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09 January 2025

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Journal:	*Biotechnology and Applied Biochemistry*
Manuscript ID	Draft
Wiley - Manuscript type:	Mini-Review Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Mazzoli, Roberto; University of Torino, Life Sciences and Systems Biology
Keywords:	Clostridium, Lactic acid bacteria, Cellulosome
Mandatory Keywords:	Biochemical Engineering, Cell Factory, Metabolic Engineering, Microbial Metabolism, Synthetic Biology
Abstract:	Lactic acid (LA) is one the most requested molecules by the chemical industry. Current expansion of LA market is mainly driven by its application as building block for the synthesis of polylactide (PLA), i.e. a family of biodegradable and biocompatible plastic polymers. PLA can potentially replace oil-derived polymers as general purpose plastic, but current LA price makes PLA not cost-competitive with traditional plastics. Nowadays, LA is mainly produced by fermentation of expensive starchy biomass. Hopefully, cheaper lignocellulosic feedstock could be used in future 2nd generation biorefinery processes. However, most efficient natural LA producers cannot ferment lignocellulose without prior biomass saccharification. Metabolic engineering may develop improved microorganisms that feature both efficient biomass hydrolysis and LA production, thus supporting consolidated bioprocessing (CBP), that is one-pot fermentation, of lignocellulose to LA. CBP could dramatically reduce LA production cost thus contributing to the expansion of more environmental sustainable plastics and commodity chemicals. The present study presents an overview of "recombinant cellulolytic strategies", mainly consisting in introducing cellulase systems in native producers of LA, and "native cellulolytic strategies" aimed at improving LA production in natural cellulolytic microorganisms. Issues and perspectives of these approaches will be discussed.

1   **Metabolic engineering strategies for consolidated production of lactic acid**

2   **from lignocellulosic biomass**

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11   Running title: Direct lactic acid production from lignocellulose

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Abstract

Lactic acid (LA) is one the most requested molecules by the chemical industry. Current expansion of LA market is mainly driven by its application as building block for the synthesis of polylactide (PLA), i.e. a family of biodegradable and biocompatible plastic polymers. PLA can potentially replace oil-derived polymers as general purpose plastic, but current LA price makes PLA not cost-competitive with traditional plastics. Nowadays, LA is mainly produced by fermentation of expensive starchy biomass. Hopefully, cheaper lignocellulosic feedstock could be used in future 2nd generation biorefinery processes. However, most efficient natural LA producers cannot ferment lignocellulose without prior biomass saccharification. Metabolic engineering may develop improved microorganisms that feature both efficient biomass hydrolysis and LA production, thus supporting consolidated bioprocessing (CBP), that is one-pot fermentation, of lignocellulose to LA. CBP could dramatically reduce LA production cost thus contributing to the expansion of more environmental sustainable plastics and commodity chemicals. The present study presents an overview of "recombinant cellulolytic strategies", mainly consisting in introducing cellulase systems in native producers of LA, and "native cellulolytic strategies" aimed at improving LA production in natural cellulolytic microorganisms. Issues and perspectives of these approaches will be discussed.

Key words: *Clostridium*, lactic acid bacteria, *Bacillus*, *Rhizopus*, consolidated bioprocessing.

Introduction

Lactic acid (LA) is one of the most requested chemicals owing to its application in several areas [1]. The most traditional utilization of LA is in the food industry, e.g. as acidifier, emulsifier, preservative and flavour-enhancing agent, but also in the production of cosmetics (such as emulsifying and moisturizing agent), pharmaceuticals (as intermediate) and in the chemical industry (e.g. for production of solvents) [1]. However, the LA application that best fits the current green economy revolution towards more sustainable and environment-friendly technologies is as building block for the synthesis of biodegradable plastic polymers (e.g. polylactide, PLA, and its co-polymers) [2]. PLA application ranges from the medical area (e.g. surgical sutures, orthopaedic and cardiovascular devices, drug delivery, tissue regeneration) owing to its biocompatibility, to use in agriculture (mulch films and bags), food and good packaging, and manufacturing of disposable cutlery, cups and trays [1,3]. PLA can therefore be considered as a general-purpose material potentially able to replace fossil-fuel derived plastics in most applications.

All these uses, especially for PLA synthesis, have driven global market expansion of LA. The global demand of LA was 1,220.0 kt in 2016 and is expected to reach 1,960.1 kt in 2025, that corresponds to an annual growth of 16.2% [1]. About 90% of LA produced worldwide is obtained by microbial fermentation of dedicated crops (mainly corn) by companies such as Corbion-Purac (The Netherlands), Galactic (Belgium) and NatureWorks LLC-Cargill (USA) [1,2]. Actually, LA production by microbial fermentation is advantageous over chemical synthesis since optically pure LA can be obtained instead of a racemic mixture of D- and L-LA [4]. This is particularly important for certain LA applications such as in the production of PLA, whose characteristics highly depend on the ratio of LA enantiomers, or in food, drink and pharmaceutical industries since D-LA can cause metabolic problems to humans and should be avoided [4,5]. However, some issues of the current processes for producing LA risks to hamper further expansion of the global LA market. In particular, the current cost of LA is relatively high ($1.30-4.0/kg) and may suffer from important fluctuations depending of the price of commodity starch or sugar

66 feedstock used for fermentation [6]. As a consequence, the current price of PLA and other LA polymers

67 is significantly higher than oil-derived plastics [1]. It has been calculated that the cost of LA should be $\leq$

68 \$0.8/kg for PLA to be economically competitive with fossil fuel-based polymers [7]. Furthermore,

69 current fermentative strategies for producing LA have major ethical concern since they represent a threat

70 to food crops, e.g. corn. Intense research has therefore been targeted at non-food feedstocks for LA

71 fermentation such as by-products of dairy industry (e.g. milk whey), food waste, glycerol, microalgae or

72 wheat bran [1,2,8]. In this scenario, lignocellulosic biomass is among the most promising feedstocks,

73 since it is the most abundantly available raw material on the Earth. Furthermore, lignocellulose includes

74 the greatest fraction of waste biomass such as agricultural/land by-products (cereal straw, sugar cane

75 bagasse, forest residues), municipal solid wastes and industrial wastes (e.g. paper mill sludge) [9].

76 However, lignocellulose is highly recalcitrant to biodegradation because of its complex composition

77 (generally consisting in 35–50% cellulose, 20–35% hemicellulose, and 10–25% lignin) and the highly-

78 ordered structure of these plant polymers [10]. Current industrial production of LA is mainly based on

79 fermentation by lactic acid bacteria (LAB) [11], but other potent natural producers of LA are bacteria

80 belonging to the *Bacillus* genus and fungi of the *Rhizopus* genus [12,13]. Unfortunately, none of these

81 microorganisms can ferment lignocellulosic material without prior biomass saccharification [12–14].

