can metabolize both arabinose and xylose through heterofermentative metabolism (Fig. 1) (Tanaka *et al.* 2002; Okano *et al.* 2009a). An additional xylose fermentation pathway featuring higher LA production yields was identified in *Lactococcus* (*Lc.*) *lactis* IO-1 (Tanaka *et al.* 2002). In this strain, at high xylose concentration, xylose catabolism is shifted from the phosphoketolase pathway to the pentose-phosphate pathway which catalyzes its homo-lactic conversion (Fig. 1) (Tanaka *et al.* 2002).

Efficient metabolism of oligosaccharides derived from partial hydrolysis of cellulose/hemicellulose is essential for optimal fermentation of these polysaccharides (Galazka *et al.* 2010; Lane *et al.* 2015). In native cellulolytic microorganisms, a significant part of these oligosaccharides are likely not saccharified in the extracellular environment (Desvaux 2006). Instead, they are transported through specific proteins into the cytoplasm where they are further

metabolized through either hydrolytic or phosphorolytic mechanism (Desvaux 2006). Notoriously, cellodextrin transport and intracellular metabolism have been engineered in important candidates for 2nd generation biorefinery such as *Saccharomyces cerevisiae* and *Yarrowia lipolytica* (Galazka *et al.* 2010; Lane *et al.* 2015). Advantageously, an increasing number of natural LAB have been shown to metabolize cellobiose and other short cellodextrins or short oligosaccharides derived from hemicellulose (e.g. xylan, β-glucan) hydrolysis (Ohara *et al.* 2006; Adsul *et al.* 2007a; Kowalczyk *et al.* 2008; Okano *et al.* 2010b; Lawley *et al.* 2013). Recently, *Lc. lactis* IL1403, i.e. one of the very reference LAB strains, has shown the natural ability to ferment up to cellotetraose/cellopentaose (Gandini *et al.* 2017). This study has indicated that this strain is equipped with membrane transporters for short cellodextrins, although they have not been identified yet. The genome of this strain is rich in genes encoding putative β-glucosidases/6-P-β-glucosidases, while no gene coding for cellodextrin phosphorylase is present (Bolotin *et al.* 2001). As regards the metabolism of partial hydrolysis products of hemicellulose, it is worth reminding the identification of three LAB strains, i.e. *Lc. lactis* IO-1, *Leu. lactis* SHO-47 and *Leu. lactis* SHO-54, that can ferment xylooligosaccharides with degrees of polymerization up to six (Ohara *et al.* 2006). Here again, it was demonstrated that these xylooligosaccharides are hydrolyzed by intracellular xylosidases, while transporters for their uptake were not identified (Ohara *et al.* 2006). Although rare, the presence of genes encoding enzymes involved in depolymerization of xylooligosaccharides, and/or arabinoxylans and/or arabinans (i.e. β-xylosidases and arabinofuranosidases) has been detected in different strains of *Lactobacillus* spp., *Pediococcus* spp., *Leuconostoc/Weissella* branch, and *Enterococcus* spp. (Michlmayr *et al.* 2013). Recently, *Lb. ruminis*, an inhabitant of human bowels and bovine rumens, has been shown to ferment tetrasaccharides derived from barley β-glucan (Lawley *et al.* 2013).

Since fermented vegetables and other environments rich in plant biomass are habitats in which LAB can be commonly found, it is likely that future analyses will identify further LAB strains equipped with basic biochemical systems for metabolizing sugars derived from plant material.

Alternative strategies for lignocellulose fermentation through LAB

Fermentation of pre-treated lignocellulosic biomass by natural LAB

Since natural LAB cannot directly hydrolyze and ferment polysaccharides present in lignocellulose physical and/or chemical and/or enzymatic pre-treatment(s) of biomass are necessary. Several examples of fermentation of different pre-treated/hydrolyzed lignocellulosic feedstocks by LAB have been reported that include de-oiled algal biomass (Overbeck *et al.* 2016), barley bran (Moldes *et al.* 2006), corncob (Guo *et al.* 2010; Bai *et al.* 2016), corn stover (Hu *et al.* 2016; Wang *et al.* 2017), de-oiled cottonseed cake (Grewal and Khare 2018), oak wood chip (Wee and Ryu 2009), paper mill sludge (Marques *et al.* 2008; Shi *et al.* 2015), sugarcane bagasse (Adsul *et al.* 2007; Laopaiboon *et al.* 2010), trimming vine shoots (Bustos *et al.* 2005; Moldes *et al.* 2006), wheat bran (Naveena *et al.* 2005; Li *et al.* 2010), wheat straw (Grewal and Khare 2018) (Table 1).

Two main technical challenges are specifically associated with this fermentation strategy: i) generation of inhibitory compounds by physico-chemical pre-treatment; ii) inefficient saccharification of biomass (for an extensive overview Abdel-Rahman *et al.* 2016). Most physico-chemical methods generate inhibitory by-products such as phenolic and furan compounds (e.g. furfural and 5-hydroxymethylfurfural), organic acids (e.g. acetic, formic, and levulinic acid) and alcohols (Zhang *et al.* 2016a). The latter may negatively interfere with the activity of cellulolytic/hemicellulolytic enzymes and/or the metabolism of fermenting strains (Abdel-Rahman *et al.* 2016). Furthermore, enzymatic hydrolysis of plant polysaccharides frequently suffers from inhibition by end-product (e.g. glucose, cellobiose) accumulation (Abdel-Rahman *et al.* 2016). For this reason, separate hydrolysis and fermentation (SHF) approach can be advantageously replaced by simultaneous saccharification and fermentation (SSF) strategy. The latter minimizes end-product inhibition of hydrolases through rapid consumption of soluble sugars by fermenting microorganisms (Lynd *et al.* 2002). Furthermore, lignocellulose fermentation suffers from the complex nature of this biomass, consisting of different polysaccharides (mainly cellulose, hemicelluloses and pectin) (Lynd *et al.* 2002). Lignocellulose hydrolysis generates sugar mixtures which may undergo inefficient fermentation caused by heterofermentation of pentoses (see previous section) and/or carbon catabolite repression (Jojima *et al.* 2010). The latter refers to inhibition of pentose metabolism by the presence of glucose leading to non-simultaneous fermentation of sugar mixtures that often leaves most sugar unutilized (Abdel-Rahman *et al.* 2016). A wide variety of solutions can be employed to overcome these limitation(s) (Abdel-Rahman *et al.* 2011; 2016). Strategies to reduce the concentration of inhibitory compounds include the choice of alternative milder physico-chemical pre-treatments (e.g. acid or alkaline treatment, steam explosion, ionic liquids) (Abdel-Rahman *et al.* 2011) and methods (e.g. chemical additives such as ion exchange resins, bioabatement) for detoxifying pre-treated biomass (Laopaiboon *et al.* 2010; Jönsson and Martín 2016). Alternatively, the use of enzymes and LAB strains with higher tolerance to these

