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Review

One-step production of biocommodities from lignocellulosic biomass by recombinant cellulolytic *Bacillus subtilis*: Opportunities and challenges

One-step consolidated bioprocessing that integrates cellulase production, cellulose hydrolysis, and product fermentation into a single step for decreasing costly cellulase use, increasing volumetric productivity, and reducing capital investment is widely accepted for low-cost production of biofuels or other value-added biochemicals. Considering the narrow margins between biomass and low-value biocommodities, good physiological performance of industrial microbes is crucial for economically viable production. *Bacillus subtilis*, the best-characterized Gram-positive microorganism, is a major industrial microorganism with numerous valuable features such as hexose and pentose utilization, low-nutrient needs, fast growth rate, high protein secretion capacity, industrial safety, *etc*. As compared with other potential consolidated bioprocessing microorganisms such as *Clostridium* spp., *Escherichia coli*, and the yeast *Saccharomyces cerevisiae*, recombinant cellulolytic *B. subtilis* strains would be a potential platform for biocommodity production from nonfood biomass. Here, we review the advances in recombinant cellulolytic *B. subtilis* development and metabolic engineering for biocommodity production, and discuss the opportunities and challenges of cellulolytic *B. subtilis* for biocommodity production.

Keywords: *Bacillus subtilis* / Biofuels / Cellulase expression / Consolidated bioprocessing / Metabolic engineering

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1 Introduction

Lignocellulosic biomass, the most abundant renewable biological resource, is a low-cost energy source [1, 2]. The production of bio-based products and biofuels from renewable lignocellulose is important for promoting rural economy, decreasing greenhouse gas emissions, and enhancing national energy security.

The high costs of cellulases are one of the largest obstacles to commercialization of second-generation biorefineries, although cellulases are among the least costly industrial enzymes (*e.g.* tens of dollars *per* kilogram of dry protein) [3–5]. In order to decrease cellulase use, increase volumetric productivity, and reduce capital investment, consolidated bioprocessing (CBP) has been proposed to integrate cellulase production, cellulose hydrolysis, and value-added biocommodity fermentation into a single step [1, 6]. Now the CBP concept is widely accepted for ultra-low-cost production of biofuels or other value-added biochemicals [1, 7–11]. For example, several biofuel companies, such as Mascoma and Qteros, have been founded based on this concept.

The margins between expenditure for purchasing biomass feedstock and revenues of low-value commodities are very small [12, 13]. Such small margins (~$50–200/ton of dry biomass) have to pay for processing costs for pretreatment (*e.g.* utilities, chemicals consumed or recycled, detoxification), bioconversion (*e.g.* cellulase, media, and utilities), product separation, waste treatment plus capital investment and

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Abbreviation: CBP, consolidated bioprocessing

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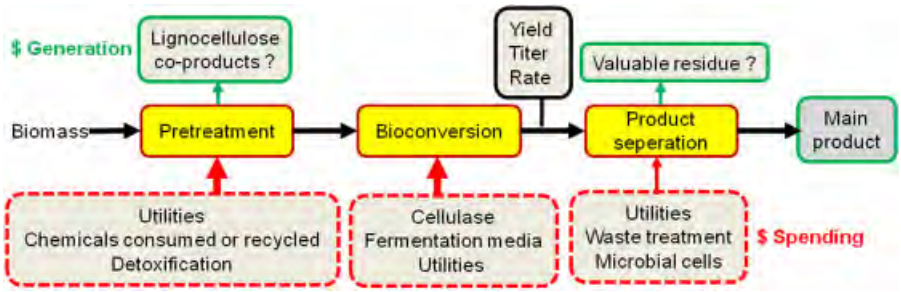

Figure 1. Multiple factors influencing biorefinery design and microorganism development. The flow scheme for processing and fermentation of biomass to product is presented in the yellow box. The boxes with the dotted red line and the solid green line represent the factors influencing the spending for biorefineries and potential revenues from the products and coproducts, respectively. The factors related to the efficiency of bioconversion and product separation are presented in the black box.

depreciation, taxes, labor, *etc.* (Fig. 1). Therefore, it is vital that we increase the overall revenues of whole biorefineries, *e.g.* by isolation of value-added lignocellulose components (*e.g.* high-quality lignin), production of other valuable residues [12], and/or reduction of any costly inputs (*e.g.* cellulase and medium nutrients).