82 Processes featuring biomass pre-treatment (through physical and/or chemical and/or enzymatic

83 approach) followed by microbial fermentation of soluble sugars to LA can be highly efficient, with LA

84 yields close to the theoretical maximum at nearly optical purity [14]. However, biomass pre-treatment

85 has significant cost and, in particular, the cost of cellulases is among the highest in the entire process

86 [7,15]. This currently makes industrial production of LA through fermentation of lignocellulose hardly

87 viable from an economic standpoint.

88 Research is therefore active in developing alternative strategies for lignocellulose fermentation with

89 lower dependence on biomass pre-treatment(s), and especially on exogenous cellulase supplementation.

90 The most ambitious process configuration in this context is the so-called consolidated bioprocessing

91 (CBP), i.e. single-pot fermentation of lignocellulosic biomass, featuring huge cost reduction (about 78%)

92 with respect to current technologies based on multiple bioreactors [16,17]. Recently, a nice example of

93 CBP using a microbial consortium consisting of a cellulolytic fungus (i.e. *Trichoderma reesei*) and a

94 LAB (i.e. *Lactobacillus pentosus*) has been reported [18]. Fermentation of whole-slurry beech wood by

95 this consortium led to production of 19.8 g/L of LA, with an estimated yield of 85.2% of the theoretical

96 maximum [18]. Utilization of designer microbial consortia for CBP of plant biomass is receiving

97 increasing attention, based also on the observation that decay of plant material in natural environments

98 is performed by syntrophic microbial communities [19]. However, industrial exploitation of this strategy

99 will require improvement of robustness, stability and reproducibility of artificial microbial consortia [19].

100 Most frequently, metabolic engineering has been employed to develop microbial strains able to both

101 directly ferment lignocellulose and produce LA with high efficiency. Construction of recombinant

102 microorganisms for CBP of lignocellulosic biomass has been mainly pursued through two alternative

103 paradigms, the native cellulolytic strategy (NCS) or the recombinant cellulolytic strategy (RCS) [17].

104 NCSs intend to introduce and/or improve the production of high-value chemical(s) in native cellulolytic

105 microorganisms. RCSs aim at engineering cellulolytic characteristics (e.g. by expression of heterologous

106 cellulases) in microbial strains that naturally produce high-value chemicals with high efficiency.

107 Examples of these strategies aimed at developing strains for CBP of plant biomass to LA will be

108 illustrated in the next sections.

109

Metabolic engineering strategies for direct production of LA from lignocellulosic

111 **biomass**

112 As regards direct production of LA from lignocellulose, most metabolic engineering approaches reported

113 so far have used the RCS paradigm, with a particular focus on LAB and some remarkable examples on

114 bacteria belonging to *Bacillus sp.*. So far, metabolic engineering aimed at improving chemical production

115   in native cellulolytic microorganisms has been mainly targeted on biofuel (e.g. ethanol, butanol)

116   production. However, these studies have provided precious hints also for improving LA production in

117   these organisms, as described in the following paragraphs.

118

119   **Native cellulolytic strategies**

120   Most metabolic engineering studies addressed at improving chemical production in native cellulolytic

121   microorganism have been performed on anaerobic bacteria, while research on fungi has been mainly

122   focused at enhancing production of cellulases [20,21] with few exceptions [22]. Generally, sugar

123   catabolism in anaerobic (hemi/)cellulolytic bacteria produces a mixture of organic organics (including

124   acetic acid, formic acid and LA), ethanol, H_2 and CO_2 (Fig. 1). Butyrate and/or butanol are produced by

125   few cellulolytic bacteria such as *Clostridium cellulovorans* or *Thermoanaerobacterium*

126   *thermosaccharolyticum* [23,24]. Frequently, LA is not among the most abundant end-products of these

127   organisms as in the case of *Clostridium cellulovorans*, *Clostridium thermocellum* or

128   *Thermoanaerobacterium saccharolyticum* [24,25]. Exceptions include *Thermoanaerobacter*

129   *thermohydrosulfuricus* WC1, i.e. a recently isolated xylan-metabolizing strain, whose main fermentation

130   product is LA [26].

131   Improvement of the production of a chemical by rational metabolic engineering is generally performed

132   by: i) enhancing the expression/activity of enzymes involved in the product biosynthesis and/or ii)

133   disrupting pathways that compete for carbon substrate and/or electrons and/or co-factors [17]. In

134   addition, organisms must be tolerant to high concentration of the chemical so as to allow high-titer

135   industrial fermentation. LA is produced by reduction of pyruvate derived from sugar catabolism and this

136   reaction is catalyzed by lactate dehydrogenase (LDH) which uses NAD(P)H as electron donor [17] (Fig.

137   1). LA production is generally considered as a sink for electrons derived from sugar catabolism. For this

138   reason, it especially competes with other metabolic pathways that consume reducing equivalents such as

139   production of alcohols (e.g. ethanol, butanol) or H_2 [17] and, more in general, is affected by the redox

140   balance of the cell [27]. Recently, improvement of LA production through engineering the transcriptional

141   promoter of *ldh* gene has been reported in *Caldicellulosyruptor bescii*, a hyperthermophilic anaerobic

142   cellulolytic bacterium [28]. However, most metabolic engineering studies affecting LA production in

143   cellulolytic microorganisms were targeted to disruption of fermentative pathways that compete for

144   reducing equivalents (production of H_2), carbon (production of acetate, formate) or both (production of

145   ethanol) as described in the following sub-sections. The last subsection will be dedicated at strategies for

146   improving acid tolerance in cellulolytic microorganisms.

147

Disruption of ethanol production

148

149   Several studies indicated that repression of ethanol synthesis leads to improvement of LA production. In

150   nature, biosynthesis of ethanol from pyruvate can occur through two pathways: (i) oxidative

151   decarboxylation via pyruvate ferredoxin/flavodoxin oxidoreductase (PFOR) and subsequent reduction of

152   acetyl-CoA to acetaldehyde (by aldehyde dehydrogenase, ALDH) and finally to ethanol (by alcohol

153   dehydrogenase, ADH); (ii) decarboxylation to acetaldehyde by pyruvate decarboxylase (PDC) and

154   acetaldehyde reduction to ethanol by ADH [17]. As far as I know, anaerobic cellulolytic bacteria

155   generally employ the first pathway, since they are not equipped with PDC [29,30] (Fig. 1). However,

156   side PDC activity of PFOR has sometimes been reported [31,32]. Multiple ADHs and ALDHs are

157   generally found in alcohol producing microorganisms, including bifunctional alcohol/aldehyde

158   dehydrogenases [25]. This complicates the identification of the genes which are the main responsible for

159   alcohol biosynthesis and has been frequently pointed out as an issue for metabolic engineering strategies

160   [25,33]. Deletion of *adhE* encoding bifunctional alcohol/aldehyde dehydrogenase has been obtained in

161   *Clostridium thermocellum*, *Thermoanaerobacter mathranii*, *Thermoanaerobacterium saccharolyticum*

162   and *T. thermosaccharolyticum* resulting in dramatic (> 95%) decrease or loss of alcohol (i.e. ethanol and

163   butanol) biosynthesis and impressive enhancement of LA production which became the most abundant

product of such engineered strains (Table 1) [23,25,34]. Interestingly, in *C. thermocellum* Δ*adhE* a spontaneous mutation of the gene encoding LDH was also observed which caused loss of allosteric regulation by fructose 1,6 bis-phosphate (F1,6BP) [25]. *C. thermocellum* LDH as most other LDH are allosteric enzymes activated by F1,6BP [35]. The mutant LDH found in strain LL1111 actually had specific activity even higher than that of the native *C. thermocellum* LDH in presence of F1,6BP [25]. However, the main cause of the increase in LA production in the engineered *C. thermocellum* strain was deletion of *adhE* and not mutation in LDH [25].