compounds (either natural or obtained through evolutionary or rational engineering) is a valuable option (Abdel-Rahman *et al.* 2016). Cellulase mixtures with different composition and different configurations of the fermentative process (e.g. SHF and SSF) can be used to optimize specific biomass hydrolysis (Abdel-Rahman *et al.* 2016). Finally, several LAB strains showing highly efficient metabolism of pentoses are known. Homolactic fermentation of xylose has been observed in *Lc. lactis* IO-1 (Tanaka *et al.* 2002) or *E. faecium* QU 50 (Abdel-Rahman *et al.* 2015). Several LAB showing relaxed carbon catabolite repression have been reported. For instance, different *Lb. brevis* strains were able to simultaneously utilize xylose and glucose derived from hydrolysis of a variety of lignocellulosic feedstocks (Guo *et al.* 2010; Grewal and Khare 2018), while *E. faecalis* RKY1 co-metabolized mixtures of sucrose, glucose, and/or fructose to LA with high yield (Reddy *et al.* 2015) and *E. faecium* QU 50 homofermentatively utilized glucose/xylose mixtures (Abdel-Rahman *et al.* 2015). Additionally, metabolic engineering strategies have been used to develop strains with improved pentose catabolism, as described in the following sections.

Actually, some studies demonstrate that very efficient bioconversion of lignocellulosic biomass into nearly optically pure LA through LAB fermentation (with LA yields close to the theoretical maximum) can be obtained by selecting optimal combination of pre-treatment, process configuration and microbial strain suitable for a specific substrate (Table 1). However, both physico-chemical and enzymatic treatments utilized in these studies have significant costs which represent relevant economic barriers at the industrial scale (Okano *et al.* 2010a). Despite extensive research efforts for reducing the cost of production of cellulases, no significant decrease has been observed since the 1990s (Olson *et al.* 2012). A recent study has estimated the cost of at-site production of cellulases as $10/kg protein (the cost of commercial cellulases is higher) (Klein-Marcuschamer *et al.* 2012). Based on calculations used by Lynd *et al.* (2017), it can be estimated that the cost of added cellulases per kg LA produced through lignocellulose fermentation cannot be lower than 0.31 $. It is worth noting that the cost of fermentative production of LA should be at or below 0.8 $ /kg for PLA to be economically competitive with fossil fuel-based plastics (Okano *et al.* 2010a). Such an economic target is therefore very challenging through processes such as those described in this section, where the cost of physico-chemical and enzymatic pre-treatment risks to be significantly too high. Some techno-economic analyses of LA production from renewable biomass have been recently summarized by de Oliveira *et al.* (2018). Costs may widely vary depending on the process configuration (e.g. type of feedstocks, method for biomass pre-treatment, LA purification process). In most cases the minimum LA sell price was higher than 0.8 $ /kg (i.e. between 0.83 and 5 $ /kg). However, a recent study reported a minimum sell price of 0.56 $ /kg for LA produced through fermentation of pre-treated (i.e. dilute acid plus enzymatic hydrolysis) corn

stover (Liu et al. 2015). Interestingly, cellulase cost was reported as the highest in the entire process (Liu et al. 2015). Research for alternative strategies for lignocellulose fermentation with lower dependence on biomass pre-treatment(s) is therefore highly recommended. Significant attention has been dedicated to the so-called CBP, i.e. single-pot fermentation of lignocellulosic biomass (Mazzoli 2012). This process configuration differs from SHF and SSF especially in that it does not involve a dedicated process step for cellulase production (Lynd *et al.* 2005). This could be obtained through cellulolytic microorganisms-LAB consortia or by engineering cellulolytic ability in LAB. It has been calculated that CBP could lower cost of biological conversion of lignocellulose by about 78 % (Lynd *et al.* 2005).

Fermentation of lignocellulosic biomass by cellulolytic microorganisms-LAB consortia

Co-cultivation of LAB with native cellulolytic microorganisms could interestingly replace saccharification of lignocellulosic biomass by exogenously supplemented cellulases. Utilization of microbial consortia including cellulolytic strains and high-value compound producing microbes has been successfully applied to convert cellulosic feedstocks to a variety of products such as ethanol or butanol (Zuroff *et al.* 2013; Brethauer and Studer 2014; Wen *et al.* 2014). To date, a single application of this strategy to production of LA by LAB fermentation has been reported (Shahab *et al.* 2018). In this study, a stable consortium between the cellulolytic fungus *Trichoderma reesei* and *Lb. pentosus* based on mutual benefits was developed (Fig. 2). *Lb. pentosus* efficiently consumes cellobiose thus avoiding inhibition of *T. reesei* cellulase activity. On the other hand, a by-product of sugar fermentation by *Lb. pentosus*, i.e. acetic acid, can serve as carbon source for *T. reesei* (Shahab *et al.* 2018). Fermentation of whole-slurry pre-treated beech wood by this consortium led to production of 19.8 g/L of LA, with an estimated yield of 85.2% of the theoretical maximum, through CBP (Shahab *et al.* 2018). This study demonstrates that this approach, that mimics microbial syntrophic communities involved in natural decay of plant material, deserves further investigation. In parallel, difficulties related to design and maintain stable artificial microbial communities represent main challenges of this strategy (Johns *et al.* 2016).

Construction of recombinant cellulolytic/hemicellulolytic LAB through metabolic engineering: state of the art and future directions.