As compared with other developing CBP microorganisms (*Clostridium* spp., *Escherichia coli*, and the yeast *Saccharomyces cerevisiae*), we propose the development of recombinant cellulolytic *Bacillus subtilis* strains, which have numerous advantages: (i) being industrially safe (generally regarded as safe microorganism by the Food and Drug Administration), (ii) having a very high-protein-secreting capability, (iii) growing very fast, (iv) having very low nutrient requirements, (v) utilizing soluble pentose (C5) and hexose (C6) sugars including glucose, xylose, mannose, cellobiose, *etc.*, (vi) having native hemicellulases, (vii) tolerating very high concentrations of salts and solvents, (viii) having an available genomic DNA sequence and well-developed recombinant DNA techniques, (ix) having well-developed fermentation technologies, (x) having no significant bias in codon usage, and (xi) being an animal-feed protein additive after fermentation. Several excellent reviews are available pertaining to *B. subtilis* systems and their industrial applications [14–16].

Considering the physiological needs for an ideal biocommodity-producing microorganism [17, 18], here we present the advantages of *B. subtilis* and discuss the potentials and challenges for developing recombinant cellulolytic *B. subtilis* strains that will produce low-value biocommodities.

2 CBP strategies

Biofuels pioneer Professor Lee Lynd has proposed that CBP microorganisms can be developed *via* both a native cellulose and a recombinant cellulose utilization strategy [1]. The recombinant cellulolytic strategy involves introducing heterologous cellulase genes into an organism whose product yield and tolerance credentials are well established (*e.g. S. cerevisiae*). The native cellulolytic strategy involves engineering the product metabolism to produce the desired products based on natural cellulolytic microorganisms (*e.g. Clostridium thermocellum* [19, 20], *C. phytofermentans* [21], *C. cellulolyticum* [22], and *Trichoderma reesei* [9]). A third pathway is a combination of recombinant cellulose utilization and product-producing ability (Fig. 2). The recombinant cellulolytic and ethanologenic combination has been conducted in some model microorganisms (*e.g. E. coli*) [23, 24] or industrially safe microorganisms (*e.g. B. subtilis*) [25].

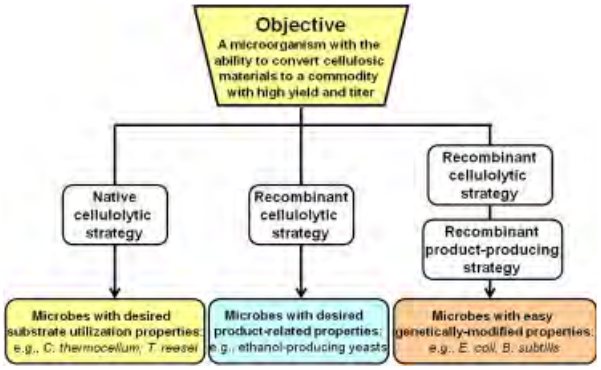

Figure 2. Three different strategies for developing CBP microorganisms.

Although a large number of cellulose utilization microorganisms have been discovered from diverse environments, attempts in creating recombinant cellulolytic microorganisms have not been successful because recombinant cellulolytic microorganisms must be capable of (i) overexpressing high-level recombinant active cellulase, (ii) secreting or displaying most active cellulases outside the (outer) membrane so that the cellulase can hydrolyze insoluble cellulose, (iii) producing several cellulase components, (iv) regulating the expression of cellulase components for a maximal synergy for hydrolysis, (v) producing sufficient ATP for cellulase synthesis, and (vi) having the right sugar transport systems for soluble cellulolytic products.