Disruption of H$_2$ production

Production of H$_2$ by hydrogenases is another typical electron-consuming reaction found in anaerobic cellulolytic microorganisms (Fig. 1). As mentioned above for ADH, also inhibiting H$_2$ production in a microbial strain may not trivial since multiple hydrogenases likely involved in different functions (e.g. redox balancing, derivation of energy from H$_2$ oxidation, proton respiration and/or proton-gradient build-up) are frequently found within one species [36]. For instance, disruption of *hyd* or *ech* gene clusters of *T. saccharolyticum*, encoding a NAD-dependent [FeFe]-hydrogenase and membrane-bound [Ni-Fe] hydrogenase, respectively, did not result in any significant reduction of H$_2$ production, while deletion of *hfs* gene cluster, likely encoding another [FeFe]-hydrogenase, resulted in >95% decrease in hydrogen accumulation [29]. Furthermore, in the Δ*hfs* strain LA was the most abundant product which is consistent with re-distribution of reducing equivalents towards alternative electron-consuming pathways in strains lacking hydrogenases (Table 1). More recently, a Δ*hydG* Δ*ech C. thermocellum*, lacking the gene encoding HydG, involved in the maturation of its four [FeFe]-hydrogenases, and the [Ni-Fe] hydrogenase Ech, was obtained which showed complex perturbation of the central carbon metabolism causing dramatic reduction of LA accumulation (Table 1) [37]. Although the exact cause of this unexpected metabolic shift was not determined, it was speculated that disruption of hydrogenases could have altered intracellular levels of possible allosteric regulators of LDH [37]. Apart from the abovementioned F1,6BP,

189 LDHs may also be activated by ATP and may be inhibited by pyrophosphate, e.g. in *Caldicellulosiruptor*

190 *saccharolyticus* [38]. Nicotinamide cofactors are other typical regulators of LDH activity such as in

191 *Caldicellulosiruptor saccharolyticus*, where NAD^+ is a competitive inhibitor [38], or in

192 *Thermoanaerobacter ethanolicus* where, curiously, LDH is inhibited by NADPH [39]. It is likely that

193 hydrogenase-deleted *C. thermocellum* features accumulation of reduced ferredoxin via PFOR which

194 could cause accumulation of other reduced electron carriers such as NADPH possibly leading to

195 inhibition of LDH [37].

196

197 ***Disruption of pyruvate dissimilation to acetyl-CoA***

198 Because of its key role in driving pyruvate dissimilation towards C2 (acetate, ethanol) and other end-

199 products of fermentation (Fig. 1), the conversion of pyruvate to acetyl-CoA should be regarded as a main

200 target for metabolic engineering strategies aimed at LA overproduction. In anaerobic (hemi)cellulolytic

201 microorganisms, pyruvate conversion to acetyl-CoA can generally occur through: i) oxidation by PFOR

202 leading to production of acetyl-CoA and reduced ferredoxin and/or; ii) pyruvate formate lyase (PFL)

203 which breaks pyruvate into formate and acetyl-CoA (Fig. 1). Genetic evidence indicated the presence of

204 the pyruvate dehydrogenase complex in some anaerobic bacteria, but functional confirmation remains to

205 be determined [30]. Reduced ferredoxin can supply electrons to hydrogenase, for biosynthesis of H_2 from

206 H^+ [30] (Fig. 1). Alternatively, a number of reactions can be used to transfer electrons from reduced

207 ferredoxin to nicotinamide cofactors (i.e. NAD^+ and $NADP^+$), such as those catalyzed by

208 ferredoxin:NAD oxidoreductase (FNOR), ion-translocating reduced ferredoxin: NAD^+ oxidoreductase

209 (RNF) and NADH-dependent reduced ferredoxin: $NADP^+$ oxidoreductase (NFN) (Fig. 1) [40]. These

210 reactions constitute a bridge between ferredoxin-dependent reactions and NAD(P)-dependent reactions,

211 such as production of ethanol or LA. Also in the case of PFL pathway, formate can possibly be a source

212 of electrons for reduction of $NAD(P)^+$ through formate dehydrogenase (FDH). So, both PFOR and PFL

213 play key roles in the metabolism.

214 Generally, multiple PFORs are encoded by the genome of anaerobic microorganisms [30]. Deletion of

215 *pforA*, encoding the primary PFOR of the hemicellulolytic *T. saccharolyticum*, resulted in a dramatic

216 decrease in growth, that is only 10% of the cellobiose initially supplied could be consumed (Table 1)

217 [30]. However, through an adaptation process, the growth performance of these recombinant strains was

218 partially restored. One of these strains, i.e. LL1141, produced more formate and LA than the parent strain.

219 In particular, LA was its major fermentation product, with a yield that was about 4.5 fold higher than that

220 of the wild type strain [30].

221 Elimination of formate production by disruption of the *pflB* and *pflA* genes, encoding PFL and PFL-

222 activating enzyme, respectively, increased LA titer up to 9.3 fold in *C. thermocellum* (Table 1) [41].

223 Increase in LA production of this strain may be due to : i) improved availability of reducing equivalents

224 (since pyruvate is forced to be converted to acetyl-CoA by PFOR in the recombinant strain); ii) possible

225 increase in intracellular concentration of LDH-allosteric activator F1,6BP [35] derived from restriction

226 on the rate of glycolytic flux when pyruvate conversion to acetyl-CoA is catalyzed by PFOR only.

227 Disruption of *pfl* cluster had moderate negative effect on the growth of *T. saccharolyticum* and

228 supplementation of formate and yeast extract was required for recovering the growth efficiency of the

229 parent strain [30]. In strain LL1164, this modification led to elimination of formate production and

230 increase of acetate and, especially, LA yield [30]. However, additional spontaneous mutation in the genes

231 encoding ferredoxin hydrogenase in this strain may have contributed the excess of reducing equivalents

232 leading to increased LA production [30].

233 Double deletion of *pfor* and *pfl* was obtained in *T. saccharolyticum* (Table 1) [30]. The engineered strain

234 consumed about 70 % of the cellobiose initially supplemented, but also required sodium acetate for

235 growth. This strain produced LA as its main fermentation product at a yield (3.5 mol/mol cellobiose

236 consumed) that corresponds to 88 % of the maximum theoretical yield.