Forefront research in development of 2nd generation biorefinery includes endowing microorganisms that produce high-value chemicals with the ability to directly ferment lignocellulose without prior physico-chemical and/or enzymatic pre-treatment through recombinant techniques (Mazzoli *et al.* 2012). The number of examples of recombinant cellulolytic strategies (RCS) addressed to LAB is growing (Mazzoli *et al.* 2014; Gandini *et al.* 2017; Stern *et al.* 2018). Natural ability to grow on lignocellulose relies on multiple-enzyme systems that mainly consist of glycosyl hydrolases and polysaccharide lyases (Lynd *et al.* 2002). Most studies have been addressed to two main paradigms for cellulose depolymerization, the non-complexed enzyme model of aerobic fungi and bacteria and the cellulosome complexes of anaerobic microorganisms (Lynd *et al.* 2002). The latter are based on scaffolding proteins (i.e. scaffoldins) that generally provide multiple functions, i.e. the ability to bind enzyme subunits (thus organizing the enzyme complex architecture), polysaccharides and cell surface through specific protein domains (Mazzoli *et al.* 2012). RCS aim at mimicking nature by engineering minimal cellulolytic systems (Mazzoli 2012) (Fig. 3). Traditionally, a minimal non-complexed system able to act efficiently on cellulosic substrates consists of an exoglucanase, an endoglucanase and a β-glucosidase (Lynd *et al.* 2002) (Fig. 3A, B). A mini-scaffoldin is also required in the case of mini- or designer-cellulosomes (Fig. 3C). However, in most studies aimed at LAB engineering with heterologous cellulases reported so far, a single cellulase was introduced (for an extensive review, Mazzoli *et al.* 2014) (Table 2). This modification may enable metabolization of short cellodextrins or partial hydrolysis of cellulose/hemicellulose but is insufficient for these recombinant strains to efficiently grow on and ferment complex lignocellulosic substrates (Mazzoli *et al.* 2014) (Table 2). Actually, most of these recombinant strains were aimed at being used as inoculants for silage fermentation (i.e. for improving silage acidification and/or digestibility) (Bates *et al.* 1989; Scheirlinck *et al.* 1989; Rossi *et al.* 2001; Ozkose *et al.* 2009) rather than as biocatalysts in biorefinery processes. More recently, construction of cellulolytic LAB for industrial production of LA has been considered. Among the most performant strains, *Lb. plantarum* engineered with Cel8A endoglucanase from *C. thermocellum* was able to grow on cellooligosaccharides long up to 5-6 glucose residues (Okano *et al.* 2010b). Several studies have reported that expression of heterologous cellulases may be toxic (Mingardon *et al.* 2011; Moraïs *et al.* 2014). Hence, expression of multiple cellulases is extremely challenging. The development of artificial syntrophic consortia (consisting of recombinant strains that biosynthesize single different cellulase-system components) has been used to circumvent this bottleneck (Moraïs *et al.* 2013; 2014; Stern *et al.* 2018). Morais and co-workers (2013) have shown the potential of simple consortia of recombinant *Lb. plantarum* strains secreting cellulase-xylanase mixtures for biomass (i.e. wheat straw) bioconversion. The same research group has significantly

improved its hemi/cellulolytic LAB consortium over time by including strains that biosynthesize different: i) surface-anchored mini-scaffoldins (each able to bind up to 4 enzymatic subunits); ii) adaptor mini-scaffoldins (each able to bind up to 2 enzymatic subunits) and; iii) endoglucanases and xylanases (Moraïs *et al.* 2014; Stern *et al.* 2018) (Fig. 3C). Synthetic *Lb. plantarum* consortia that display mini-cellulosomes incorporating up to six enzymatic subunits could be developed, which is a remarkable result (Stern *et al.* 2018). Although these enzyme complexes showed improved hydrolysis of wheat straw, they were unable to support growth of *Lb. plantarum* on wheat straw as the sole carbon source. This result is likely related to the amount and/or type of sugars released by the specific designer cellulosomes which seems insufficient/unsuitable for *Lb. plantarum* growth (Stern *et al.* 2018). Additionally, it has to be reminded that management of these consortia at the industrial scale may not be trivial. Recently, a cellulase system consisting of a β-glucosidase and an endoglucanase has been engineered in a single *Lc. lactis* strain through construction of an artificial operon (Gandini *et al.* 2017). This strain could directly convert cellooligosaccharides up to at least cellooctaose to L-LA with high yield. However, the basal expression triggered by the used promoter (P32) did not show to be very high, and further improvement of this strain towards application in biorefinery will be required, e.g. through increased cellulase expression (Gandini *et al.* 2017).

Attempts to improve hemicellulose metabolism in LAB include few examples of expression of heterologous xylanases (Raha *et al.* 2006; Morais *et al.* 2013; Gandini *et al.* 2017) (Table 3). Morais *et al.* (2013) demonstrated that xylanase-expressing *Lb. plantarum* improved cellulose accessibility. Most other metabolic engineering studies have concerned the improvement of pentose conversion into LA through disruption of the phosphoketolase pathway and introduction or enhancement of the pentose phosphate pathway (Okano et al 2009 a; b; Shinkawa *et al.* 2011; Qiu *et al.* 2017) (Table 2). These studies obtained impressive results since engineered strains were able of nearly homolactic fermentation of xylose and/or arabinose (Table 3). Additionally, some engineered strains showed the ability to co-ferment glucose/xylose mixtures without carbon catabolite repression (Yoshida *et al.* 2011; Zhang *et al.* 2016b).

Although the number of RCS targeted to LAB engineering is growing, research progress on these organisms is still far behind that obtained in other microbial models, such as *S. cerevisiae*. All abovementioned examples suffer from multiple limits which hamper application of such recombinant LAB to industrial fermentation of real cellulosic substrates. In most cases, inducible promoters have been used to control the transcription of heterologous cellulases (Table 2). Inducible promoters have been preferred so as to delay cellulase expression in the late exponential phase, thus avoiding major growth inhibition by cellulase expression. However, utilization of inducible

promoters is not cost-sustainable at the industrial scale, since large amounts of expensive inducer should be employed. A further problem may be represented by the limited amount of cellulases which are secreted by the recombinant cellulolytic LAB obtained so far (Table 2) (Mazzoli *et al.* 2014). As a basis for comparison, cellulase activity of native cellulosome-producing *Clostridium thermocellum* on cellulosic substrates can range between 100 and 1000 U/L (Krauss *et al.* 2012; You *et al.* 2012). In many state-of-the-art recombinant cellulolytic LAB, measured cellulolytic activities are around or under the lower side of this range (Table 2) and are strongly dependent on specific cellulase (Stern *et al.* 2018). Although available genetic tools for LAB are relatively abundant, those enabling strong constitutive expression of proteins have long been restricted to few choices, such as the lactococcal P32 and P45 promoters (Table 2). Luckily, new constitutive promoters with different strengths are being discovered for both Lactococci (Zhu *et al.* 2015) and Lactobacilli (Duong *et al.* 2011; Tauer *et al.*; 2014). Alternatively, generation of libraries of synthetic constitutive promoters diplaying a wide range of strenght (Jensen and Hammer 1998; Rud *et al.* 2006) seems a potent tool to mimicking native cellulase systems in which the highest synergism is obtained for non-equimolar expression of different enzymes (Mazzoli *et al.* 2012). Additional tools to increase cellulase/hemicellulose expression in LAB include improvement of mRNA stability (Narita *et al.* 2006; Okano *et al.* 2010) or increase of translation efficiency through design of synthetic genes with optimized codon usage (Johnston *et al.* 2014; Dong *et al.* 2015; Li *et al.* 2016). The most challenging factor in heterologous expression of cellulases consists in finding efficient secretion strategy (Mazzoli *et al.* 2012). Saturation of transmembrane transport mechanisms of the host and accumulation of misfolded or aggregated proteins is the most probable factor causing toxicity of heterologous cellulases (Illmen *et al.* 2011; Morais *et al.* 2014). Mechanisms of cellulase secretion in native cellulolytic microorganisms are almost completely unknown. Based on analysis of signal peptide sequence, a recent study postulated that only about 6% of the known cellulases is secreted through established mechanisms (e.g. the Sec or Tat pathway) (Yan and Wu 2014). In this scenario, studies on heterologous cellulase expression have often been based on trial-and-error approach so as to find enzymes compatible with the host (Illmen *et al.* 2011; Mingardon *et al.* 2011). Luckily, mechanisms of protein secretion in cellulolytic clostridia and LAB have shown some similarities since a number of components of cellulase systems of clostridia, with their original signal peptide, could be efficiently secreted by *Lb. plantarum* or *Lc. lactis* (Okano *et al.* 2010b; Wieczoreck and Martin 2010; Morais *et al.* 2013; Gandini *et al.* 2017). Alternatively, original signal peptides of cellulases can be replaced with sequences (i.e. signal peptides, propeptides) promoting efficient protein secretion in the host of interest (Dong *et al.* 2015; Lim *et al.* 2017). Typically, the native (or engineered) signal peptide of

