Efficient Lactic Acid Production from Dilute Acid Pretreated Lignocellulosic Biomass by a Synthetic Consortia of Engineered *Pseudomonas Putida* and *Bacillus Coagulans*

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Research Article

Keywords: Lignocellulosic hydrolysate, Detoxification, *Pseudomonas putida*, *Bacillus coagulans*, Microbial consortium, Lactic acid

Posted Date: September 29th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-923947/v1>

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Abstract

Background

Lignocellulosic biomass is an attractive and sustainable alternative to petroleum-based feedstock for the production a range of biochemicals, and a pretreatment is generally regarded to be indispensable for its bio-refinery. Nevertheless, various inhibitors that severely hindered the growth and fermentation of microorganisms were produced inevitably during the pretreatment of lignocellulose. Presently, a single microorganism that can tolerate toxic mixtures of pretreatment hydrolysate while effectively transforming sugar components into valuable compound is less well reported. Alternatively, microbial co-culture provides a simpler and more efficacious way to realize this goal via distributing metabolic tasks among proper strains.

Results

In this study, a novel synthetic microbial consortia, which is composed of a responsible for detoxification bacterium engineered *Pseudomonas putida* KT2440 and a lactic acid production specialist *Bacillus coagulans* NL01, was developed to directly produce lactic acid from high-toxic lignocellulosic hydrolysate. The engineered *P. putida* with deletion of sugar metabolism pathway was suggested to be unable to consume the major fermentable sugars of lignocellulosic hydrolysate, but can rapidly remove inhibitors in hydrolysate. With detoxification using engineered *P. putida* for 24 h, the pretreated hydrolysate was fermented into 35.8 g/L of lactic acid by *B. coagulans* with a yield of 90%. The fermentation performance of microbial co-culture was significantly improved than that single culture of *B. coagulans* without lactic acid production.

Conclusions

The microbial coculture system constructed by this study demonstrated strong potential of the process for biosynthesis of valuable product from lignocellulosic hydrolysate containing high concentration of complex inhibitors by specifically recruited consortia of robust microorganisms with desirable characteristics and also provided a feasible and attractive method for bioconversion of lignocellulosic biomass to other value-added biochemicals.

Background

Biochemicals produced from renewable resources through microbial fermentation is gaining rapidly growing interest due to the exhaustion of fossil fuels and the associated environmental issues [1]. Among these biochemicals, lactic acid and its derivatives are increasingly important building blocks, which have broad applications in food and chemical industries [2]. Currently, the biological approach for lactic acid production is very mature. But the cost of traditional substrates limits its use in the large-scale development of poly-lactic acid (PLA) as green alternative to petroleum-based plastics [3]. Lignocellulosic biomass

mainly consisting of cellulose, hemicellulose and lignin, is the most abundant renewable carbon source on earth with low cost [4]. It is of great significance to realize the effective biotransformation from lignocellulose-derived sugars to lactic acid. However, owing to its recalcitrance and irregular bond structure, few microorganisms can directly utilize lignocellulose to a product of interest [5] and thus pretreatment steps are indispensable to improve its digestibility and release more fermentable sugars for microorganism fermentation [6]. Nevertheless, many pretreatment studies showed that various inhibitors that severely impede microbial growth and fermentation would be inevitably formed during the deconstruction of lignocellulose, mainly including organic acids, furan aldehydes and phenolic compounds [7]. These inhibitors constitute a severe obstacle to the efficient use of lignocellulosic sugars and the effective product of biochemicals. Therefore, developing low-cost approaches and microorganisms to overcome barriers of inhibitors from biomass for fermentation would contribute to the scale-up and commercialization of lignocellulosic biorefineries.