237

238 ***Engineering the redox state of the cell***

239   As previously mentioned, even reduced ferredoxin, e.g. produced by PFOR, can indirectly serve as

240   electron donor for LA production by LDH, through the activity of FNORs (Fig. 1) [42]. Improvement of

241   the expression of FNORs seems therefore an appealing strategy to increase NAD(P)H availability in the

242   cell and accumulation of reduced fermentation end-products such as ethanol or LA. Although no major

243   improvement of LA production was reported, overexpression of *rnf* operon triggered moderate increase

244   in ethanol production in some recombinant *C. thermocellum* strains (Table 1) [42]. More in details, the

245   effect of *rnf* overexpression was dependent on the genetic background, so that no change in ethanol

246   accumulation was observed in the wild type strain, while 30% increase occurred in the Δ*hydG* strain, that

247   is the strain where the four [FeFe]-hydrogenases were inactivated [42]. This study indicated that

248   improvement of FNOR activity is a valuable strategy to increase NAD(P)H availability, but also pointed

249   out at the complexity of electron metabolism in cellulolytic anaerobic bacteria and at important gaps in

250   its current understanding.

251   The global redox-responsive transcription factor Rex has been recently the target of metabolic

252   engineering strategies aimed at improving the production of reduced catabolites, particularly ethanol, in

253   anaerobic cellulolytic bacteria. Rex acts as a gene transcription repressor in response to low intracellular

254   [NAD(P)H]/[NAD(P)$^+$] ratio [43]. Targets of Rex generally include genes involved in energy conversion,

255   redox metabolism, glycolysis, fermentation and NAD biosynthesis [43]. Successful deletion of *rex* gene

256   has been reported in the hyperthermophilic anaerobic bacterium *Caldicellulosyruptor bescii* [27] and in

257   *Thermoanaerobacterium saccharolyticum* [44]. *C. bescii* Δ*rex* metabolic profile indicated more reduced

258   intracellular redox status and increased accumulation of a number of catabolites including LA (Table 1)

259   [27]. Deletion of *rex* in *T. saccharolyticum* deregulated the expression of ADH genes *adhE* and *adhA*

260   leading more than two-fold increase of ethanol yield but LA yield was reduced (Table 1) [44]. The diverse

261   metabolic effect of *rex* deletion observed in different microbial strains may depend on several metabolic

262   constraints specific to each bacterial model, including the fact that specific targets of Rex regulation,

263 although often including enzymes such as hydrogenases, PFORs and LDH may vary from strain to strain

264 [43].

265

Disruption of acetate production

267 Acetate is a common and abundant fermentation product of cellulolytic microorganisms. Acetate is

268 produced from acetyl-CoA by a two-reaction pathway catalyzed by phosphate acetyltransferase (PTA)

269 and acetate kinase (ACK). Production of acetate from acetyl-CoA has been frequently found essential in

270 anaerobic bacteria since it features ATP synthesis through substrate level phosporylation (Fig. 1) [45,46].

271 Actually, a number of studies failed in obtaining disruption of acetate producing genes in *C.*

272 *cellulolyticum* or in *T. thermosaccharolyticum* [23,47] or led to strains with severe growth deficiency, as

273 in the case of *C. thermocellum* Δ*pta* [48]. However, a more recent study on a *C. thermocellum* reported

274 deletion of *pta* gene with dramatic decreased production of acetate and significant improvement (about

275 1.6 fold) of LA titer [49]. An alternative approach using antisense RNA instead of traditional gene

276 disruption was also able to repress *pta* expression in *C. cellulolyticum*, although it was not effective on

277 *ack* espression [50]. However, 15 % reduction in acetate titer in *pta*-repressed strain was accompanied

278 by more the 50% reduction in LA titer. This unexpected result indicates that LA and acetate production

279 could be connected by some metabolic regulatory network yet to be determined in this strain [50].

280

Improvement of acid tolerance

282 One of the main limits towads LA production through native cellulolytic microorganisms is that known

283 anaerobic cellulolytic bacteria, such as *C. thermocellum*, typically do not grow at pH values lower than

284 pH 6.0 [51,52]. Low extracellular pH is toxic because it causes dissipation of the proton gradient across

285 the cytoplasmic membrane. In this condition, weak acids such as LA become protonated and can cross

286 the cytoplasmic membrane. Since cytoplasm is more alkaline, weak acids dissociate protons which

acidify cytoplasm and collapse the ΔpH [52]. As far as I know, no information on LA tolerance by native

cellulolytic microorganisms has been reported. However, accumulation of LA during fermentation is

known to inhibit natural LA producers and cause decrease in LA productivity [4]. Both issues, i.e. limited

tolerance to acidic pH and LA, have been traditionally fixed through fermentation process engineering.

Neutralizing agents are generally used during LA fermentation but this increases the cost of the process

both because of consumption of high amounts of neutralizing agent and because this complicates

downstream process of LA purification from the medium [53]. Alternatively, severe drop in pH and

accumulation of LA in the growth medium has been prevented by continuous removal of LA by several

strategies such as electrodialysis, solvent extraction, adsorption, and membrane bioreactors [4].

However, these methods complicate the fermentation process owing to associated technical problems

[54]. Improving acidic pH/LA tolerance of native cellulolytic bacteria has therefore the same importance

as increasing their LA production towards application of these strains in industrial production of LA.

Improving tolerance of a strain to a chemical or an environmental condition can be pursued through

different approaches, such as evolutionary engineering or rational metabolic engineering [17].

A recent transcriptomic/metabolomic study has identified possible protein targets for improving acidic

pH tolerance of *C. thermocellum* [52] that include: i) improving the expression of F_1F_0-ATPase, owing

to its function in pumping protons out of the cell at the expense of ATP; ii) up-regulating proton-pumping

PP_i-ase; iii) improving the expression of protein chaperones and heat-shock proteins such as GrpE, Hsp

20 and Hsp33. A further promising target for engineering acid tolerance in this strain seems nitrogen

metabolism. Acidic pH induces intracellular glutamate accumulation, which could be exploited by

introducing a heterologous glutamate decarboxylase [52]. Actually, bacterial glutamate decarboxylases

are generally involved in neutralizing pH acidity, through proton-consuming decarboxylation of

glutamate to γ-aminobutyrate [55]. Furthermore, inactivation of Glutamine synthase might also reduce

the need for buffering fermentation media of *C. thermocellum* cultures [52,56]. Recently, a combination

of random chemical mutagenesis and evolutionary engineering has been used to increase acid tolerance

312   in the anaerobic cellulolytic bacterium *Fibrobacter succinogenes* [57]. Improvement of acid tolerance

313   was moderate since the pH limit was lowered from 6.10 to 5.65, nonetheless this study showed that it is

314   possible to generate more acid-tolerant cellulolytic microorganisms.

315   No study on LA tolerance of native cellulolytic microorganisms and/or on how to improve it has been

316   reported so far. However, several investigations have been performed on more established strains for

317   industrial LA production, such as LAB, which can inspire research on cellulolytic microorganisms.