In spite of intensive efforts in heterologous expression of cellulases in noncellulolytic microorganisms [26–32], recombinant self-catalysis cellulolytic microorganisms are not yet available because they cannot grow on cellulose by utilizing their own cellulase without the help of organic nutrients such as yeast extract, peptone, or amino acids [33]. For example, van Zyl and his coworkers [30] have constructed a

recombinant *S. cerevisiae* strain with the *T. reesei* endoglucanase and the *S. fibuligera* β-glucosidase that can grow on regenerated amorphous cellulose in the presence of 0.5% yeast extract and 1% tryptone. However, a costly rich medium for biocommodity production is economically prohibited [34, 35]. The supplementary yeast extract in the medium works as both the carbon source and the nitrogen source at the same time. Their addition drastically decreases the burdens of synthesis of amino acids and cellular proteins and secretion of a large amount of cellulases. For low-value biocommodity production, significant addition of any costly nutrients, even 0.1 g/L yeast extract, is economically prohibitive.

Based on the native cellulolytic strategy, metabolic pathways can be modified for increasing the desired product yields, such as in *C. cellulolyticum* [22]. But limited genetic engineering tools prevent fast and efficient metabolic engineering for most cellulolytic microorganisms [36, 37].

It is unclear whether an ideal biocommodity-producing organism will be engineered using a native, isolated strain, or a recombinant model organism as the starting point [17]. *E. coli*, a model microorganism, has been engineered to produce ethanol in high yields and secrete some active cellulases [23, 24]. When considering industrial needs for biocommodity production (Table 1), recombinant ethanologenic *E. coli* has several weaknesses, such as relatively high-nutrient requirements, weak ability of secreting active cellulases, sensitivity to end-product inhibition, and the need of waste treatment for residual cells. We urge that *B. subtilis*, instead, would be a good platform as a recombinant cellulolytic industrial microorganism that can produce the desired biocommodity with high yields and can exert strong physiological robustness and fitness for industrial bioprocesses (Table 1).

3 Cellulase expression and engineering in *B. subtilis*

B. subtilis is a sporulating Gram-positive soil bacterium [38]. Its natural habitat contains a wide variety of different polymeric carbohydrates from plants, animals, and microbes. Not surprisingly, *B. subtilis* produces a large number of polysaccharide-degrading enzymes, such as α-amylase, pullulanase, endo-β-1,4-mannanase, levanase, glucan-1,4-α-maltohydrolase, pectate lyases, β-1,4-endoglucanase, β-1,3-1,4-endoglucanase, and endo-1,4-β-xylanases, so that it can break down polysaccharides into soluble carbohydrates [39]. Based on the Carbohydrate-Active Enzymes database [40], *B. subtilis* 168 has more than 100 carbohydrate-active enzymes which belong to 40 families of glycoside hydrolases, glycosyl transferases, polysaccharide lyases, and carbohydrate esterases (www.cazy.org/b68.html). Beside the carbohydrate-active enzymes, *B. subtilis* can produce the bacterial expansin YoaJ [41], which can enhance the enzymatic hydrolysis of cellulose but has no hydrolytic activity [42, 43].

B. subtilis has a number of validated and putative transporters, which enable this organism to take up mono-, di-, and oligosaccharides, amino sugars and their *N*-acetyl derivates, sugar-containing opines, glyconic and glycuronic acids, and sugar-derived polyalcohols [40]. Several monomer sugars including glucose, fructose, galactose, mannose, xylose, and arabinose can be utilized as carbon sources. Such native sugar transportation and utilization abilities make this organism a good CBP candidate without genome modifications for sugar uptake. It was reported that the model strain *B. subtilis* 168 is unable to grow on xylose as a sole carbon source because this strain cannot actively transport xylose, but the xylose transportation ability can be easily restored [44].

Although it natively produces a large number of polysaccharide-degrading enzymes, *B. subtilis* is noncellulolytic. It has been found that *B. subtilis* 168 cannot grow in chemically defined M9 minimal medium with amorphous cellulose or pretreated biomass as the sole carbon source [25]. For developing an efficient recombinant cellulolytic *B. subtilis*, the expression level or specific activities of native or heterologous cellulases should be further improved in *B. subtilis*.

