

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Metabolic engineering strategies for consolidated production of lactic acid from lignocellulosic biomass

This is a pre print version of the following article:

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1728135> since 2020-04-25T09:11:35Z

Published version:

DOI:10.1002/bab.1869

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)



Metabolic engineering strategies for consolidated production of lactic acid from lignocellulosic biomass

Journal:	<i>Biotechnology and Applied Biochemistry</i>
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:	<p>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.</p>

1 **Metabolic engineering strategies for consolidated production of lactic acid**
2 **from lignocellulosic biomass**

3

4 Mazzoli R.*

5

6 Structural and Functional Biochemistry. Laboratory of Proteomics and Metabolic Engineering of
7 Prokaryotes. Department of Life Sciences and Systems Biology. University of Torino. Via Accademia
8 Albertina 13. 10123 Torino. Italy.

9

10

11 Running title: Direct lactic acid production from lignocellulose

12

13

14

15 *Corresponding author: Roberto Mazzoli,

16 Department of Life Sciences and Systems Biology. University of Torino. Via Accademia Albertina 13.

17 10123 Torino. Italy.

18 Fax +39 011 6704508

19 E-mail : roberto.mazzoli@unito.it

20

21 **Abstract**

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

37

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

39

40

41 **Introduction**

42 Lactic acid (LA) is one of the most requested chemicals owing to its application in several areas [1]. The
43 most traditional utilization of LA is in the food industry, e.g. as acidifier, emulsifier, preservative and
44 flavour-enhancing agent, but also in the production of cosmetics (such as emulsifying and moisturizing
45 agent), pharmaceuticals (as intermediate) and in the chemical industry (e.g. for production of solvents)
46 [1]. However, the LA application that best fits the current green economy revolution towards more
47 sustainable and environment-friendly technologies is as building block for the synthesis of biodegradable
48 plastic polymers (e.g. polylactide, PLA, and its co-polymers) [2]. PLA application ranges from the
49 medical area (e.g. surgical sutures, orthopaedic and cardiovascular devices, drug delivery, tissue
50 regeneration) owing to its biocompatibility, to use in agriculture (mulch films and bags), food and good
51 packaging, and manufacturing of disposable cutlery, cups and trays [1,3]. PLA can therefore be
52 considered as a general-purpose material potentially able to replace fossil-fuel derived plastics in most
53 applications.

54 All these uses, especially for PLA synthesis, have driven global market expansion of LA. The global
55 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
56 annual growth of 16.2% [1]. About 90% of LA produced worldwide is obtained by microbial
57 fermentation of dedicated crops (mainly corn) by companies such as Corbion-Purac (The Netherlands),
58 Galactic (Belgium) and NatureWorks LLC-Cargill (USA) [1,2]. Actually, LA production by microbial
59 fermentation is advantageous over chemical synthesis since optically pure LA can be obtained instead of
60 a racemic mixture of D- and L-LA [4]. This is particularly important for certain LA applications such as
61 in the production of PLA, whose characteristics highly depend on the ratio of LA enantiomers, or in food,
62 drink and pharmaceutical industries since D-LA can cause metabolic problems to humans and should be
63 avoided [4,5]. However, some issues of the current processes for producing LA risks to hamper further
64 expansion of the global LA market. In particular, the current cost of LA is relatively high (\$1.30-4.0/kg)
65 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

110 **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, H₂ and CO₂ (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 H₂ [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 H₂), 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

148 ***Disruption of ethanol production***

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

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

171

172 ***Disruption of H₂ production***

173 Production of H₂ by hydrogenases is another typical electron-consuming reaction found in anaerobic
174 cellulolytic microorganisms (Fig. 1). As mentioned above for ADH, also inhibiting H₂ production in a
175 microbial strain may not trivial since multiple hydrogenases likely involved in different functions (e.g.
176 redox balancing, derivation of energy from H₂ oxidation, proton respiration and/or proton-gradient build-
177 up) are frequently found within one species [36]. For instance, disruption of *hyd* or *ech* gene clusters of
178 *T. saccharolyticum*, encoding a NAD-dependent [FeFe]-hydrogenase and membrane-bound [Ni-Fe]
179 hydrogenase, respectively, did not result in any significant reduction of H₂ production, while deletion of
180 *hfs* gene cluster, likely encoding another [FeFe]-hydrogenase, resulted in >95% decrease in hydrogen
181 accumulation [29]. Furthermore, in the Δhfs strain LA was the most abundant product which is consistent
182 with re-distribution of reducing equivalents towards alternative electron-consuming pathways in strains
183 lacking hydrogenases (Table 1). More recently, a $\Delta hydG \Delta ech$ *C. thermocellum*, lacking the gene
184 encoding HydG, involved in the maturation of its four [FeFe]-hydrogenases, and the [Ni-Fe] hydrogenase
185 Ech, was obtained which showed complex perturbation of the central carbon metabolism causing
186 dramatic reduction of LA accumulation (Table 1) [37]. Although the exact cause of this unexpected
187 metabolic shift was not determined, it was speculated that disruption of hydrogenases could have altered
188 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 H₂ 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 $\Delta hydG$ 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* Δrex 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