318   Rational engineering of stress tolerant LAB have been based on overexpression of proteins that are up-

319   regulated upon acid exposure, such as molecular chaperones [58,59] and DNA repair proteins [60].

320   Overexpression of the molecular chaperone DnaK [61] or of the DNA repair protein RecO [60] in

321   *Lactococcus lactis* improved tolerance to multiple stresses, including LA, and also enhanced LA

322   production. Rational engineering has been used also for improving LA tolerance of a weak LA producer,

323   i.e. *Saccharomyces cerevisiae* [62]. A gene deletion library indicated that several genes affect LA

324   tolerance in this microorganism [62]. Disruption of these genes increased LA resistance and LA

325   productivity. Furthermore, multiple gene disruption had cumulative effects [62]. Adaptive evolution

326   approach was recently used to improve LA tolerance of *Leuconostoc mesenteroides* up to 70 g/L [63].

327   Improved LA tolerance phenotype corresponded also in this case to increased LA production (titer up to

328   76.8 g/L) that was 2-fold higher than in the wild type strain. Analysis of *L. mesenteroides* mutants

329   revealed increased intracellular content of ammonia and a mutation in the gene encoding ε subunit F_0F_1

330   ATPase likely causing more efficient ATP-dependent proton extrusion activity [63].

331

Recombinant cellulolytic strategies

333   RCSs take advantage from current understanding of the cellulase systems found in native

334   cellulolytic microorganisms. The latter consist of multiple enzymes with different substrate specificity

335   and catalytic mechanisms that act synergistically [51]. Most metabolic engineering strategies have taken

336   inspiration from the two most extensively studied paradigms of cellulase systems, i.e. the non-complexed

14

337 enzyme model of aerobic fungi and bacteria and the cellulosome complexes of anaerobic microorganisms

338 [51]. Cellulosomes provide significant advantage in terms of catalytic efficiency, because close

339 proximity of different enzyme subunits improves their synergism. Moreover, cellulosomes are generally

340 tethered to the bacterial surface, which further promotes their synergistic activity through cellulosome-

341 cell proximity [64]. Additional characteristics of cellulosomes with respect to non-complexed systems,

342 are provided by one to several scaffolding proteins (i.e. scaffoldins), that is proteins generally consisting

343 of multiple domains that are specifically involved in binding enzyme subunits (via cohesion domains) or

344 polysaccharides (via carbohydrate binding modules, CBM) or the cell surface (e.g. via S-layer homology

345 domains or sortase recognition motifs) [64]. Consistently, cellulosomal enzymes contain an additional

346 domain, i.e. a dockerin, which is required for binding cohesin modules. Because of the complexity of

347 these native enzyme systems, RCSs face significant challenges. The minimal requirement for efficient

348 depolymerization of cellulosic substrate is a system consisting of 3 enzyme activities (i.e. an

349 exoglucanase, an endoglucanase and a β-glucosidase) and, additionally, a scaffoldin for cellulosome-

350 inspired complexes [51]. However, expression of heterologous cellulases is often toxic because of

351 saturation of protein secretion pathways in the host [65–67]. These issues have severely hampered

352 advances of RCSs.

353 As far as production of LA from lignocellulose is concerned, most examples of RCSs have been

354 targeted on LAB (for an extensive review refer to [14]). LAB can produce LA with high yield,

355 productivity and optical purity [8] through fermentation of several mono-, di- and oligo-saccharides [14].

356 Furthermore, some strain is very acid tolerant and the vast majority of them is GRAS, i.e. generally

357 recognized as safe, which avoid possible adverse health effects on either consumers or industrial

358 production workers. Concerns of RCSs in LAB are represented by the fact that the large majority of

359 engineered LAB described so far expresses a single heterologous cellulase or hemicellulase which is not

360 sufficient for these strains to grow on complex lignocellulosic substrates [8]. As far as I know, only one

361 recent study reported engineering of a cellulase system consisting of a β-glucosidase and an

362 endoglucanase in a single *Lactococcus lactis* strain [68]. However, the latter strain could ferment

363 cellooligosaccharides up to at least cellooctaose to L-LA with high yield, but could not grow on more

364 complex cellulosic substrates.

365 Research has therefore been oriented towards alternative strategies able to reduce the burden of

366 producing and secreting heterologous proteins. This can be accomplished by designing engineered

367 microbial consortia where each strain expresses a single heterologous enzyme or protein. The studies of

368 the group directed by Profs. Mizrahi and Bayer in Israel have leaded this research approach on LAB.

369 Different proteins have been introduced in *Lactobacillus plantarum* including endoglucanases, xylanases

370 and different scaffolding proteins [66,69,70]. Over years these studies have gradually improved their

371 achievements leading to assembly of a *L. plantarum* consortium that display mini-cellulosomes

372 consisting of up to six enzymatic components (Fig. 2A) [70]. This outstanding result, leading to engineer

373 enzymes complexes with significant hydrolysis of wheat straw, was nonetheless insufficient to enable *L.*

374 *plantarum* consortium to grow on wheat straw as the sole carbon source. It has been hypothesized that

375 the enzyme mixture used to engineer the *L. plantarum* consortium could release insufficient/unsuitable

376 soluble sugars through biomass hydrolysis for this strain [70]. This focus the attention on the importance

377 of choosing suitable enzymes for RCSs. This is not trivial, since a rationale that can predict which enzyme

378 partners can function with the best synergism in a certain microbial strain is currently unavailable.

379 Moreover, the choice of enzyme candidates for RCSs is often limited to those who are efficiently secreted

380 by the microbial host [65]. So far, the number of strategies to solve or reduce issues in protein secretion

381 is relatively little [67]. In most cases they consist in engineering the signal peptide of cellulases by

382 replacing it with host-specific signal peptides [70–73]. Inactivation of housekeeping protease(s), such as

383 the unique exported protease HtrA of *L. lactis*, may be an alternative solution to increase cellulase

384 secretion yield [71]. Peculiar mechanisms of protein folding requiring specific chaperon(s) have been

385 hypothesized for some cellulases and especially for cellulosomal components [74], but no study have

386 identified them. Actually, almost no information on mechanisms of cellulase secretion in native

387   cellulolytic microorganisms is currently available [75]. This represent a significant hurdle towards

388   engineering of cellulase systems in heterologous microorganisms and will require a considerable amount

389   of research.

390   Apart from numerous examples of RCSs focused on the expression of heterologous enzymes for

391   plant polysaccharide depolymerization, it is worth reminding some studies aimed at improving the

392   metabolism of monosaccharides released by hemicellulose hydrolysis in LAB [76–79]. Actually,

393   hemicellulose is mainly composed by pentoses which are fermented to LA with low yield by most LAB

394   [14]. Strains able of almost homolactic fermentation of xylose and/or arabinose were obtained by

395   inactivation of the phosphoketolase pathway and introduction or enhancement of the pentose phosphate

396   pathway [76–79]. Other studies have been addressed to relieving carbon catabolite repression of pentose

397   metabolism, leading to recombinant strains able to simultaneously ferment glucose/xylose mixtures

398   [80,81].