B. subtilis is a perfect host for the production of secretory proteins [45]. Many of the genes for extracellular proteins from bacteria to humans have been cloned and expressed in *B. subtilis*, as summarized in the reviews [15, 46]. The titers of several secretory homologous enzymes are as high as 30 g/L [14, 15, 47, 48]. Overproduction of heterologous proteins by *B. subtilis* is more challenging. Although an expression and secretion system has been developed for one protein, it does not guarantee that this system will function well for another protein. For example, the family 48 cellobiohydrolase from *C. phytofermentans* has been successfully expressed and secreted as the major extracellular

Table 1. Comparison of the potential CBP microorganisms for the production of low-value biocommodities.[a)]

Key feature	*S. cerevisiae*	*E. coli*	*C. thermocellum*	*B. subtilis*
C5 sugar utilization	−/+	+++	−	+++
Oligosaccharide utilization	−	−/+	+++	+++
Protein secretion capacity	+	−/+	+++	+++
Ease of genetic modification	+++	+++	−/+	+++
Medium cost benefits	++	−	++	+++
Resistant to product inhibition	+++	−	−/+	+++
Resistant to salt/toxic inhibition	+	−	−	+++
Value of cell residues	++	−	−	+++
Growth rate	++	+++	+	+++
Anaerobic fermentation	+++	+++	+++	++
Culture temperature (°C)	~30	~37	~60	30–45

[a)] +++, excellent;++, good;+, moderate;−/+, doable; and −, bad.

protein in *B. subtilis* [49] using the high-level expression and secretion system [50]. But the same promoter and signal peptide cannot allow *B. subtilis* to produce a large amount of secretory *C. phytofermentans* family 9 processive endoglucanase [51]. In theory, each step among gene transcription, mRNA stability and degradation, protein synthesis, and translocation may be optimized for high-level expression of heterologous proteins [52, 53].

B. subtilis has served for the production of some cellulases [54]. In general, two methods can be used to express cellulase genes in *B. subtilis*: from a vector or from the chromosome [15]. By using a vector, the plasmid stability associated with the high costs of antibiotics and the potential risk of spreading the antibiotic resistance gene are major concerns to large-scale biocommodity production. Integrating target genes into the chromosome is a better choice, but the transcription and translation elements usually need to be optimized for high-level expression or for increasing the copy number of the target gene in the chromosome, as has occurred for the hypersecretion of the heterologous cellulases in *B. subtilis* [55, 56].

By using a protease-deficient *B. subtilis* as the host, Doi and colleagues have constructed *in vitro* mini-cellulosomes that use recombinant cellulosomal enzymes and truncated scaffoldin components from *C. cellulovorans* [57]. The reconstituted cellulosomes exhibit synergistic activity on cellulosic substrates. The ability of two *B. subtilis* strains to cooperate in the synthesis of an enzyme complex (an *in vivo* mini-cellulosome) has been demonstrated, and the mini-cellulosomes formed by "intercellular complementation" show their respective enzymatic activities [58]. Doi has proposed that *B. subtilis* would be an efficient cellulosome producer [59].

Recently, we have demonstrated that a recombinant *B. subtilis* can grow on amorphous cellulose or COSLIF-pretreated biomass (corn stover, common reed, and switch) in chemically defined minimal M9 media through overexpression and secretion of its intrinsic glycoside hydrolase family 5 endoglucanase [25]. The one-step production of ethanol or lactate from cellulose or pretreated biomass has also been demonstrated [25]. To our limited knowledge, this strain may be the first recombinant cellulolytic microorganism that can express enough cellulase to support itself to truly grow on cellulose as the sole carbon source without any other organic nutrient. But this strain cannot hydrolyze pretreated biomass with high glucan digestibility. It is believed that, for good recombinant cellulolytic microorganisms, several different types of cellulases (for example, family 5, 9, and 48 glucanases) should be coexpressed and should work together synergistically. However, coexpression of many cellulases in the same *Bacillus* strain remains challenging. A binary plasmid system [60–63] may be used, but this system needs further validation for coexpression of several cellulases in *B. subtilis*. Here, we propose development of a recombinant cellulolytic *B. subtilis* by combining the plasmid and chromosome integration expression systems as shown in Fig. 3A. In this strategy, the major cellulolytic component (such as family 5 endoglucanse) can be expressed from a plasmid that has both segregational and structural stabilities [15, 64]. Since the T7 system has been demonstrated to be functional in *B. subtilis* [65], the other important cellulases (such as family 9 and 48 glucanases) can be integrated into a different locus of the genome and driven by the promoter-specific T7 RNA polymerase for their high-level expression (Fig. 3A). It is likely that