Usp45, the main secreted protein of *Lc. lactis*, has been used for promoting the secretion of heterologous proteins in *Lc. lactis* (Morello *et al.* 2008; Ng and Sarkar 2013), including cellulase system components from different microorganisms (Wieczoreck and Martin 2010; Wang *et al.* 2014; Liu *et al.* 2017), while Lp3050 or Lp2588 leader peptides have been used to enable secretion of cellulosomal components in *Lb. plantarum* (Stern *et al.* 2018). All these tools can significantly help development of RCS of LAB, however, they cannot guarantee their success that currently still mainly depends on specific protein/host combination. Signal peptides and propeptides likely play additional roles in protein translocation, maturation and folding which need better understanding (Harwood and Cranenburgh 2008; Mazzoli *et al.* 2012; Yan and Wu 2014). Furthermore, unusual mechanisms of protein folding have been speculated for some cellulases which may require assistance by specific chaperon(s) (Mingardon *et al.* 2011). For instance, co-expression of chaperon-like *B. subtilis* PrsA protein was able to improve secretion yield of heterologous amylase and penicillinase in *Lc. lactis* (Lindholm *et al.* 2006). Increase of secretion yield of heterologous cellulases may also be obtained by inactivation of housekeeping protease(s), as demonstrated by *Lc. lactis* mutants defective in the unique exported housekeeping protease HtrA (Wieczoreck and Martin 2010). Co-expression of protease inhibitors found as integral components of some clostridial cellulosomes (Meguro *et al.* 2011; Xu *et al.* 2014) could be an alternative strategy worth being tested.

Apart from improving the amount of cellulolytic enzymes, future directions in construction of recombinant cellulolytic LAB should focus on improving synergism of designer cellulase systems. Expression of multiple enzymes with highly complementary activities, preferably in a single strain, is essential for developing strains aimed at CBP of complex substrates. Apart from "traditional" cellulase activities (i.e. exoglucanases, endoglucanases and β-glucosidases, Fig. 3), attention should be addressed also to recently discovered cellulose-active proteins, such as microbial expansins (Chen *et al.* 2016) and lytic polysaccharide monooxygenases (LPMOs) (Liang *et al.* 2014). The latter could significantly improve depolymerization of most recalcitrant polysaccharides, such as crystalline cellulose. Gene integration into the LAB chromosome seems the most suitable strategy to construct genetically stable strains that co-express multiple cellulases. An extensive literature on integrative gene expression systems in LAB is available, although it is mainly focused on lactobacilli and *Lc. lactis* (for extensive reviews refer to Gaspar *et al.* 2013 and Bravo and Landete 2017). Molecular tools for unlabelled (i.e. without insertion of antibiotic resistance markers) gene integration in the LAB genomic DNA include homologous recombination (e.g. pORI, pSEUDO and Cre-lox systems) or single-stranded DNA recombineering (Gaspar *et al.* 2013; Bravo and Landete 2017). Some of them have already been used to improve pentose

metabolism in different LAB strains (Table 3), but more extensive application to expression of heterologous hemi/cellulase systems seems necessary for significant progress of RCS in LAB.

Surface-display of proteins is also a valuable tool for increasing cellulase activity in LAB. This strategy mimics some of the most efficient cellulose depolymerization systems found in nature (e.g. cellulosome), where cellulase activity is improved by rapid metabolism of cellulose hydrolysis products promoted by enzyme-cell proximity (Wieczoreck and Martin 2010). So far, studies in this direction have been reported by two research groups only, i.e. that of Prof. Martin in Canada (Wieczoreck and Martin 2010; 2012) and that coordinated by Profs. Mizrahi and Bayer in Israel (Morais *et al.* 2014; Stern *et al.* 2018). While direct binding of glycosyl hydrolases to the LAB surface may cause allosteric hindrance and diminish enzyme/protein activity (Morais *et al.* 2014; Stern *et al.* 2018), surface display of mini-cellulosomes seems a good compromise for improving enzyme-cell synergism without major negative effects on cellulase flexibility and activity (Morais *et al.* 2014). Furthermore, cellulosomes were shown to improve enzyme stability (Stern *et al.* 2018). Multiple tools for protein surface-display in LAB through covalent (i.e. sortase-mediated) and non-covalent (e.g. LysM domains) binding have been reported (Okano *et al.* 2008; Wieczoreck and Martin 2010; Morais *et al.* 2014; Zadravec *et al.* 2015) and can be used to further developing these strategies.

Conclusion

LAB have long been used for industrial purposes and show good characteristics for future application also to 2nd generation biorefinery. Generally, they can metabolize several monosaccharides which are components of plant biomass, including both hexoses and pentoses. Some of them can directly ferment short cello- or xylo-oligosaccharides or co-ferment hexoses and pentoses without carbon catabolite repression. Successful examples of LAB fermentation of hydrolyzed lignocellulosic feedstocks (e.g. algal cake, corncob, corn stover, paper mill sludge, sugarcane bagasse, trimming vine shoots, wheat straw) have been reported. However, the high cost of physico-chemical pre-treatment and of the high amounts of commercial cellulases needed for biomass saccharification are major barriers towards industrial application of these technologies. Waiting for development of cheaper pre-treatments or cellulase-production processes, research for alternative lignocellulose-LAB fermentation strategies is in progress. Synthetic consortia of cellulolytic microorganisms and LAB may eliminate the need for exogenous cellulases through an approach that mimics natural microbial communities involved in plant biomass decay. The main

challenge here is represented by maintaining such stable consortia at the industrial scale, but the studies reported in the literature encourage pursuing research along this, so far scarcely investigated, path. Recombinant strategies aim at engineering LAB with heterologous cellulase systems able to directly ferment lignocellulose without any external help. This strategy promises huge process cost reduction, but is highly challenging. Despite the relatively high number of gene tools available for LAB, RCS suffer from intrinsic toxicity of many heterologous cellulases and from lignocellulose recalcitrance requiring expression of multiple synergistic enzyme activities. Recombinant LAB obtained so far cannot grow on cellodextrins longer than 8-9 glucose units and intense research efforts will be needed towards direct fermentation of lignocellulosic feedstocks.