Currently, a single naturally microbial that can detoxify or tolerate the non-sugar ingredients of pretreatment hydrolysate while validly transforming the sugar mixtures into valuable products is less well reported [8]. Adaptive evolution was considered as a powerful tool to improve substrate utilization and tolerance to toxic of biochemicals producing bacteria [9]. A series of investigations have been conducted to achieve the production of biochemicals from the pretreatment hydrolysates by using evolved strains [10, 11]. However, the evolved process is time-consuming and labor-intensive, and the phenotypes of evolved strains are unstable. In the recent decades, genetically engineered hosts that can both tolerance inhibitors and produce a range of different biofuels or biochemicals from lignocellulose have been reported [12]. The common strategy is the introduction of inhibitor tolerant elements into product-generating microbes [13, 14]. But owing to the complexity of lignocellulosic hydrolysates and the inherently challenging of engineering and optimizing heterogeneous functional modules in a single strain, low titers product and productivity were obtained by using mono-culture based on genetically engineered microorganisms [15]. Alternatively, microbial co-culture provides a more feasible and efficacious way to realize this objective [16].

In recent years, synthetic microbial consortium has garnered considerable interest especially in the field of industrial biotechnology because of its ability to perform complex biotransformation by division of labor [17]. Furthermore, microbial consortium synergies existed that can result in more efficient substrate utilization and increased product yield through functional modularity. Designed and established of microbial consortia with the capability of detoxification and biochemical production from pretreated lignocellulosic feedstock hold many appealing properties in practical lignocellulose biorefining. Zhu et al. developed an individual species co-culture system of *Saccharomyces cerevisiae* for bioethanol production from pretreated biomass. After process optimization, ethanol yield of co-culture was greatly improved than pure culture [18]. Biodetoxification of *Amorphotheca resinae* ZN1 provides a promising approach to remove inhibitors in hydrolysates. Dry dilute acid pretreatment corn stover are treated with *A. resinae* ZN1, subsequent cascade hydrolysis and fermentation, the production of gluconic acid and xylonic acid can be achieved by *Gluconobacter oxydans* [19]. A co-culture system of *Acinetobacter baylyi* ADP1 and *Clostridium tyrobutyricum* for removal of model inhibitors from mock hydrolysate and the production of hydrogen was successfully constructed [20]. These studies have illustrated the potential of co-culture strategy in the

transformation of lignocellulosic biomass. Despite this progress, the microbial consortia in conversion of lignocellulosic hydrolysate still confronted with many difficulties, such as long detoxification time, considerable loss of sugars and inferior toxicity tolerance. The development and application of mixed cultures for pretreated biomass with high content of inhibitors has thus far been unachieved [21].

In this study, a novel synthetic microbial consortium composed of engineered *P. putida* KT2440 and *B. coagulans* NL01 directly converted undetoxified lignocellulosic hydrolysate to lactic acid was developed (Fig. 1). The sugar catabolism pathway of *P. putida* KT2440 was disabled by deleting the gene encoding glucose dehydrogenase, *gcd*, and gene encodes for glucose transporter, *gtsABCD* operon from its genome. The engineered strain could simultaneously digest multiple fermentation inhibitors in lignocellulosic hydrolysate while preserving the sugars for subsequent fermentation. *B. coagulans* utilize sugars reserved in the hydrolysate to produce lactic acid. The successful construction of the co-cultivation system suggests a promising route towards the development and utilization of lignocellulosic feedstock.

Results And Discussion

Undetoxified corn stover hydrolysate fermentation by *B. coagulans*

B. coagulans as a potential industrial lactic acid producing strain has been received widespread attention. It could effectively convert various monosaccharides (pentose and hexose) derived from lignocellulose into high optical purity L-lactic acid through homolactic fermentation under thermophilic conditions [22]. To exploit the effective utilization of lignocellulosic feedstocks by *B. coagulans*, the undetoxified dilute acid pretreatment corn stover hydrolysate consisting of glucose (22.2 g/L), xylose (122 g/L), arabinose (~ 16 g/L), five carbohydrate degradation products, ten phenolic compounds and other unidentified inhibitors (Table 1) was used in this work. The hydrolysate was diluted to different concentrations (20%, 30% and 40% (v/v)) and then directly employed for lactic acid fermentation by *B. coagulans* NL01.