266 ***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 phosphorylation (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* Δpta [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* expression [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

281 ***Improvement of acid tolerance***

282 One of the main limits towards 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

287 acidify cytoplasm and collapse the ΔpH [52]. As far as I know, no information on LA tolerance by native
288 cellulolytic microorganisms has been reported. However, accumulation of LA during fermentation is
289 known to inhibit natural LA producers and cause decrease in LA productivity [4]. Both issues, i.e. limited
290 tolerance to acidic pH and LA, have been traditionally fixed through fermentation process engineering.
291 Neutralizing agents are generally used during LA fermentation but this increases the cost of the process
292 both because of consumption of high amounts of neutralizing agent and because this complicates
293 downstream process of LA purification from the medium [53]. Alternatively, severe drop in pH and
294 accumulation of LA in the growth medium has been prevented by continuous removal of LA by several
295 strategies such as electro dialysis, solvent extraction, adsorption, and membrane bioreactors [4].
296 However, these methods complicate the fermentation process owing to associated technical problems
297 [54]. Improving acidic pH/LA tolerance of native cellulolytic bacteria has therefore the same importance
298 as increasing their LA production towards application of these strains in industrial production of LA.
299 Improving tolerance of a strain to a chemical or an environmental condition can be pursued through
300 different approaches, such as evolutionary engineering or rational metabolic engineering [17].

301 A recent transcriptomic/metabolomic study has identified possible protein targets for improving acidic
302 pH tolerance of *C. thermocellum* [52] that include: i) improving the expression of F_1F_0 -ATPase, owing
303 to its function in pumping protons out of the cell at the expense of ATP; ii) up-regulating proton-pumping
304 PP_1 -ase; iii) improving the expression of protein chaperones and heat-shock proteins such as GrpE, Hsp
305 20 and Hsp33. A further promising target for engineering acid tolerance in this strain seems nitrogen
306 metabolism. Acidic pH induces intracellular glutamate accumulation, which could be exploited by
307 introducing a heterologous glutamate decarboxylase [52]. Actually, bacterial glutamate decarboxylases
308 are generally involved in neutralizing pH acidity, through proton-consuming decarboxylation of
309 glutamate to γ -aminobutyrate [55]. Furthermore, inactivation of Glutamine synthase might also reduce
310 the need for buffering fermentation media of *C. thermocellum* cultures [52,56]. Recently, a combination
311 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

332 **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

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 led 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

431 **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

470 **Acknowledgements**

471 This study was granted by “Fondo Finanziamento delle Attività Base di Ricerca”.

472

473 **Conflict of interest**

474 The author declares no conflict of interest

475

476

References

- [1] Alves de Oliveira, R., Komesu, A., Vaz Rossell, C.E., and Maciel Filho, R. (2018). *Biochem. Eng. J.* **133**, 219–239.
- [2] Abdel-Rahman, M.A., Tashiro, Y., and Sonomoto, K. (2013). *Biotechnol. Adv.* **31**, 877–902.
- [3] Abdel-Rahman, M.A., Tashiro, Y., and Sonomoto, K. (2011). *J. Biotechnol.* **156**, 286–301.
- [4] Abdel-Rahman, M.A., and Sonomoto, K. (2016). *J. Biotechnol.* **236**, 176–192.
- [5] Jem, K.J., van der Pol, J.F., and de Vos, S. in Jem, K.J., van der Pol, J.F., and de Vos, S. Eds. (2010) *Plastics from bacteria*, Springer, Berlin, Heidelberg, pp. 323–346.
- [6] Bidy, M.J., Scarlata, C.J., and Kinchin, C.M. (2016). *NREL Rep.*, 10.2172/1244312.
- [7] Okano, K., Tanaka, T., Ogino, C., Fukuda, H., and Kondo, A. (2010). *Appl. Microbiol. Biotechnol.* **85**, 413–423.
- [8] Mazzoli, R., Bosco, F., Mizrahi, I., Bayer, E.A., and Pessione, E. (2014). *Biotechnol. Adv.* **32**, 1216–1236.
- [9] Sims, R.E.H., Mabee, W., Saddler, J.N., and Taylor, M. (2010). *Bioresour. Technol.* **101**, 1570–1580.
- [10] Gray, K.A., Zhao, L., and Emptage, M. (2006). *Curr. Opin. Chem. Biol.* **10**, 141–146.
- [11] Sauer, M., Porro, D., Mattanovich, D., and Branduardi, P. (2008). *Trends Biotechnol.* **26**, 100–108.
- [12] Zhang, Z.Y., Jin, B., and Kelly, J.M. (2007). *Biochem. Eng. J.* **35**, 251–263.
- [13] Poudel, P., Tashiro, Y., and Sakai, K. (2016). *Biosci. Biotechnol. Biochem.* **80**, 642–654.
- [14] Tarraran, L., and Mazzoli, R. (2018). *FEMS Microbiol. Lett.* **365**, doi: 10.1093/femsle/fny126.