In conclusion, interesting progress towards LAB application in 2nd generation biorefinery has been made. Since finding alternative energies is currently a global priority, it can be hoped that new economic resources will help further developments in this research area. In this perspective, each alternative strategy presented in this review represents a promising opportunity.

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Table 1. Examples of LAB fermentation of pre-treated lignocellulosic biomass. Strategies for physico-chemical and/or enzymatic pretreatment of biomass are summarized. n.r., not reported; SHF, separate hydrolysis and fermentation; SSF simultaneous saccharification and fermentation. In this table, the term SSF has been employed for processes featuring simultaneous saccharification and fermentation of all the soluble sugars derived from biomass hydrolysis that, depending of the biomass composition, may be hexoses or pentoses or mixtures (i.e. co-fermentation).

Biomass	Physico-chemical treatment(s)	Enzymatic treatment	Microorganisms	Fermentation mode	LA enantiomer	LA (g/L)	Yield $Y_{P/S}$ (g/g)	Productivity (g/L/h)	References
Algal cake (de-oiled algal biomass)	-	Porcine pepsin (37°C, 3 h) plus α-amylase (37°C, 16 h) plus endo-1,4-β-D-glucanase (50°C, 24h) from *Aspergillus niger*	*Lb. casei* 12A	SHF Batch	L- (and traces of D-)	11.17	-	-	Overbeck *et al*., 2016
Barley bran	Biomass was dried, milled and hydrolyzed with 3% H_2SO_4 (130°C, 15 min)	-	*Lb. pentosus* CECT-4023T	SHF Batch	n.r.	33	0.57[a]	0.60	Moldes *et al*., 2006
Birch wood xylan	-	Xylanase (1.25 g/L) (60°C, 20 min)	*Leu. lactis* SHO-47	SHF Batch	D-	2.3	-	-	Ohara *et al*., 2006
Corncob	Biomass was dried, milled and hydrolyzed with 2% H_2SO_4 (130°C, 15 min)	-	*Lb. pentosus* CECT-4023T	SHF Batch	n.r.	26	0.53[a]	0.34	Moldes *et al*., 2006
Corncob	Biomass was mashed and hydrolyzed with 0.1% H_2SO_4 (80°C, 1 h) and 0.8% H_2SO_4 (110°C, 2h)	-	*Lb. brevis* S3F4	SHF Batch	n.r.	39.1	0.69[a]	0.81	Guo *et al*., 2010
Corncob residue	-	Commercial cellulase mixture (15 FPU/g biomass)	*Sporolactobacillus inulinus YBS1-5*	SHF Fed-Batch	D-	107.2	0.85[b]	1.19	Bai et al., 2016
Corn stover	Biomass was mashed	-	*Lb. brevis* S3F4	SHF Batch	n.r.	18.2	0.74[a]	0.76	Guo *et al*.,

	and hydolyzed with 2% H_2SO_4 at a 10% (w/v) (121°C, 1h)								2010
Corn stover	Biomass was dried, sieved and treated with 5% NaOH (75°C, 3 h)	Commercial cellulase, β-glucosidase, and xylanase mixture (30 FPU/g biomass)	*Lb. pentosus* FL0421	SSF Fed-batch	n.r.	92.30	0.66[c]	1.92	Hu *et al*., 2016
Corn stover	Biomass was crushed, sieved, dried and treated with 1.5% solid acid (120°C, 80 min)	Commercial cellulase mixture (30 FPU/g biomass)	*Lactobacillus delbrueckii delbrueckii sp. bulgaricus CICC21101*	SSF Batch	D-	18	-		Wang *et al*. 2017
Deoiled cottonseed cake	Biomass was ground, sieved and mixed with ionic liquid (120°C, 2 h)	Immobilized cellulases (25 FPU/g biomass) from *Trichoderma reesei*	*Lactobacillus brevis MTCC 4460*	SSF Batch	n.r.	-	0.22[c]	-	Grewal and Khare, 2018
Detoxified *Eucalyptus globulus*	Biomass was dried, milled, and hydrolyzed with 3% H_2SO_4 (130°C, 1h). Hydrolysate was neutralized with $CaCO_3$ and stirred with 15% w/v of charcoal (room temperature, 1 day)	-	*Lb. pentosus* CECT-4023T	SHF Batch	n.r.	14.5	0.70[a]	0.28	Moldes *et al*., 2006
Oak wood chip	Biomass was treated with 0.5% H_2SO_4 (room temperature, overnight) and steam explosion (215°C, 5')	Commercial cellulase mixture (20 IU/g) supplemented with β-glucosidase (30 IU/g) (50°C, 48 h)	*Lactobacillus* sp. RKY2	SHF Continuous cell recycle (dilution rate 0.16 h^{-1})	n.r.	42	0.95[b]	6.7	Wee and Ryu, 2009
Recycled paper sludge	Biomass was neutralized with 0.3 g HCl/g biomass	-	*Lb. rhamnosus* ATCC 7469	SSF Batch	n.r.	73	0.97[a]	2.9	Marques *et al*., 2008
Softwood pre-	Softwood particles	Commercial cellulases	*Lactobacillus*	SSF Batch	n.r.	60	0.83[d]	0.62	Shi *et al*.,

hydrolysate plus paper mill sludge	were sieved and pre-treated with hot-water	(15 FPU/g glucan) plus pectinases (15 mg protein/g mannan)	*rhamnosus ATCC-10863*						2015
Sugarcane bagasse	Biomass shreds (1–3 mm size) were pre-treated with steam and alkali	Enzyme preparation from *Penicillium janthinellum*	*Lb. delbrueckii* mutant Uc-3	SSF Batch	L-	67	0.83[e]	0.93	Adsul *et al*., 2007°
Sugarcane bagasse	Biomass was dried, milled, treated with 10% NH_4OH and hydrolyzed with 0.5 % HCl (100°C, 5h). Hydrolysate was detoxified by amberlite treatment.	-	*Lc. lactis* IO-1 JCM 7638	SHF Batch	n.r.	10.9	-	0.14	Laopaiboon *et al*., 2010
Sugarcane bagasse	Biomass was ground, sieved and mixed with ionic liquid (120°C, 2 h)	Immobilized cellulases (25 FPU/g biomass) from *Trichoderma reesei*	*Lactobacillus brevis MTCC 4460*	SSF Batch	n.r.	-	0.52[c]	-	Grewal and Khare, 2018
Trimming vine shoots	Biomass was dried, milled and hydrolyzed with 3% H_2SO_4 (130 °C, 15 min)	-	*Lb. pentosus* CECT-4023T	SHF Batch	n.r.	46	0.78[a]	0.933	Bustos *et al*., 2005
Trimming vine shoots	Substrate was dried, milled and hydrolyzed with 3% H_2SO_4 (130 °C, 15 min).	-	*Lb. pentosus* CECT-4023T	SHF Batch	n.r.	24	0.76[a]	0.51	Moldes *et al*., 2006
Wheat bran	Biomass was pre-reduced and sterilized	-	*Lb. amylophilus* GV6	Solid state fermentation	L-	-	0.23[c]	-	Naveena *et al*., 2005b
Wheat bran	Biomass was treated with 1.5% H_2SO_4 (ratio 1:4 w/v) (80°C, 20 h)	-	*Lb. rhamnosus* LA-04-1	SHF Batch	L-	75	0.99[b]	3.75	Li *et al*., 2010b
Wheat straw	Biomass was ground, sieved and mixed with ionic liquid (120°C, 2 h)	Immobilized cellulases (25 FPU/g biomass) from *Trichoderma*	*Lactobacillus brevis MTCC 4460*	SSF Batch	n.r.	-	0.49[c]	-	Grewal and Khare, 2018