Table 1
Composition of sugars, weak acids, furans, and phenolic compounds present in concentrated undetoxified corn stover dilute-acid pretreatment hydrolysate.

	products	concentration
Sugars (g/L)	Glucose	22.36 ± 0.24
	Xylose	121 ± 1.08
	Arabinose	15.20 ± 0.28
Weak acids (g/L)	Formic acid	1.35 ± 0.24
	Acetic acid	14.50 ± 0.25
	Levulinic acid	2.96 ± 0.05
Furans (g/L)	Furfural	0.4 ± 16
	HMF	0.99 ± 0.04
Phenolic compounds (mg/L)	3'4-Dihydroxybenzoic acid	34.25 ± 6.02
	4-hydroxybenzoic acid	41.34 ± 0.99
	Vanillic acid	66.35 ± 5.33
	Syringic acid	24.36 ± 1.60
	4-hydroxybenzaldehyde	59.35 ± 1.01
	Vanillin	86.65 ± 2.03
	P-coumaric acid	133.90 ± 2.64
	Syringaldehyde	55.29 ± 2.21
	Ferulic acid	214.20 ± 0.99
	Cinnamic acid	0
	Total phenolics	5.33

The growth and lactic acid fermentation capacity of *B. coagulans* in fermentation medium containing different concentrations of pretreatment hydrolysate were evaluated. As illustrated in Fig. 2a and 2b, the growth and xylose utilization of *B. coagulans* decreased as the concentration of the hydrolysate was elevated. At a concentration of 20% (v/v) hydrolysate with 31.93 g/L monomeric sugars, the bacteria showed obvious growth after an initial 12 h lag phase. Which can completely uptake the primary monosaccharides composed of xylose, glucose and arabinose within 72 h, and achieve the maximum OD₆₀₀ 6.6. Whereas, the growth and lactic acid fermentation of the *B. coagulans* were completely ceased at a higher hydrolysate concentration (30% (v/v)). Even after prolonged fermentation time to 96 h, the growth of *B. coagulans* was still seriously hindered. The same results were observed with 40% (v/v) hydrolysate.

These results demonstrated that *B. coagulans* had a certain tolerance to hydrolysate, but the tolerance ability of strain was not high. It was merely suited for hydrolysate with low inhibitors and low corresponding sugar concentration. It is well known that substrate titer is one of vital factors impacting the final product concentration [23]. However, the concentration of inhibitors in the hydrolysate would enhance with the increase of the concentration of sugar. Furthermore, synergistic effects of between inhibitors under high contents render of lignocellulosic hydrolysates more toxic. The poor fermentation performance indicated that the tolerance of *B. coagulans* to higher concentration of hydrolysate was very inferior. These features limit the potential of using it as a platform strain to handle the carbohydrate products of lignocellulosic waste [24]. To promote the effective conversion of lignocellulosic sugars in hydrolysate, the introduction of an outstanding detoxified strain capable of transforming and degrading inhibitors while preserving the sugars is highly desirable for removing the inhibitory and toxic compounds to improve the fermentation of *B. coagulans*.

Metabolic engineering of *P. putida* to block sugar metabolism and growth characterization of knockout strains

P. putida KT2440 has emerged as a promising candidate for a future lignocellulose-based biotechnology process due to its outstanding toxicity tolerance to numerous by-products of biomass hydrolysis [25–27]. This was confirmed here by incubating of wild-type *P. putida* in LB medium supplemented with major inhibitors found in lignocellulosic hydrolysate (Fig. S1). Besides, it is a robust microorganism capable of natively catabolizing glucose other than toxins. Unfortunately, the excessive utilization of sugar during the detoxification will adversely affect subsequent fermentation and reduce process efficiency. With the intent of developing a prominent detoxified strain capable of removing inhibitors in hydrolysate and retaining monomeric sugars, we engineered *P. putida* to delete its sugar metabolism. There are three glucose catabolism pathways in *P. putida* KT2440 (Fig. 3). The first route is that glucose is transported to cytoplasm from periplasm by means of an ATP dependent ABC transporter (PP_1015-PP_1018), encoded by the *gtsABCD* operon and is then phosphorylated by glucokinase to glucose-6-phosphate and subsequently converted by glucose-6-phosphate dehydrogenase to 6-phosphogluconate. The second is a periplasmic oxidation pathway mediated by PQQ-dependent glucose dehydrogenase *gcd* (PP_1444). Glucose is oxidized to gluconate by *gcd* in the periplasm, which is then transported to the cytoplasm and subsequently phosphorylated, later reduced to 6-phosphogluconate. Besides, previous studies revealed that xylose is also oxidized to xylonate by *gcd* [28]. The third route is the 2-ketogluconate loop, which involves the oxidation of gluconate to 2-ketogluconate. The resulting 2-ketogluconate is imported into the cytoplasm via the outer membrane and subsequently phosphorylated, in which the oxidation pathway and the direct phosphorylation route converge at the node of 6-phosphogluconate and that function simultaneously [28, 29]. Accordingly, the *gcd* and *gtsABCD* were selectively removed.