- [15] Liu, G., Sun, J., Zhang, J., Tu, Y., and Bao, J. (2015). *Bioresour. Technol.* **198**, 803–810.
- [16] Lynd, L.R., Van Zyl, W.H., McBride, J.E., and Laser, M. (2005). *Curr. Opin. Biotechnol.* **16**, 577–583.
- [17] Mazzoli, R. (2012). *Comput. Struct. Biotechnol. J.* **3**, e201210007.
- [18] Shahab, R.L., Luterbacher, J.S., Brethauer, S., and Studer, M.H. (2018). *Biotechnol. Bioeng.* **115**, 1207–1215.
- [19] Jiang, Y., Wu, R., Zhou, J., He, A., Xu, J., Xin, F., Zhang, W., Ma, J., Jiang, M., and Dong, W. (2019). *Biotechnol. Biofuels* **12**, 155.
- [20] Benocci, T., Aguilar-Pontes, M.V., Kun, R.S., Lubbers, R.J.M., Lail, K., Wang, M., Lipzen, A., Ng, V., Grigoriev, I. V., Seiboth, B., *et al.* (2019). *Biotechnol. Biofuels* **12**, 81.
- [21] Li, C., Lin, F., Zhou, L., Qin, L., Li, B., Zhou, Z., Jin, M., and Chen, Z. (2017). *Biotechnol. Biofuels* **10**, 228.
- [22] Li, J., Lin, L., Sun, T., Xu, J., Ji, J., Liu, Q., and Tian, C. (2019). *Metab. Eng.*, pii: S1096-7176(19)30005-9.
- [23] Bhandiwad, A., Guseva, A., and Lynd, L. (2013). *Adv. Microbiol.* **03**, 46–51.
- [24] Yang, X., Xu, M., and Yang, S.T. (2015). *Metab. Eng.* **32**, 39–48.
- [25] Lo, J., Zheng, T., Hon, S., Olson, D.G., and Lynd, L.R. (2015). *J. Bacteriol.* **197**, 1386–1393.
- [26] Verbeke, T.J., Spicer, V., Krokhin, O. V., Zhang, X., Schellenberg, J.J., Fristensky, B., Wilkins, J.A., Levin, D.B., and Sparling, R. (2014). *Appl. Environ. Microbiol.* **80**, 1602–1615.
- [27] Sander, K., Chung, D., Hyatt, D., Westpheling, J., Klingeman, D.M., Rodriguez, M., Engle, N.L., Tschaplinski, T.J., Davison, B.H., and Brown, S.D. (2019). *Microbiologyopen* **8**, e00639.
- [28] Williams-Rhaesa, A.M., Awuku, N.K., Lipscomb, G.L., Poole, F.L., Rubinstein, G.M., Conway,

- J.M., Kelly, R.M., and Adams, M.W.W. (2018). *Extremophiles* **22**, 629–638.
- [29] Shaw, A.J., Hogsett, D.A., and Lynd, L.R. (2009). *J. Bacteriol.* **191**, 6457–6464.
- [30] Zhou, J., Olson, D.G., Lanahan, A.A., Tian, L., Murphy, S.J.L., Lo, J., and Lynd, L.R. (2015). *Biotechnol. Biofuels* **8**, 138.
- [31] Ma, K., Hutchins, A., Sung, S.J.S., and Adams, M.W.W. (1997). *Proc. Natl. Acad. Sci. U. S. A.* **94**, 9608–9613.
- [32] Eram, M.S., Oduaran, E., and Ma, K. (2014). *Archaea* **2014**, 349379.
- [33] Bao, T., Zhao, J., Li, J., Liu, X., and Yang, S.T. (2019). *Bioresour. Technol.* **285**, 121316.
- [34] Yao, S., and Mikkelsen, M.J. (2010). *J. Mol. Microbiol. Biotechnol.* **19**, 123–133.
- [35] Özkan, M., Yilmaz, E.I., Lynd, L.R., and Özcengiz, G. (2004). *Can. J. Microbiol.* **50**, 845–851.
- [36] Calusinska, M., Happe, T., Joris, B., and Wilmotte, A. (2010). *Microbiology* **156**, 1575–1588.
- [37] Biswas, R., Zheng, T., Olson, D.G., Lynd, L.R., and Guss, A.M. (2015). *Biotechnol. Biofuels* **8**, 20.
- [38] Willquist, K., and van Niel, E.W.J. (2010). *Metab. Eng.* **12**, 282–290.
- [39] Bryant, F.O. (1991). *J. Enzyme Inhib. Med. Chem.* **5**, 235–248.
- [40] Cui, J., Olson, D.G., and Lynd, L.R. (2019). *Metab. Eng.* **51**, 32–42.
- [41] Rydzak, T., Lynd, L.R., and Guss, A.M. (2015). *J. Ind. Microbiol. Biotechnol.* **42**, 1263–1272.
- [42] Lo, J., Olson, D.G., Murphy, S.J.L., Tian, L., Hon, S., Lanahan, A., Guss, A.M., and Lynd, L.R. (2017). *Metab. Eng.* **39**, 71–79.
- [43] Ravcheev, D.A., Li, X., Latif, H., Zengler, K., Leyn, S.A., Korostelev, Y.D., Kazakov, A.E., Novichkov, P.S., Osterman, A.L., and Rodionov, D.A. (2012). *J. Bacteriol.* **194**, 1145–1157.