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[a]g of LA /g of total sugar in the hydrolysate

[b]g of LA / g of glucose in the hydrolysate

[c]g of LA / g of biomass

[d]g of LA / g of total hexose sugars

[e]g of LA / g of cellulose in the biomass

Table 2. Examples of recombinant cellulolytic strategies (RCS) on lactic acid bacteria (LAB). Recombinant LAB strains listed here were engineered with heterologous cellulase/hemicellulose systems. *Lb.*, *Lactobacillus*; *Lc.*, *Lactococcus*

Strains	Heterologous protein(s) expressed	Transcriptional promoter	Gene cloning strategy	Heterologous protein expression/secretion level[a]	Improved phenotypic properties of the strain	References
Lb. gasseri ATCC 33323	Ce8lA endoglucanase from *Clostridium thermocellum*	inducible (lacA promoter)	Plasmid	722 U/L (CMC)[b]	Hydrolysis of CMC	Cho et al., 2000
Lb. jonhsonii NCK 88	Cel8A endoglucanase from *C. thermocellum*	inducible (lacA promoter)	Plasmid	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	Not indicated	Chromosome integration	34.24/43.61 U/L (CMC)[b]	Increased silage acidification	Rossi et al., 2001
Lb. plantarum Lp80	Cel8A endoglucanase from *C. thermocellum*	Not indicated	Chromosome integration	≈ 90 U/L (CMC)[b]	Hydrolysis of CMC	Scheirlinck et al., 1989
Lb. plantarum NCDO 1193	Cel5E endoglucanase from *C. thermocellum*	Not indicated	Plasmid	1996 U/L (CMC)[b]	Hydrolysis of CMC	Bates et al., 1989
Lb. plantarum NCIMB 8826 (Δ*ldh1*)	Cel8A endoglucanase from *C. thermocellum*	Constitutive (ClpC core promoter)	Plasmid	6.03 U/L (barley β-glucan)[b]	Growth on cellohexaose	Okano et al., 2010b
Lb. plantarum WCFS1	Cel6A endoglucanase from *Thermobifida fusca*	Inducible (sakacin P promoter)	Plasmid	280 U/L (PASC)[b]	Hydrolysis of sodium hypochlorite-pretreated wheat straw	Morais et al., 2013
Lb. plantarum WCFS1	Xyn11A endoxylanase from *T. fusca*	Inducible (sakacin P promoter)	Plasmid	3360 U/L (oat spelt xylan)[b]	Hydrolysis of sodium hypochlorite-pretreated wheat straw	Morais et al., 2013
Lb. plantarum WCFS1	Cel6A endoglucanase plus Xyn11A endoxylanase from *T. fusca* plus chimeric scaffoldin-AT (synthetic consortium)	Inducible (sakacin P promoter)	Plasmid		Hydrolysis of sodium hypochlorite-pretreated wheat straw	Morais et al., 2014
Lb. plantarum WCFS1	Chimeric GH5 and GH9 endoglucanases and GH10 and GH11 xylanases from *Clostridium papyrosolvens* plus chimeric adaptor and anchoring scaffoldins (synthetic	Inducible (sakacin P promoter)	Plasmid	0.2-59.1 nM[b, c]	Hydrolysis of sodium hypochlorite-pretreated wheat straw	Stern et al., 2018

	consortium)					
Lb. reuteri XC1	CelW endoglucanase from *Bacillus subtilis* WL001 and phyW phytase from *Aspergillus fumigatus* WL002 (artificial operon)	Constitutive (LdhL promoter)	Plasmid	960 U/L (CMC)[b]	Hydrolysis of CMC	Wang et al., 2014
Lc. lactis HtrA NZ9000	Fragments of CipA scaffoldin from *C. thermocellum*	Inducible (nisA promoter)	Plasmid	9 x 10^3 scaffolds/cell[d]	Scaffoldins displayed on the cell surface	Wieckzoreck and Martin, 2010
Lc. lactis IL1403	BglA β-glucan glucohydrolase and EngD Endoglucanase/Xylanase from *Clostridium cellulovorans* (artificial operon)	Constitutive (P32 promoter)	Plasmid	1.220 U/L (pNGP)[b]; 157 U/L (Azo-CMC)[b]	Hydrolysis of CMC; Growth on cellooctaose	Gandini et al., 2017
Lc. lactis strains IL1403 and MG1363	Cellulase from *Neocallimastix sp.*	Inducible (lacZ promoter)	Plasmid	5.9 U (CMC)[b, e]	Hydrolysis of CMC	Ozkose et al., 2009
Lc. lactis MG1316	Xylanase from *Bacillus coagulans* ST-6	Constitutive (P32 promoter)	Plasmid	≈87 U/L (xylan)[c]	Hydrolysis of RBB-xylan	Raha et al., 2006
Lc. lactis MG1316	Egl3 endoglucanase from *Trichoderma reesei*	Constitutive (P32 promoter)	Plasmid	1118 U/L (CMC)[b]	Improved metabolization of paper and wheat straw	Liu et al, 2017

[a]Maximum values reported in each study. Substrates used for determining enzyme activity are indicated in parentheses. Azo-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; pNGP, p-nitrophenyl-b-D-glucopyranoside (pNGP); RBB-xylan, remazol brilliant blue xylan .