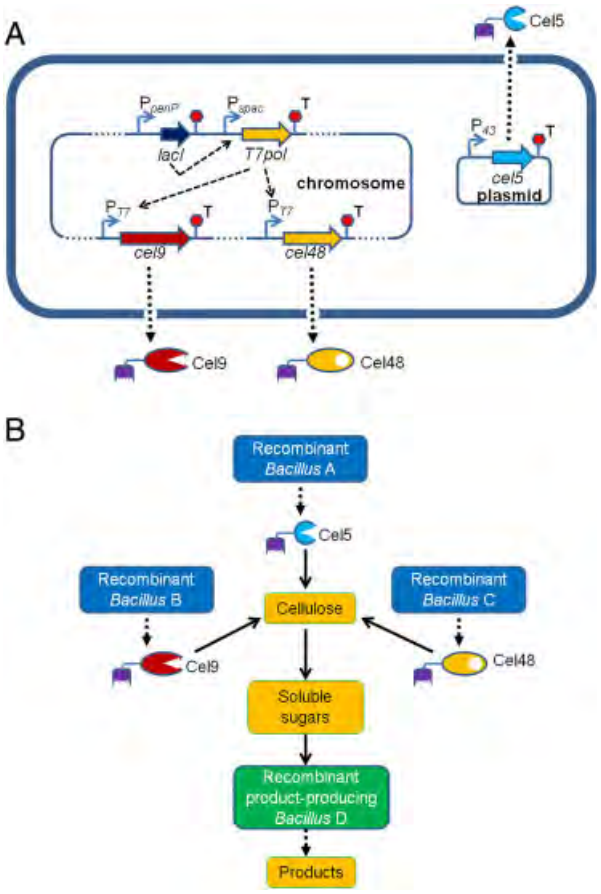

Figure 3. Scheme of recombinant cellulolytic *B. subtilis* strains or their consortium. (A) Construction of a recombinant cellulolytic *B. subtilis* strain by coexpression of several cellulases. Family 5 endoglucanase (Cel5) is expressed from *cel5* on the self-replicate plasmid which is under the control of the constitutive promoter P_{43}. Family 9 endoglucanase (Cel9) and family 48 exoglucanase (Cel48) are expressed from *cel9* and *cel48* which are integrated on the chromosome. *cel9* and *cel48* are under the control of the IPTG-inducible T7 promoter which is recognized by T7 RNA polymerase. (B) Consortium of recombinant cellulolytic *B. subtilis* strains. Cel5, Cel9, and Cel48 are expressed from individual *B. subtilis* strains, and these strains synergistically degrade cellulose into soluble sugars. The soluble sugars are fermented by the recombinant product-producing strain to the final product.

we can put all the cellulase genes into the chromosome, like in most native cellulolytic microorganisms, in the near future. Several new *B. subtilis* marker-free delivery systems [66–68] can be used to integrate many cellulase genes in the same chromosome precisely and rapidly. High-level expression of several heterologous cellulases may cause the "crowded traffic" for protein secretion [45, 52, 69]. To address this problem, the host and secretion system must be optimized systematically, *e.g.* overexpression of molecular chaperons [70] or utilization of other secretion systems, such as Tat and SRP [15, 71, 72].

Directed evolution is very useful for cellulase performance improvement or for increasing cellulase expression and secretion in *B. subtilis*. Different from *E. coli* or yeast systems, *B. subtilis* is a

perfect host for screening secretory enzyme variants [73, 74]. The expression levels of subtilisin and amylase have been successfully improved by using the directed evolution technology [75, 76]. Recently, we have developed a novel powerful *B. subtilis* platform for fast screening of cellulase variants with better performance on natural solid cellulosic substrates [77].