- [44] Zheng, T., Lanahan, A.A., Lynd, L.R., and Olson, D.G. (2018). *PLoS One* **13**, e0195143.
- [45] Levin, D.B., Carere, C.R., Cicek, N., and Sparling, R. (2009). *Int. J. Hydrogen Energy* **34**, 7390–7403.
- [46] Cai, G., Jin, B., Saint, C., and Monis, P. (2011). *J. Biotechnol.* **155**, 269–274.
- [47] Li, Y., Tschaplinski, T.J., Engle, N.L., Hamilton, C.Y., Rodriguez, M., Liao, J.C., Schadt, C.W., Guss, A.M., Yang, Y., and Graham, D.E. (2012). *Biotechnol. Biofuels* **5**, 2.
- [48] Tripathi, S.A., Olson, D.G., Argyros, D.A., Miller, B.B., Barrett, T.F., Murphy, D.M., McCool, J.D., Warner, A.K., Rajgarhia, V.B., Lynd, L.R., *et al.* (2010). *Appl. Environ. Microbiol.* **76**, 6591–6599.
- [49] Argyros, D.A., Tripathi, S.A., Barrett, T.F., Rogers, S.R., Feinberg, L.F., Olson, D.G., Foden, J.M., Miller, B.B., Lynd, L.R., Hogsett, D.A., *et al.* (2011). *Appl. Environ. Microbiol.* **77**, 8288–8294.
- [50] Xu, T., Li, Y., He, Z., Van Nostrand, J.D., and Zhou, J. (2017). *Front. Microbiol.* **8**, 1744.
- [51] Lynd, L.R., Weimer, P.J., van Zyl, W.H., and Pretorius, I.S. (2002). *Microbiol. Mol. Biol. Rev.* **66**, 739–739.
- [52] Whitham, J.M., Moon, J.W., Rodriguez, M., Engle, N.L., Klingeman, D.M., Rydzak, T., Abel, M.M., Tschaplinski, T.J., Guss, A.M., and Brown, S.D. (2018). *Biotechnol. Biofuels* **11**, 98.
- [53] Upadhyaya, B.P., DeVeaux, L.C., and Christopher, L.P. (2014). *Trends Biotechnol.* **32**, 637–644.
- [54] Boontawan, P., Kanchanathawee, S., and Boontawan, A. (2011). *Biochem. Eng. J.* **54**, 192–199.
- [55] Laroute, V., Yasaro, C., Narin, W., Mazzoli, R., Pessione, E., Cocaïgn-Bousquet, M., and Loubière, P. (2016). *Front. Microbiol.* **7**, 1050.

- [56] Rydzak, T., Garcia, D., Stevenson, D.M., Sladek, M., Klingeman, D.M., Holwerda, E.K., Amador-Noguez, D., Brown, S.D., and Guss, A.M. (2017). *Metab. Eng.* **41**, 182–191.
- [57] Wu, C.W., Spike, T., Klingeman, D.M., Rodriguez, M., Bremer, V.R., and Brown, S.D. (2017). *Sci. Rep.* **7**, 2277.
- [58] Wu, R., Zhang, W., Sun, T., Wu, J., Yue, X., Meng, H., and Zhang, H. (2011). *Int. J. Food Microbiol.* **147**, 181–187.
- [59] Wu, C., He, G., and Zhang, J. (2014). *J. Ind. Microbiol. Biotechnol.* **41**, 1533–1540.
- [60] Wu, C., Zhang, J., Du, G., and Chen, J. (2013). *Bioresour. Technol.* **143**, 238–241.
- [61] Abdullah-Al-Mahin, Sugimoto, S., Higashi, C., Matsumoto, S., and Sonomoto, K. (2010). *Appl. Environ. Microbiol.* **76**, 4277–4285.
- [62] Suzuki, T., Sakamoto, T., Sugiyama, M., Ishida, N., Kambe, H., Obata, S., Kaneko, Y., Takahashi, H., and Harashima, S. (2013). *J. Biosci. Bioeng.* **115**, 467–474.
- [63] Ju, S.Y., Kim, J.H., and Lee, P.C. (2016). *Biotechnol. Biofuels* **9**, 240.
- [64] Mazzoli, R., Lamberti, C., and Pessione, E. (2012). *Trends Biotechnol.* **30**, 111–119.
- [65] Ilmén, M., Den Haan, R., Brevnova, E., McBride, J., Wiswall, E., Froehlich, A., Koivula, A., Voutilainen, S.P., Siika-Aho, M., La Grange, D.C., *et al.* (2011). *Biotechnol. Biofuels* **4**, 30.
- [66] Moraïs, S., Shterzer, N., Lamed, R., Bayer, E.A., and Mizrahi, I. (2014). *Biotechnol. Biofuels* **7**, 112.
- [67] Song, X., Li, Y., Wu, Y., Cai, M., Liu, Q., Gao, K., Zhang, X., Bai, Y., Xu, H., and Qiao, M. (2018). *FEMS Yeast Res.* **18**, doi: 10.1093/femsyr/foy090.
- [68] Gandini, C., Tarraran, L., Kalemasi, D., Pessione, E., and Mazzoli, R. (2017). *Biotechnol. Bioeng.* **114**, 2807–2817.