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

[c]Protein quantification through ELISA-based binding assays on cultures with OD_{600nm}=1

[d]Proteins displayed on the cell surface

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

Table 3. Recombinant LAB showing improved pentose metabolism. Tkt, transketolase; XylA, xylose isomerase; XylB, xylulose kinase

Strains	Heterologous protein(s) expressed	Transcriptional promoter	Gene cloning strategy	Improved phenotypic properties of the strain	References
Lb. plantarum NCIMB 8826 (*Δldh1-xpk1*)	Tkt from *Lc. lactis* IL1403 (replacing endogenous phosphoketolase Xpk1)	Not indicated	Chromosome integration	Almost homolactic (D-LA) fermentation of arabinose	Okano et al. 2009a
Lb. plantarum NCIMB 8826 (*Δldh1-xpk1-xpk2*)	Tkt from *Lc. lactis* IL1403 (replacing endogenous phosphoketolase Xpk1 and Xpk2); XylA and XylB from *Lb. pentosus* NRIC 1069	Not indicated	Chromosome integration	Almost homolactic (D-LA) fermentation of xylose	Okano et al. 2009b
Lc. lactis IL1403 (*Δpkt*)	XylA and XylB from *Lc. lactis* IO-1 and endogenous tkt replacing endogenous phosphoketolase (*pkt*)	Inducible (xylose) for XylAB. Not indicated for *tkt*	Plasmid and Chromosome integration	Almost homolactic (L-LA) fermentation of xylose	Shinkawa et al., 2011
Ped. acidilactici TY112 (*ΔldhD-pkt-ackA2)*	Transaldolase, tkt (replacing endogenous phosphoketolase, *pkt*), XylA and XylB (replacing endogenous acetate kinase, *ackA2*) from *Pediococcus acidilactici* DSM20284	Constitutive (P*ldhD*)	Chromosome integration	Almost homolactic (L-LA) fermentation of xylose	Qiu et al., 2018

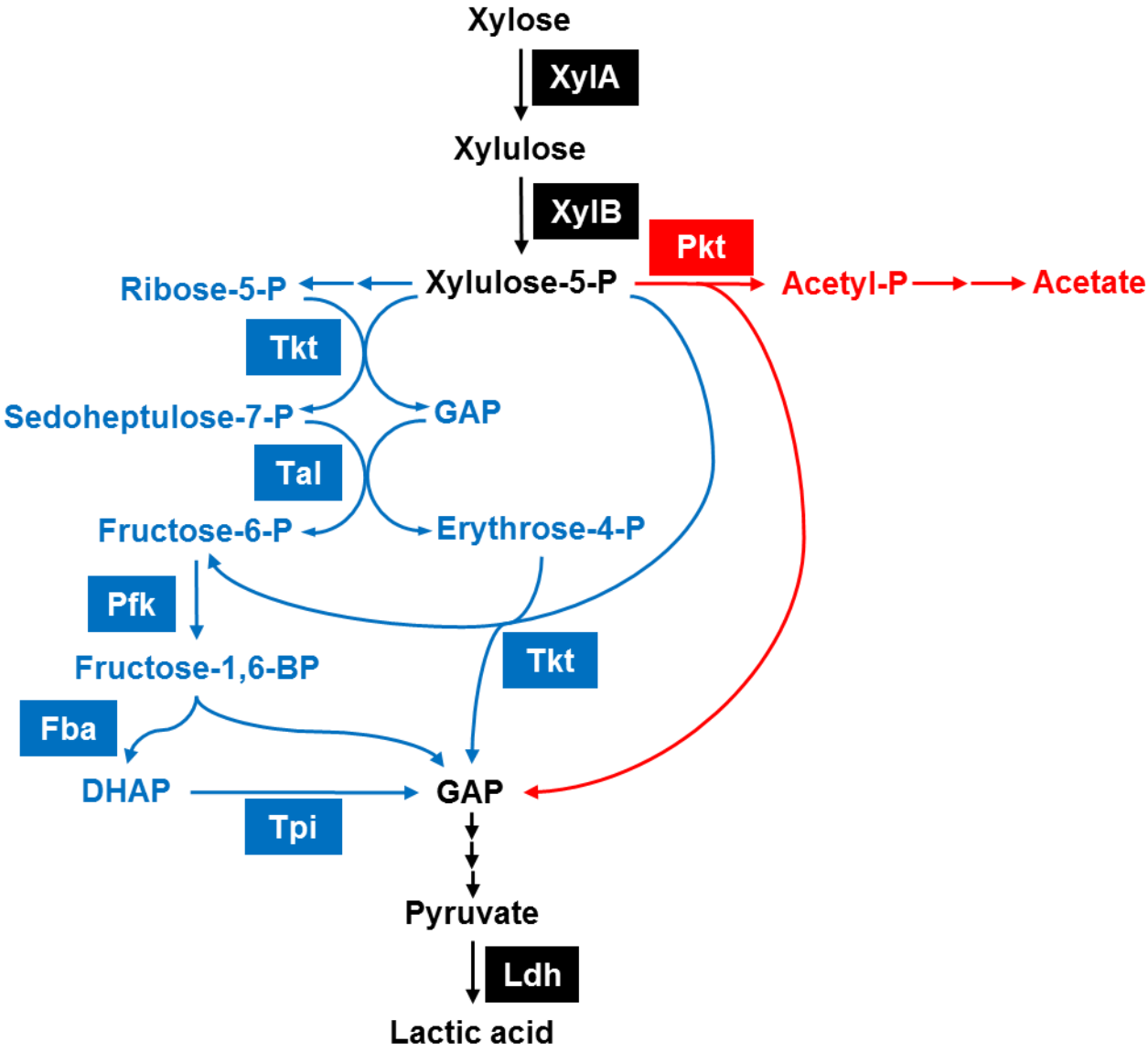

Figure 1. Heterolactic (red) and homolactic (blue) pathways for xylose dissimilation in LAB. DHAP, dihydroxyacetone phosphate; Fba, fructose bisphosphate aldolase; GAP, glyceraldehyde-3-phosphate; Ldh, lactate dehydrogenase; Pkt, phosphoketolase; Pfk, 6-phosphofructokinase; Tal, transaldolase; Tkt, transketolase; Tpi, triose phosphate isomerase; XylA, xylose isomerase; XylB xylulokinase.