399   Apart from inability of LAB to directly use lignocellulose, industrial production of LA by using LAB

400   has additional drawbacks related to LAB requirement of complex nutrients, such as amino acids,

401   nucleotides and/or and vitamins, for their growth. This significantly increases the cost of both the growth

402   medium and LA recovery. Research has therefore targeted other efficient native LA producers with lower

403   nutritional requirements such as bacteria belonging to *Bacillus* genus and *Rhizopus sp.* filamentous fungi

404   [2,13]. In both groups of microorganisms, several strains that naturally secrete cellulases and

405   hemicellulases have been reported, although, as far as I know, no strain able to grow on lignocellulosic

406   biomass without prior biomass saccharification has been reported. Efficient LA producers among bacilli

407   include *B. coagulans, B. licheniformis, B. stearothermophilus, B. subtilis*, and *B. thermoamylovorans*

408   strains [2,13]. Additionally, *B. subtilis* features high efficient secretion properties which have been

409   exploited for the production of heterologous proteins [82,83]. Further improvement of heterologous

410   protein secretion by *B. subtilis* has been attained through engineering of *B. subtilis* strain WB800, which

411   lacks eight extracellular proteases [84,85]. Valuable examples of RCSs have been reported on *B. subtilis*

412 and other bacilli, including the construction of artificial consortia of cellulase-engineered strains [86,87].

413 Remarkably, assembly of minicellulosomes in a single *B. subtilis* strain dates back to 2004 [88].

414 Recently, artificial operons encoding eight cellulosomal subunits of *C. thermocellum* have been

415 assembled and transformed in *B. subtilis* [89]. Operons included genes for the full-length adaptor

416 scaffoldin CipA (featuring 9 cohesin domains), the anchoring scaffoldin SdbA, and six enzymatic

417 subunits featuring exoglucanase, endoglucanase and xylanase activity (Fig. 2B). This allowed secretion

418 and partial surface-display of large designer cellulosomes in a single recombinant strain. Improved

419 saccharification of raw cellulosic materials by recombinant *B. subtilis* was reported, although no mention

420 was made about the fact that this improved phenotype was able to support *B. subtilis* growth on these

421 substrates [89]. However, no examples of RCSs have targeted LA-producing *Bacillus* strains. Fungi of

422 the *Rhizopus* genus, especially *R. oryzae* have been investigated as regards industrial production of LA

423 also because of easier downstream process for separation of biomass with respect to planktonic bacteria.

424 On the other side, they generally show lower LA yield (because of accumulation of other products, e.g.

425 ethanol and fumaric acid) and productivity [2,12]. *Rhizopus sp.* strains may also be able to produce

426 cellulases [90] but cannot directly use lignocellulosic biomass without prior hydrolysis treatment [91].

427 Recently, gene manipulation tools for *R. oryzae* such as transformation of heterologous genes, gene

428 knockout and RNA interference have been developed [92]. However, no attempts of expression of

429 heterologous cellulase in this strain has been reported so far.

430

Conclusions

432 Now more than ever, awareness of the effects than fossil fuel exploitation has on global warming and

433 climate change is widespread in the population. Furthermore, alarm regarding current diffusion of

434 microplastics in nearly every ecosystem on the Earth is increasing [93]. Development of alternative

435 technologies for producing commodity chemicals aimed at replacing traditional processes based on oil

436 refinery is a global priority. Industrial interest in LA has dramatically increased recently owing to its

437 application for the synthesis of biodegradable plastic polymers, namely PLA. However, current LA

438 fermentative processes are relatively expensive, thus PLA use as general purpose plastic is not cost-

439 competitive with fossil-derived polymers yet. The use of lignocellulosic biomass as feedstock for LA

440 fermentation could significantly lower LA price, but research towards simpler and cheaper process for

441 plant biomass bioconversion is necessary. Metabolic engineering could significantly help reducing the

442 cost of lignocellulose fermentation by developing recombinant microorganisms able to catalyze single-

443 reactor fermentation of plant biomass.

444 Metabolic engineering strategies aimed at direct production of LA from lignocellulose are at still

445 relatively early stage of development, especially if compared to production of biofuels. Most examples

446 concern RCSs targeted to engineer heterologous cellulase systems in LAB. RCSs are extremely

447 challenging, because of issues in expressing and secreting heterologous cellulases and the innate intricacy

448 of the native cellulolytic systems. Although expression of multicomponent designer cellulosomes has

449 been achieved in some LAB or bacilli, no direct production of LA from plant biomass has been reported

450 in these strains, so far. Ideally, improved efforts should be dedicated to understanding mechanisms of

451 protein secretion, and, in particular, cellulase secretion, together with better comprehension of cellulase

452 synergistic activity. This knowledge would greatly benefit to rational development of RCSs.

453 Improvement of LA production in native cellulolytic strains is even at earlier infancy. Gene manipulation

454 of these strains has been generally addressed at increasing their production of liquid biofuels, but these

455 studies have indicated metabolic key points that could be useful also for enhancing LA production.

456 Advantages of NCSs over RCSs include the fact that: i) gene tools have been developed for an increasing

457 number of microbial models such as *C. thermocellum*, *C. cellulolyticum*, *C. cellulovorans*, *C. bescii*, *T.*

458 *saccharolyticum* where they have been exploited at different extent for engineering their metabolic

459 pathways; ii) NCS should not face hurdles linked to the expression of heterologous cellulases. In some

460 cases, LA yield very close to the theoretical maximum has been reported in engineered strains, although

461 at the expense of growth efficiency (Table 1). Furthermore, these investigations have revealed more

462 sophisticated interconnection between different metabolic pathways than previously expected. As a

463 consequence, up-regulation of LDH and disruption of parasite pathways may be not sufficient to

464 significantly improve LA production in certain strains, because of possible effect of allosteric regulators

465 or competitive inhibitors. Taking into account these variables certainly complicates NCSs. Furthermore,

466 intense research effort aimed at improving acid tolerance of native cellulolytic microorganisms is

467 necessary towards development of cellulolytic strains able to produce high LA titers required by

468 industrial processes.