- [69] Moraïs, S., Shterzer, N., Grinberg, I.R., Mathiesen, G., Eijssink, V.G.H., Axelsson, L., Lamed, R., Bayer, E.A., and Mizrahi, I. (2013). *Appl. Environ. Microbiol.* **79**, 5242–5249.
- [70] Stern, J., Moraïs, S., Ben-David, Y., Salama, R., Shamshoum, M., Lamed, R., Shoham, Y., Bayer, E.A., and Mizrahi, I. (2018). *Appl. Environ. Microbiol.* **84**, pii: e00282-18.
- [71] Wieczorek, A.S., and Martin, V.J.J. (2010). *Microb. Cell Fact.* **9**, 69.
- [72] Wang, L., Yang, Y., Cai, B., Cao, P., Yang, M., and Chen, Y. (2014). *Int. J. Mol. Sci.* **15**, 12842–12860.
- [73] Liu, Q., Shao, T., Dong, Z., and Bai, Y. (2017). *Process Biochem.* **62**, 135–143.
- [74] Mingardon, F., Chanal, A., Tardif, C., and Fierobe, H.P. (2011). *Appl. Environ. Microbiol.* **77**, 2831–2838.
- [75] Yan, S., and Wu, G. (2014). *Appl. Microbiol. Biotechnol.* **98**, 5329–5362.
- [76] Okano, K., Yoshida, S., Tanaka, T., Ogino, C., Fukuda, H., and Kondo, A. (2009). *Appl. Environ. Microbiol.* **75**, 5175–5178.
- [77] Okano, K., Yoshida, S., Yamada, R., Tanaka, T., Ogino, C., Fukuda, H., and Kondo, A. (2009). *Appl. Environ. Microbiol.* **75**, 7858–7861.
- [78] Shinkawa, S., Okano, K., Yoshida, S., Tanaka, T., Ogino, C., Fukuda, H., and Kondo, A. (2011). *Appl. Microbiol. Biotechnol.* **91**, 1537–1544.
- [79] Qiu, Z., Gao, Q., and Bao, J. (2018). *Bioresour. Technol.* **249**, 9–15.
- [80] Yoshida, S., Okano, K., Tanaka, T., Ogino, C., and Kondo, A. (2011). *Appl. Microbiol. Biotechnol.* **92**, 67–76.
- [81] Zhang, Y., Vadlani, P. V., Kumar, A., Hardwidge, P.R., Govind, R., Tanaka, T., and Kondo, A. (2016). *Appl. Microbiol. Biotechnol.* **100**, 279–288.

- [82] Wong, S.L. (1995). *Curr. Opin. Biotechnol.* **6**, 517–522.
- [83] Ling Lin Fu, Zi Rong Xu, Wei Fen Li, Jiang Bing Shuai, Ping Lu, and Chun Xia Hu (2007). *Biotechnol. Adv.* **25**, 1–12.
- [84] Wu, S.C., Yeung, J.C., Duan, Y., Ye, R., Szarka, S.J., Habibi, H.R., and Wong, S.L. (2002). *Appl. Environ. Microbiol.* **68**, 3261–3269.
- [85] Wu, X.C., Lee, W., Tran, L., and Wong, S.L. (1991). *J. Bacteriol.* **173**, 4952–4958.
- [86] Arai, T., Matsuoka, S., Cho, H.Y., Yukawa, H., Inui, M., Wong, S.L., and Doi, R.H. (2007). *Proc. Natl. Acad. Sci. U. S. A.* **104**, 1456–1460.
- [87] Kalbarczyk, K.Z., Mazeau, E.J., Rapp, K.M., Marchand, N., Koffas, M.A.G., and Collins, C.H. (2018). *ACS Synth. Biol.* **7**, 2413–2422.
- [88] Cho, H.Y., Yukawa, H., Inui, M., Doi, R.H., and Wong, S.L. (2004). *Appl. Environ. Microbiol.* **70**, 5704–5707.
- [89] Chang, J.J., Anandharaj, M., Ho, C.Y., Tsuge, K., Tsai, T.Y., Ke, H.M., Lin, Y.J., Ha Tran, M.D., Li, W.H., and Huang, C.C. (2018). *Biotechnol. Biofuels* **11**, 157.
- [90] Kupski, L., Pagnussatt, F.A., Buffon, J.G., and Furlong, E.B. (2014). *Appl. Biochem. Biotechnol.* **172**, 458–468.
- [91] Thongchul, N., Navankasattusas, S., and Yang, S.T. (2010). *Bioprocess Biosyst. Eng.* **33**, 407–416.
- [92] Meussen, B.J., De Graaff, L.H., Sanders, J.P.M., and Weusthuis, R.A. (2012). *Appl. Microbiol. Biotechnol.* **94**, 875–886.
- [93] Akdogan, Z., and Guven, B. (2019). *Environ. Pollut.* **254**, 113011.