The chromosome of *B. subtilis* has a large number of dispensable regions [78]. Wong *et al.* have created a series of *B. subtilis* strains without extracellular proteases so that these strains show greatly improved quality and quantity of the heterologously expressed proteins [70]. Ogasawara and coworkers have enhanced recombinant cellulase productivity by large genome size reduction in *B. subtilis* and have systematically studied the effect of cellulase production by eliminating spore-related genes, protease genes, sigma factors, and genes related to catabolite inhibition, glucose metabolism, and the intercellular signaling mechanisms [79, 80].

To avoid the possible problems for coexpression of several cellulases in one strain, another strategy is to develop a *B. subtilis* coculture system (Fig. 3B). In this way, an individual cellulase will be expressed very well in one strain (either free or cell associated). Several strains in the coculture system will express different types of cellulases to synergistically degrade cellulose to soluble sugars, and another genetically engineered strain may be responsible for fermenting the soluble sugars into the desired product (Fig. 3B). However, developing a stable bacterial consortium for industrial applications remains highly challenging. To establish a stable coculture for fermenting cellulosic materials, cell–cell communication and regulation must be fine tuned [33, 81].

4 Industrial production and metabolic engineering of *B. subtilis*

Bacillus species are used for producing enzymes, insecticides, antibiotics, purine nucleotides, poly-γ-glutamic acid, D-ribose, polyhydroxybutyrate, *etc.*, through aerobic fermentation, which has been extensively reviewed by Schallmey *et al.* [14]. More recently, the production of recombinant proteins in *B. subtilis* was systematically reviewed by Schumann [15]. Although it was believed not to be able to grow in the absence of molecular oxygen as a terminal electron acceptor, *B. subtilis* can grow in the absence of oxygen using nitrate ammonification or various fermentation processes. Under anaerobic conditions, lactate, acetate, and 2,3-butanediol are major fermentation products [82]. Recently, *B. subtilis* has been reported to generate hydrogen from biowastes [83, 84] and electricity through microbial fuel cells [85] in the absence of oxygen.

The *B. subtilis* 168 genome, published in 1997, has a size of 4215 kb containing 4106 genes encoding proteins and 109 RNA genes (SubtiList: http://genolist.pasteur.fr/SubtiList/) [86]. Recently, the European Systems Biology Consortium has established a comprehensive model of the *B. subtilis* metabolism and its regulation (SubtiPathways: http://subtipathways.uni-goettingen.de/), a useful online tool for its gene regulation [87]. The European Integrated *Bacillus* Systems Biology (BaSysBio) group has announced the development of a powerful recombineering system in *B. subtilis* by employing phage recombinases (www.basysbio.eu/). It is anticipated that we will soon be able to modify *B. subtilis* as easily as and as fast as *E. coli* and yeast.

Figure 4 shows that biocommodities would be produced by *B. subtilis*, based on the validated or proposed pathways in the literature [82, 88, 89], the available genome sequence and its great plasticity, and abundant information in biochemistry and physiology. The initial attempts for ethanologenic *B. subtilis* were not successful [90, 91]. Later, Martinez and coworkers successfully constructed a recombinant ethanologenic *B. subtilis* that can produce ethanol at high titer and yield by knocking out the 2,3-butanediol pathway, introducing a heterologous transhydrogenase and placing the *Zymomonas*