469

Acknowledgements

472

Conflict of interest

474 The author declares no conflict of interest

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Strategy	Strain	Gene modification	Y_{LA} (mol/mol glucose equivalent)(fold increase vs WT)	Notes	Reference
Disruption of ethanol production	*T. mathranii*	Δ*adhE*	≈ 1.5 (≈ 4.5)	Growth rate was only 34% of WT	[34]
	T. thermosaccharolyticum	Δ*adhE*	1.90 (63.3)	Growth rate was only 11% of WT	[23]
	T. saccharolyticum	Δ*adhE*	0.67 (5.6)	Final biomass was 60% lower than WT	[25]
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Disruption of H$_2$ production	*T. saccharolyticum*	Δhsf	0.83 (1.66)	Final biomass was about 50% lower than WT	[29]
	C. thermocellum	$\Delta hydG\ \Delta ech$	≈ 0	Final biomass and growth rate were only slightly lower than WT. Y_{LA} of WT was $\approx$ 0.25 mol/mol cellobiose	[37]
Disruption of acetyl-CoA production	*T. saccharolyticum*	$\Delta pforA$	0.91 (4.53)	The strain also improved through adaptive evolution. The final biomas was about 50% lower than WT.	[30]
	T. saccharolyticum	Δpfl	1.18 (5.89)	The strains required formate and yeast extract supplementation for optimal growth. Spontaneous mutation in gene encoding ferredoxin hydrogenase may have contributed to increased Y_{LA}	[30]
	C. thermocellum	Δpfl	0.15 (7.5)	The strain grew at final biomass similar to WT but growth rate was only 33% of WT	[41]

	T. saccharolyticum	Δ*pforA*, Δ*pfl*	1.76 (8.80)	The strains required formate, acetate and yeast extract supplementation for optimal growth.	[30]
Engineering redox state	*C. thermocellum*	Overexpression of *rnf*, Δ*hydG*	0.01 (1.21)	The strains produced 30% more ethanol	[42]
	C. bescii	Δ*rex*	n.r.	LA final titer was at least 124% more abundant than in WT	[27]
	T. saccharolyticum	Δ*rex*	0.02-0.08 (0.05-0.18)	LA production was repressed. Growth rate was only 19-32 % of WT and final biomass may be reduced up to 53 %.	[44]
Disruption of acetate production	*C. thermocellum*	Δ*pta*	≈ 0.33 (≈ 3)	-	[49]
	C. cellulolyticum	i-*pta*	≈ 0.19 (≈ 0.45)	*pta* expression was repressed by antisense RNA. LA production was repressed.	[50]

Figure captions

Figure 1. Overview of the central carbon catabolism of anaeobic (hemi)cellulolytic bacteria. Cellulose is channeled to Embden Meyerhof Parnas pathway by sequential conversion to : i) glucose 6 phosphate by using hydrolysis followed by ATP-dependent phosphorylation or phosporolytic mechanism (i.e. by using P_i); ii) Fructose 1,6 bisphosphate that can be obtained from fructose 6 phosphate by using ATP- or PPi-dependent phosphorylation. Pyruvate can be obtained from PEP by ADP-dependent pyruvate kinase or by pyruvate phosphate dikinase by using AMP + PP_i. End-products of fermentation are reported in red or green. Blue solid arrows are used for reactions involving nicotinamide ($(NAD(P)H/NAD(P)^+$) cofactors. Orange solid arrows are used for reactions involving energy carriers (ATP, ADP, PP_i). Dashed lines are used for activators (green) or inhibitors (red) of lactate dehydrogenase (LDH) activity. Abbreviations: Acetyl-P, acetyl phosphate; ACK, acetate kinase; ADH, alcohol dehydrogenase; ALDH aldehyde dehydrogenase; F1,6BP, fructose 1,6 bisphosphate; Fd, ferredoxin; FNOR, ferredoxin:NAD oxidoreductase; H_2ase, hydrogenase; LDH, lactate dehydrogenase; NFN, NADH-dependent reduced ferredoxin: $NADP^+$ oxidoreductase; PFL, pyruvate-formate liase; PFOR, pyruvate ferredoxin/flavodoxin oxidoreductase; PP_i, pyrophosphate; PTA, phosphotransacetylase; Rex, global redox-responsive transcription factor; RNF, ion-translocating reduced ferredoxin: NAD^+ oxidoreductase.

Figure 2. Scheme representing the most sophisticated examples of RCSs in microbial strains aimed at consolidated bioprocessing of lignocellulosic biomass to LA. A) Consortium of engineered *L. plantarum* strains where each strain secretes a different cellulosomal component leading to assembly of designer cellulosomes on the cell surface (modified from [70]). Cellulosomal components introduced in *L. plantarum* include wild-type and chimeric cellulase and hemicellulases from *C.*