Table 1. Effects of gene modification on LA production yield in native (hemi)/cellulolytic microorganisms. Abbreviations: *adhE*, gene encoding bifunctional alcohol/aldehyde dehydrogenases involved in ethanol production; *ech*, gene encoding [NiFe]-hydrogenase; *hydG*, gene encoding [FeFe]-hydrogenase maturase; *hsf*, gene cluster encoding [FeFe]-hydrogenase; *pfl* gene cluster encoding pyruvate formate lyase; *pforA* gene encoding pyruvate ferredoxine/flavodoxine oxidoreductase; *pta* gene encoding phosphate acetyltransferase; *rex*, gene encoding global redox-responsive transcription factor Rex; *rnf*, gene cluster encoding ion-translocating reduced ferredoxin: NAD⁺ oxidoreductase. n.r. not reported.

Strategy	Strain	Gene modification	Y _{LA} (mol/mol glucose equivalent)(fold increase vs WT)	Notes	Reference
Disruption of ethanol production	<i>T. mathranii</i>	$\Delta adhE$	≈ 1.5 (≈ 4.5)	Growth rate was only 34% of WT	[34]
	<i>T. thermosaccharolyticum</i>	$\Delta adhE$	1.90 (63.3)	Growth rate was only 11% of WT	[23]
	<i>T. saccharolyticum</i>	$\Delta adhE$	0.67 (5.6)	Final biomass was 60% lower than WT	[25]
	<i>C. thermocellum</i>	$\Delta adhE$	0.78 (56)	Final biomass was 27% lower than WT	[25]

Disruption of H ₂ production	<i>T. saccharolyticum</i>	Δhsf	0.83 (1.66)	Final biomass was about 50% lower than WT	[29]
	<i>C. thermocellum</i>	$\Delta hydG \Delta ech$	≈ 0	Final biomass and growth rate were only slightly lower than WT. Y_{LA} of WT was ≈ 0.25 mol/mol cellobiose	[37]
Disruption of acetyl-CoA production	<i>T. saccharolyticum</i>	$\Delta pforA$	0.91 (4.53)	The strain also improved through adaptive evolution. The final biomass was about 50% lower than WT.	[30]
	<i>T. saccharolyticum</i>	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]
	<i>C. thermocellum</i>	Δpfl	0.15 (7.5)	The strain grew at final biomass similar to WT but growth rate was only 33% of WT	[41]

	<i>T. saccharolyticum</i>	$\Delta pforA, \Delta pfl$	1.76 (8.80)	The strains required formate, acetate and yeast extract supplementation for optimal growth.	[30]
Engineering redox state	<i>C. thermocellum</i>	Overexpression of <i>rnf</i> , $\Delta hydG$	0.01 (1.21)	The strains produced 30% more ethanol	[42]
	<i>C. bescii</i>	Δrex	n.r.	LA final titer was at least 124% more abundant than in WT	[27]
	<i>T. saccharolyticum</i>	Δrex	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	<i>C. thermocellum</i>	Δpta	$\approx 0.33 (\approx 3)$	-	[49]
	<i>C. cellulolyticum</i>	<i>i-pt</i>	$\approx 0.19 (\approx 0.45)$	<i>pta</i> expression was repressed by antisense RNA. LA production was repressed.	[50]

Figure captions

Figure 1. Overview of the central carbon catabolism of anaerobic (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 phospholytic mechanism (i.e. by using P_i); ii) Fructose 1,6 bisphosphate that can be obtained from fructose 6 phosphate by using ATP- or PP_i -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; H₂ase, 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.*

papyrosolvans, 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. papyrosolvans* 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*.