The growth and the consumption of fermentable sugars of *P. putida* and its knockout strains were characterized. As a reference, the cultivation of *P. putida* KT2440 in M9 minimal medium using glucose or xylose as a carbon source was performed. As shown in Fig. 4a, about 5 g/L of glucose can be used up by *P. putida* KT2440 in 6 h, and reach a remarkable maximum cell OD₆₀₀ of 4.5. However, when the carbon source of *P. putida* KT2440 was switched to xylose, although the xylose concentration was significantly

reduced and dropped to 0 at 30 h, the growth of the strain was not detected (Fig. 4c). The consumed xylose was oxidized to xylonate instead of being used as a carbon source for strain growth. The results obtained are consistent with previous study [30]. The growth performance of the *P. putida* pK18MS- Δgcd was evaluated under the same carbon source conditions. When incubating with glucose, *P. putida* KT2440 pK18MS- Δgcd showed a significantly slower growth than wild type and the consumption rate of glucose was remarkably decreased, which was not completely consumed until 36 h (Fig. 4b). The ability of the *P. putida* KT2440 pK18MS- Δgcd to utilize xylose was also tested (Fig. 4d). No growth of strain and reduced sugar concentration can be examined during the entire cultivation. The deletion of *gcd* could greatly retard the glucose metabolism and completely hinder the consumption of xylose. These results suggested that *gcd* is of paramount importance for glucose and xylose uptake in *P. putida*.

The successful knockout strain *P. putida* KT2440 pK18MS- Δgcd - $\Delta gtsABCD$ was verified using glucose or xylose as a substrate. As predicted, the strain did not illustrate any detectable growth and consumption of sugar during 4 days of the cultivation on glucose or xylose (Fig. 4e & Fig. 4f). Removal of *gcd* and *gtsABCD* from *P. putida* KT2440's genome rendered it unable to utilize glucose and xylose. Biological detoxification of lignocellulosic hydrolysate has been proved to be a prospective approach for inhibitors removal and many microorganisms that either degrade or convert inhibitors have been identified. However, most of detoxified microorganisms consume main fermentable sugars, which causing a substantial loss of the sugar content in the lignocellulose hydrolysate. Efficient biological detoxification required microorganism that metabolized all inhibitors while preserving the sugar fraction intactly [31].

Inhibitors tolerance analysis of engineered *P. putida* KT2440 pK18MS- Δgcd - $\Delta gtsABCD$

The ability of engineered *P. putida* KT2440 pK18MS- Δgcd - $\Delta gtsABCD$ to metabolize and convert common inhibitors encountered in lignocellulose hydrolysates was investigated. Firstly, the effect of acetate, which was formed as a major unwanted byproduct from the breakdown of hemicellulose, was tested by culturing cells in M9 minimal medium comprising different titers of sodium acetate ranging from 0–10 g/L. As presented in Fig. 5a, the knockout strain could still grow well with different concentrations of sodium acetate as a carbon source. The supplementation of high concentrations sodium acetate (5 g/L and 10 g/L) only slightly delayed the growth of cells in the early stage, but did not affect their complete catabolism by the strain. Besides, the maximum achieved biomass of the strain was positively correlated with the concentration of the initial substrate provided. With 1 g/L sodium acetate, the maximum OD₆₀₀ 0.77 was detected. Using 5 g/L sodium acetate, higher growth rate was measured. The cells reached a maximum OD₆₀₀ of 3.1 at the highest applied 10 g/L of sodium acetate. During cultivation, the consumption of sodium acetate at different concentrations was also indicated in Fig. 5b. Up to 10 g/L sodium acetate could be totally depleted by engineered *P. putida* in 48 h. Although acetate itself is not a fierce inhibitor, its elimination could lessen the whole toxicity of hydrolysate [20]. Similarly, the utilization of levulinic acid by the knockout strain was assessed. The knockout strain did not exhibit lag phase at the tested concentrations (Fig. 5c). The strain could grow rapidly with levulinic acid. A maximum OD₆₀₀ of 2.5 could be achieved starting at 5 g/L levulinic acid. Correspondingly, the consumption of levulinic acid can also be seen from Fig. 5d. Levulinic acid was rapidly consumed in a short period. Even though previous studies have reported that weak acids can inhibit cell growth and metabolism due to uncoupling and intracellular