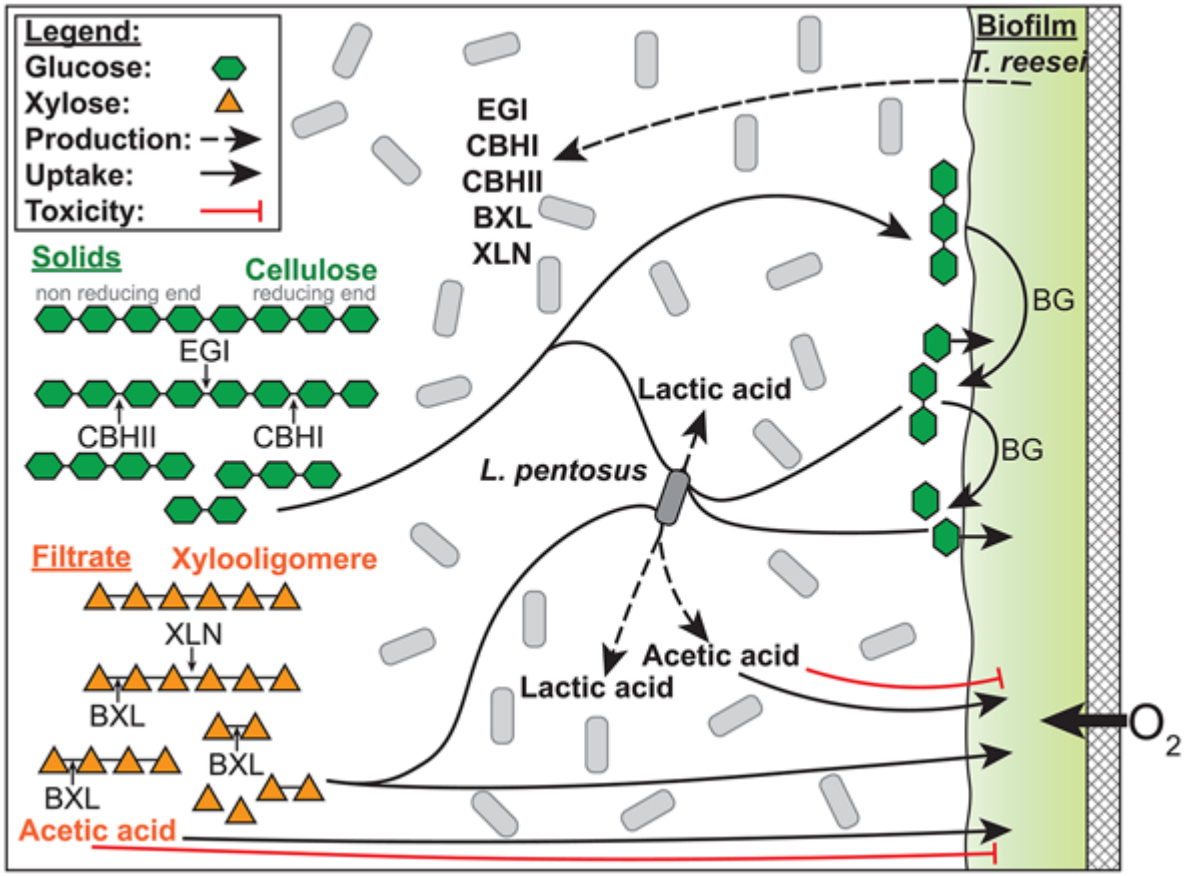

Figure 2. Schematic representation of *T. reesei*/*Lb. pentosus* consortium developed by Shahab et al. (2018). *T. reesei* grows as a biofilm on the surface of an oxygen permeable membrane and secretes cellulases and hemicellulases (EGI: endoglucanase I, CBHI: cellobiohydrolase I, CBHII: cellobiohydrolase II, BXL: β-xylosidase, XLN: β-endoxylanase). Soluble saccharides produced by *T. reesei* enzymes are fermented by *Lb. pentosus* to lactic and acetic acid. Acetic acid can serve as energy source for *T. reesei* (modified from Shahab *et al.* 2018).

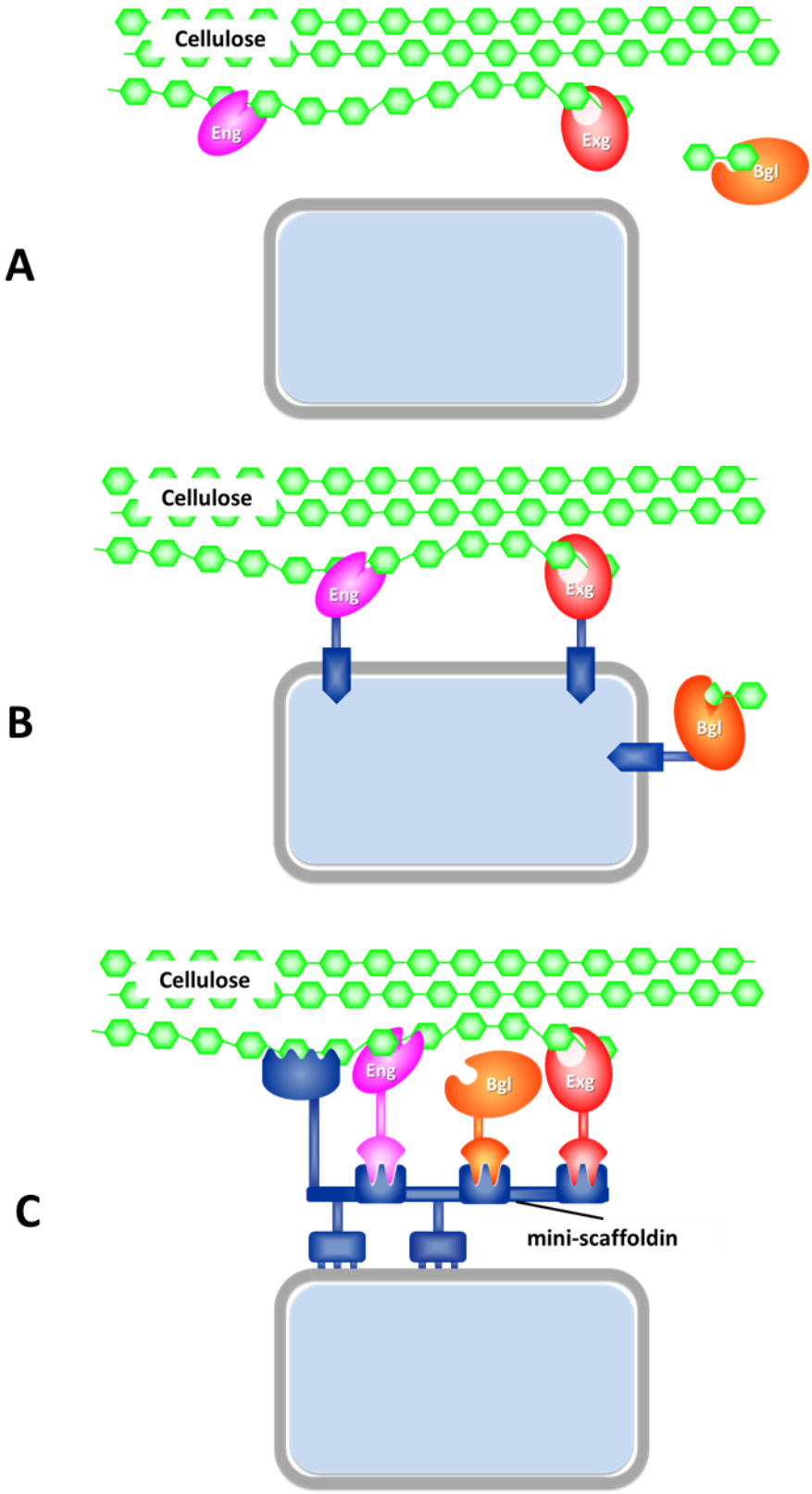

Figure 3. Paradigms for recombinant cellulolytic strategies reported in LAB. Recombinant cells A) secreting minimal non-complexed cellulase system or biosynthesizing B) surface-displayed cellulases or C) surface-displayed designer cellulosomes are depicted. Bgl, β-glucosidase; Eng, endoglucanase; Exg, exoglucanase.