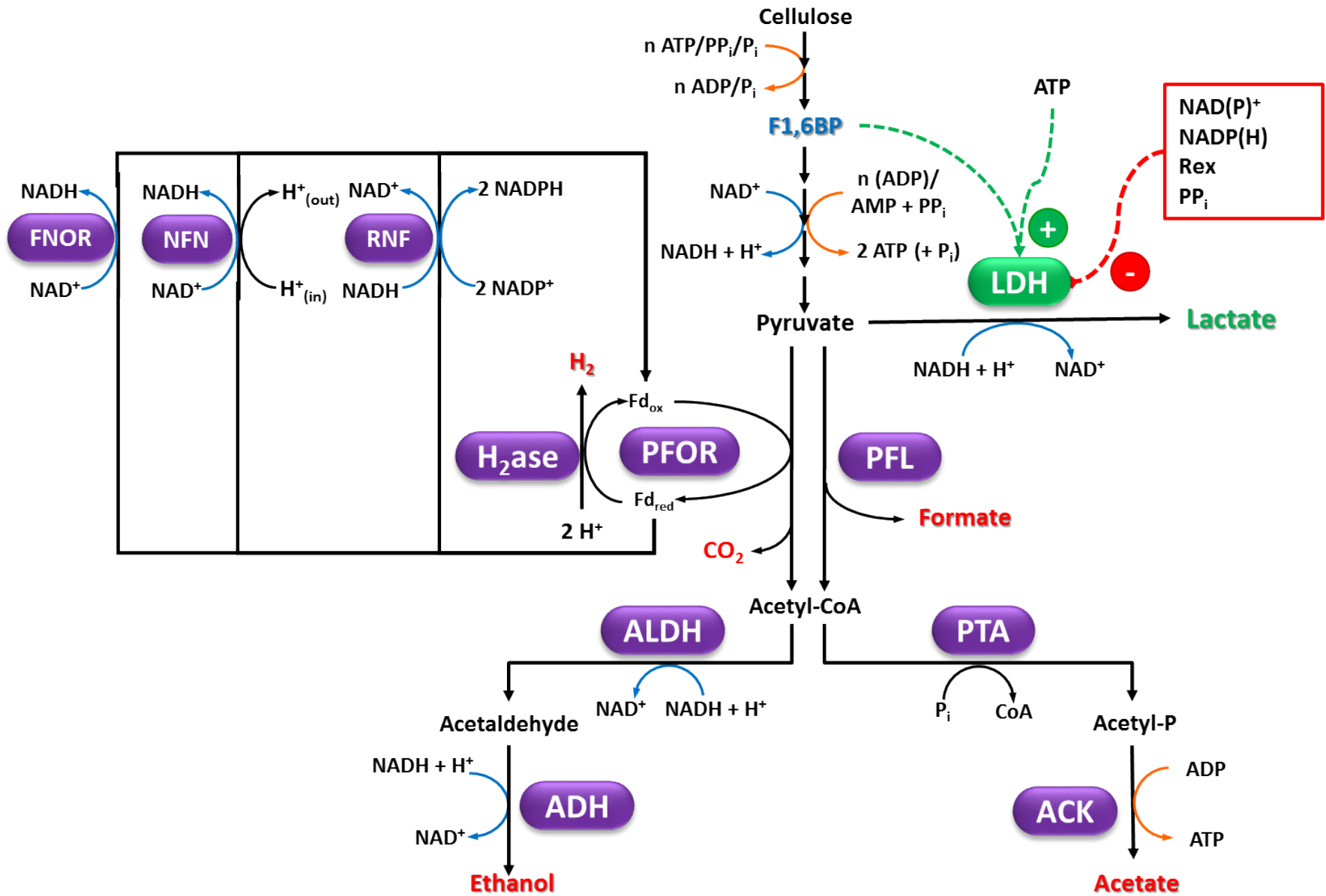
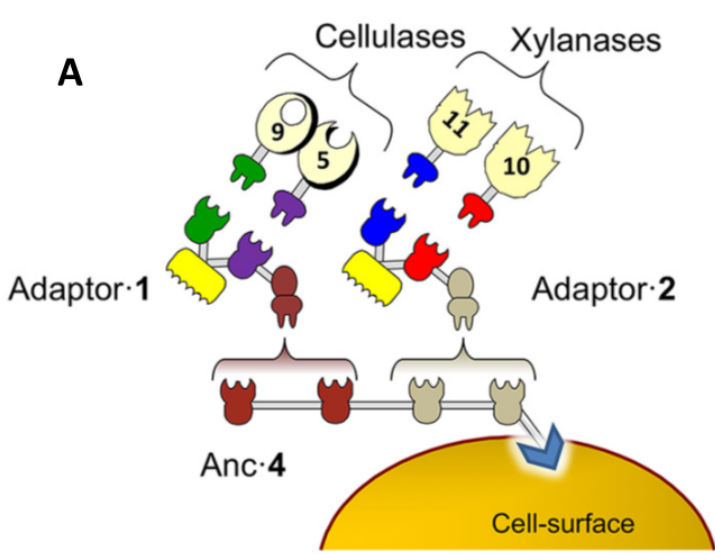


Figure 1. Overview of the central carbon catabolism of anaerobic (hemi)cellulolytic bacteria. Cellulose is channeled to Embden Meyerhof Parnas pathway by sequential conversion to : i) glucose 6 phosphate by using hydrolysis followed by ATP-dependent phosphorylation or phosphorolytic mechanism (i.e. by using P_i); ii) Fructose 1,6 bisphosphate that can be obtained from fructose 6 phosphate by using ATP- or PP_i -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_2 ase, hydrogenase; LDH, lactate dehydrogenase; NFN, NADH-dependent reduced ferredoxin: NADP⁺ oxidoreductase; PFL, pyruvate-formate lyase; PFOR, pyruvate ferredoxin/ferredoxin oxidoreductase; PP_i , pyrophosphate; PTA, phosphotransacetylase; Rex, global redox-responsive transcription factor; RNF, iron-translocating reduced ferredoxin: NAD⁺ oxidoreductase



Key to symbols

Dockerins (type I)	Dockerins (type II & III)	Cohesins	CatalyticCBM modules	Sortase motif	Linkers	His-tag
a - <i>A. cellulolyticus</i>	c - <i>C. papyrosolvans</i>	t - <i>C. thermocellum</i>				
b - <i>B. cellulosolvans</i>	r - <i>R. flavefaciens</i>	Type I Cohesin				
g - <i>A. fulgidus</i>	(type III)	Type II module				