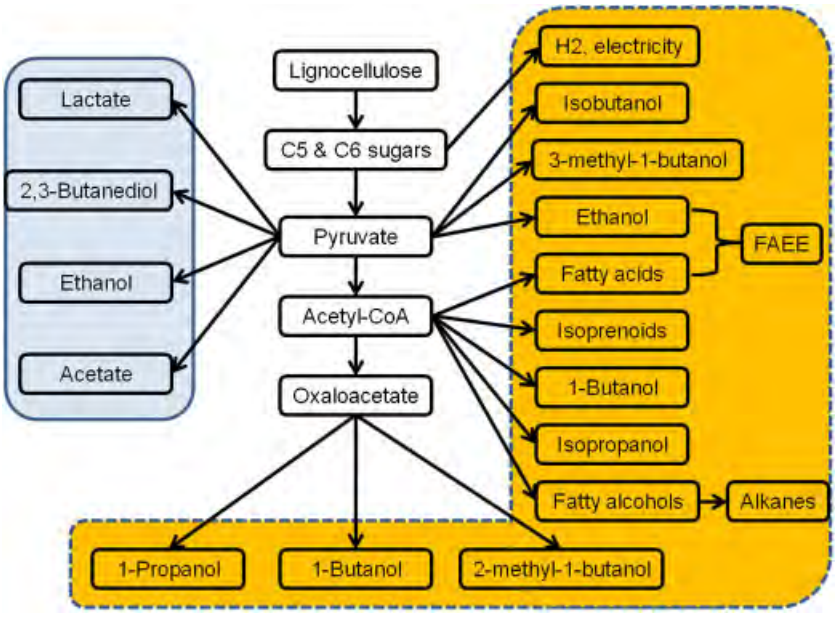

Figure 4. The intermediary metabolism of *B. subtilis* for producing potential biocommodities.

mobilis pyruvate decarboxylase gene (*pdc*) and the alcohol dehydrogenase II gene (*adhB*) under the control of the strong *ldh* native promoter [92]. Martinez and coworkers have demonstrated that *B. subtilis* produces highly pure L-lactic acid with high yields from glucose and cellobiose [93]. In a word, recent advances in systems biology, synthetic biology, protein engineering, and genetic engineering enable us to modify *B. subtilis* faster and more efficiently than before.

5 Concluding remarks

Considering the narrow margins between biomass and low-value biocommodities, good physiological performance of industrial microbes is crucial for economically viable production. The ideal microorganism for biocommodity production from nonfood biomass will possess multiple capabilities, such as cellulose hydrolysis, fast substrate utilization, fast and deregulated pathways for sugar transport, good tolerance to inhibitors and high-titer products, and high and efficient metabolic fluxes to a sole product with high yield. By considering three key facts – (i) industrial needs for physiological aspects of microorganisms, (ii) well-developed genetic engineering tools, and (iii) tremendous information on a microorganism's metabolism, biochemistry and physiology – we suggest that recombinant cellulolytic *B. subtilis* strains would be important platforms for engineering and producing cellulases as well as for producing diverse biocommodities from nonfood biomass or waste in one step. The biggest obstacle, and the critical step, for creating a CBP *B. subtilis* is to greatly improve its cellulolytic activity for the highly efficient hydrolysis of recalcitrant biomass. At the current stage, major efforts should be focused on the improvement of the cellulase expression level and performance in *B. subtilis*, *e.g.* by using directed evolution technology.

It is expected that other *Bacillus* species that have similar properties, such as thermophilic *Geobacillus*, alkaliphilic or acidophilic *Bacillus*, *Brevibacillus*, *etc.*, can also be developed to CBP microorganisms or platforms for cellulase performance improvement by directed evolution to get the desired properties for industrial applications (such as thermostability, alkaliphilic, or acidophilic properties). *Geobacillus* spp. are thermophilic aerobic or facultatively anaerobic bacilli. Members of the genus are capable of growth between 40 and 70°C and can ferment lignocellulose-derived hexose and pentose sugars and oligosaccharides to generate lactate, formate, acetate, and ethanol as products [94]. Thermophilic *Geobacillus* spp. have a number of potential advantages for development of the fermentation process. The high-growth temperatures promote higher rates of feedstock conversion, reduce cooling costs, and lower the risk of contamination in fermentation. However, for the other *Bacillus* species, *B. subtilis*, the progress may be slowed down by the non-availability of the genomic information, efficient genetic engineering tools and expression systems. Gene cloning and expression systems have been established in *Geobacillus* [95–99]. More recently, metabolic engineering has been successfully conducted in *G. thermoglucosidasius* for high-yield ethanol production [100].

The well-developed genome engineering tools and tremendous information on the *B. subtilis* metabolism, biochemistry, and physiology ensure the future success in metabolic engineering of this microorganism for producing other value-added biocommodities by using the heterologous pathways. However, since *B. subtilis* is not as robust in anaerobic fermentation as *E. coli* and yeast, the metabolic engineering process must be systematically evaluated and designed. It is clear that the fundamentally positive characteristics of *B. subtilis* will make it the preferred host for the one-step production of many new and advanced products from lignocellulosic biomass.

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Conflict of interest

The authors have declared no conflict of interest.

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