papyrosolvens, designer adaptor scaffoldins (Adaptor 1, 2), i.e. an intermediate type of scaffoldin able to bind both enzyme subunits and additional scaffoldins, and anchoring scaffoldins (e.g. Anc 4), that is proteins that can tether the protein complex to the cell surface. Numbers shown on the enzyme components (i.e. 5, 9, 10, 11) correspond to the glycosyl hydrolase (GH) family of their catalytic domain. Chimeric enzymes were obtained by fusing the catalytic modules of *C. papyrosolvens* with type I dockerin domains derived from other microorganisms. Adaptor scaffoldins were designed with: i) divergent cohesin modules for selective integration of different dockerin-containing enzymes; and ii) different type II and III dockerin modules for selective attachment of cohesin-containing anchoring scaffoldins. Anchoring scaffoldins are covalently attached to the cell surface through sortase recognition motif. B) eight-component cellulosome engineered on the surface of a single *B. subtilis* strain through introduction of artificial operons (adapted from [89]). The designer cellulosome consists of the cell-surface anchor SdbA, the adaptor scaffoldin CipA (comprising nine cohesins, coh, and one CBM), two exoglucanases (CelK, CelS), two endoglucanases (CelA, CelR) and two xylanases (XynC, XynZ) derived from *C. thermocellum.*
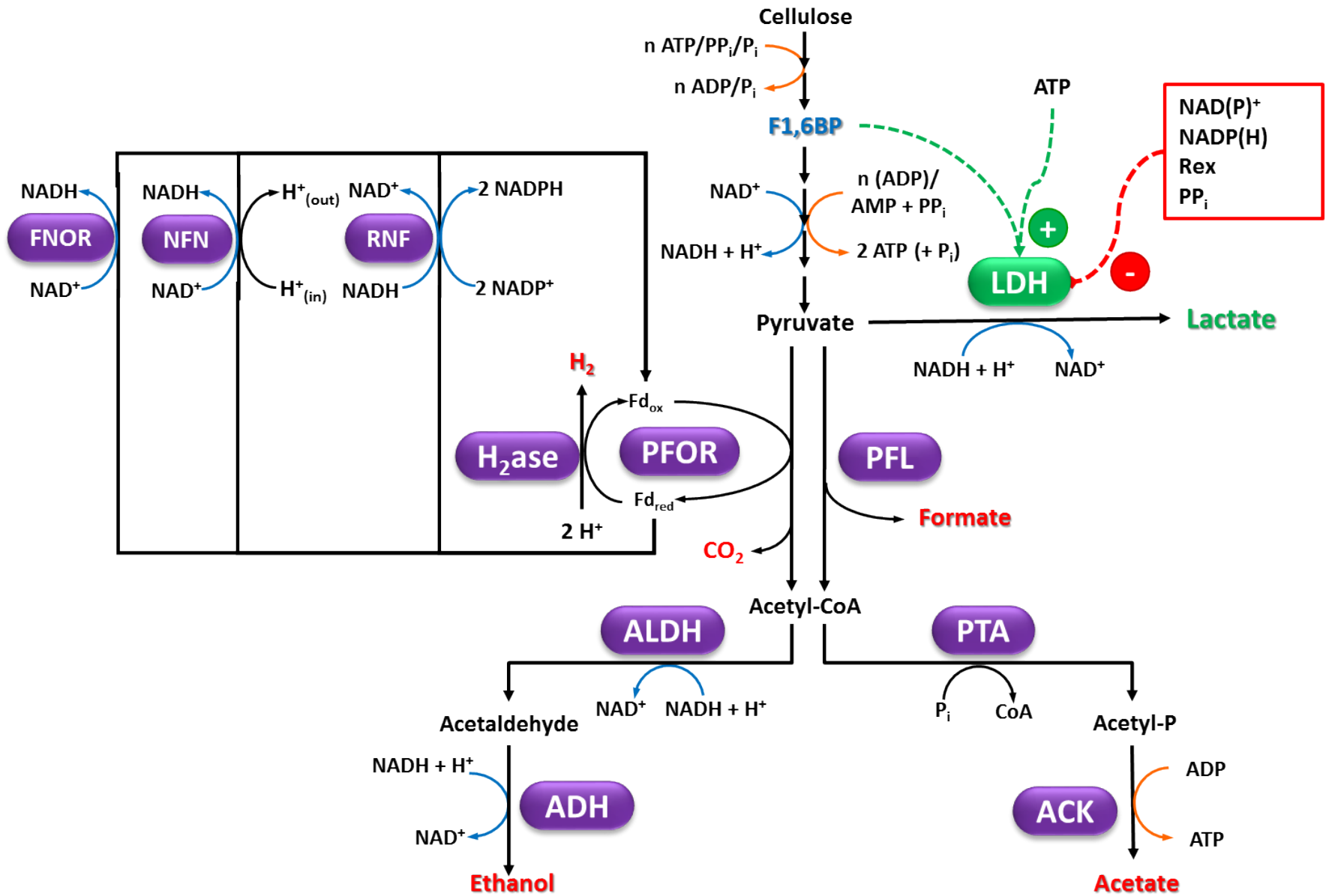
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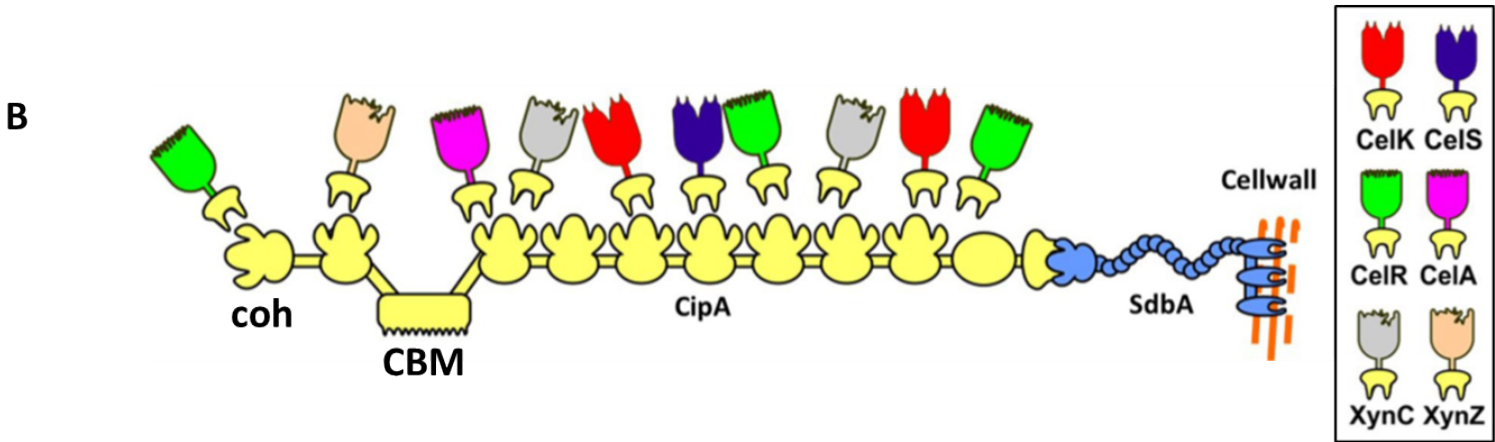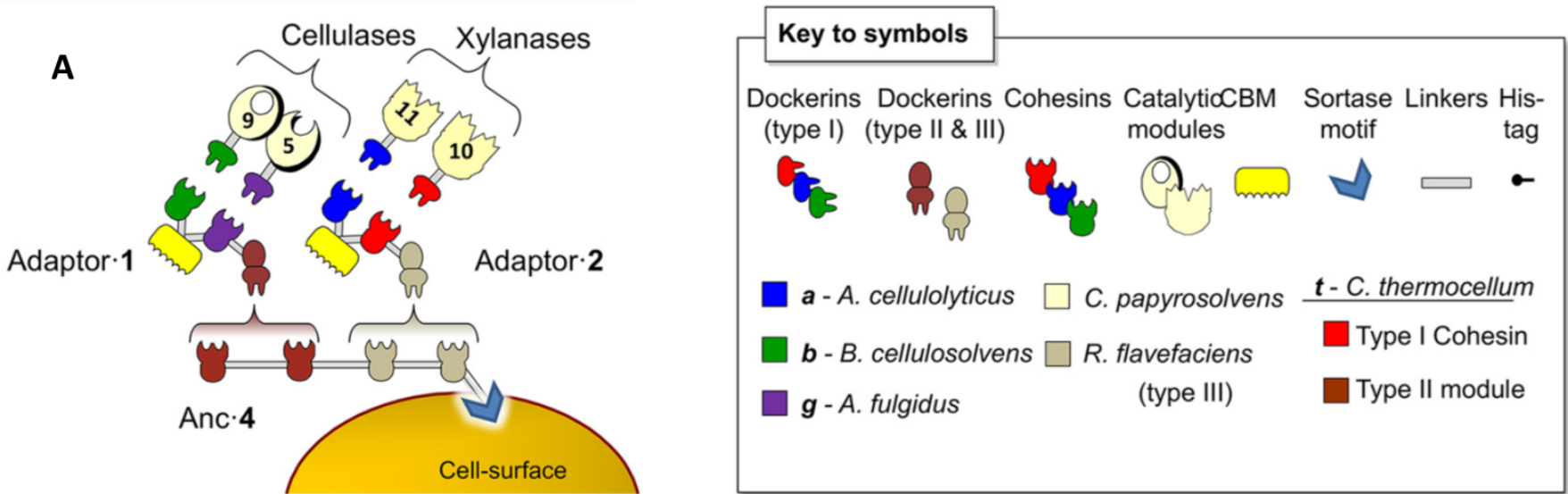
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