B

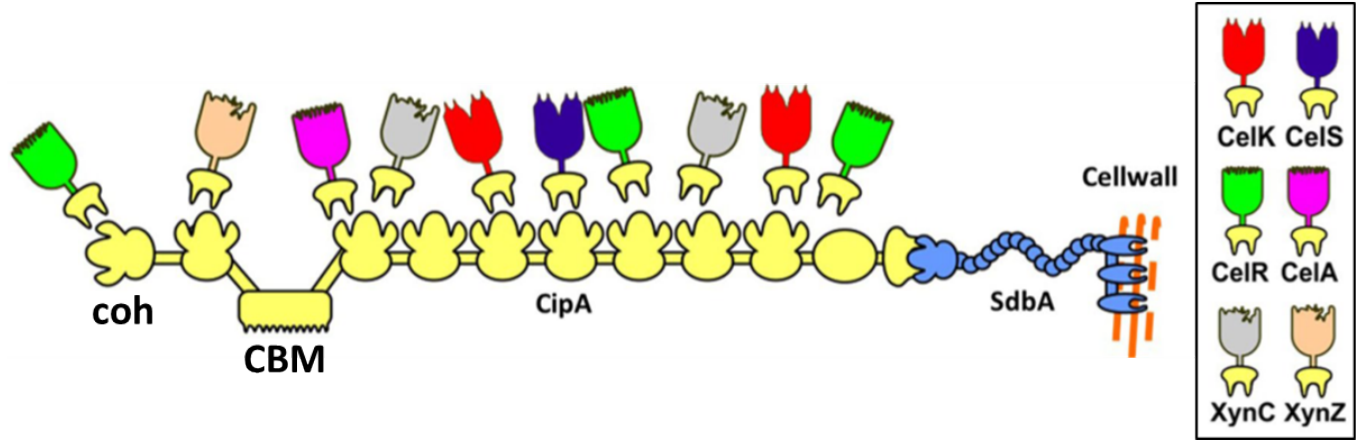


Figure 2. Schemerepresentingthe most sophisticatedexamples of RCSsin microbial strains aimed at consolidated bioprocessing of lignocellulosic biomass to LA. A) Consortium of engineered *L. plantarum* strains where each strain secretes a different cellulosomal component leading to assembly of designer cellulosomes on the cell surface (modified from Stern et al., 2018). 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 Chang et al., 2018). The

Table 1. Effects of gene modification on LA production yield in native (hemi)/cellulolytic microorganisms. Abbreviations: *adhE*, gene encoding bifunctional alcohol/aldehyde dehydrogenases involved in ethanol production; *ech*, gene encoding [NiFe]-hydrogenase; *hydG*, gene encoding [FeFe]-hydrogenase maturase; *hsf*, gene cluster encoding [FeFe]-hydrogenase; *pfl* gene cluster encoding pyruvate formate lyase; *pforA* gene encoding pyruvate ferredoxine/flavodoxine oxidoreductase; *pta* gene encoding phosphate acetyltransferase; *rex*, gene encoding global redox-responsive transcription factor Rex; *rnf*, gene cluster encoding ion-translocating reduced ferredoxin: NAD⁺ oxidoreductase. n.r. not reported.

Strategy	Strain	Gene modification	Y _{LA} (mol/mol glucose equivalent)(fold increase vs WT)	Notes	Reference
Disruption of ethanol production	<i>T. mathranii</i>	$\Delta adhE$	≈ 1.5 (≈ 4.5)	Growth rate was only 34% of WT	[34]
	<i>T. thermosaccharolyticum</i>	$\Delta adhE$	1.90 (63.3)	Growth rate was only 11% of WT	[23]
	<i>T. saccharolyticum</i>	$\Delta adhE$	0.67 (5.6)	Final biomass was 60% lower than WT	[25]
	<i>C. thermocellum</i>	$\Delta adhE$	0.78 (56)	Final biomass was 27% lower than WT	[25]

Disruption of H ₂ production	<i>T. saccharolyticum</i>	Δhsf	0.83 (1.66)	Final biomass was about 50% lower than WT	[29]
	<i>C. thermocellum</i>	$\Delta hydG \Delta ech$	≈ 0	Final biomass and growth rate were only slightly lower than WT. Y_{LA} of WT was ≈ 0.13 mol/mol glucose equivalent	[37]
Disruption of acetyl-CoA production	<i>T. saccharolyticum</i>	$\Delta pforA$	0.91 (4.53)	The strain also improved through adaptive evolution. The final biomass was about 50% lower than WT.	[30]
	<i>T. saccharolyticum</i>	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]
	<i>C. thermocellum</i>	Δpfl	0.15 (7.5)	The strain grew at final biomass similar to WT but growth rate was only 33% of WT	[41]

	<i>T. saccharolyticum</i>	$\Delta pforA, \Delta pfl$	1.76 (8.80)	The strains required formate, acetate and yeast extract supplementation for optimal growth.	[30]
Engineering redox state	<i>C. thermocellum</i>	Overexpression of <i>rnf</i> , $\Delta hydG$	0.01 (1.21)	The strains produced 30% more ethanol	[42]
	<i>C. bescii</i>	Δrex	n.r.	LA final titer was at least 124% more abundant than in WT	[27]
	<i>T. saccharolyticum</i>	Δrex	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	<i>C. thermocellum</i>	Δpta	$\approx 0.33 (\approx 3)$	-	[49]
	<i>C. cellulolyticum</i>	<i>i-pt</i>	$\approx 0.19 (\approx 0.45)$	<i>pta</i> expression was repressed by antisense RNA. LA production was repressed.	[50]