anion accumulation, we found that engineered *P. putida* grew well in the presence of carbohydrate derived acids [32]. FAL and HMF that commonly generated from high-temperature processing of lignocellulose are considered major inhibitors in microbial conversion processes [33]. The conversion performance of the engineered *P. putida* KT2440 pK18MS- Δgcd - $\Delta gtsABCD$ towards furan aldehydes was examined. As shown in Fig. 5e and 5f, the engineered strain could convert the representative concentrations of HMF and FAL into the corresponding carboxylic acids, 5-hydroxymethyl-2-furancarboxylic acid (HMFA) and furoic acid (FA), which are less toxic. In addition, a different conversion profile by engineered *P. putida* was noticed. Compared with FAL, the conversion rate of HMF were faster within 12 h (0.847 mM/h for HMF and 0.557 mM/h for FAL). The complete conversion of HMF was observed at 12 h of cultivation, whereas full conversion of FAL was observed at 24 h.

These results demonstrated that the genetically modified *P. putida* remained robust to the elimination and transformation of toxic inhibitors. It has extraordinary potential for the removal of inhibitors in the biomass pretreatment hydrolysate.

Production of lactic acid from undetoxified corn stover hydrolysate by using synthetic consortia of engineered *P. putida* and *B. coagulans*

Based on the above results, a co-cultivation reaction was performed for L-lactic acid production from undetoxified 30% (v/v) real hydrolysate that has the same composition as described above. During co-culture process, to gain detailed insights into the behavior of engineered *P. putida* KT2440 pK18MS- Δgcd - $\Delta gtsABCD$ on the complex mixtures of the hydrolysate, the detoxification reaction of the hydrolysate was set up using 1 g/L *P. putida* KT2440 pK18MS- Δgcd - $\Delta gtsABCD$ and no detoxified strain was supplemented as control. The concentration changes of the substances (fermentable sugars, organic acids, furans and phenol compounds) before and after the reaction were detected. After 24 h reaction, the concentration of major sugars such as glucose, xylose and arabinose in lignocellulosic hydrolysate remained nearly constant under both conditions (Table S1). However, there are considerable difference in the degradation and conversion of inhibitors. In the presence of detoxified strain, 4.3 g/L of acetic acid and 0.9 g/L of levulinic acid could be completely metabolized in 12 h. Besides, the removal rate of formic acid and HMF reached 73% and 100%, respectively (Fig. 6a). Furthermore, the concentration changes of total phenol and several typical monophenol inhibitors were also quantified. As shown in Table S2 and Fig. 6c, we observed that the concentration of total phenol and monophenol inhibitors have enormous degrees of reduction. The detoxification for 24 h by *P. putida* KT2440 pK18MS- Δgcd - $\Delta gtsABCD$, 25% of total phenolic derivatives was found to be removed. This result is comparable to the electrochemical detoxification reported in the literature [23]. Additionally, most of the representative monophenol compounds especially ferulic acid, *p*-coumaric acid, vanillin, 4-hydroxybenzaldehyde and syringaldehyde could be rapidly degraded to very low concentration. A control experiment confirmed that the concentration of the organic acids, furan aldehydes (Fig. 6b), ten representative phenolic compounds (Fig. 6d) as well as total phenolic derivatives (Table S2) did not vary significantly in the absence of detoxified strain pK18MS- Δgcd - $\Delta gtsABCD$ under the same investigated conditions. These results indicated that *P. putida* KT2440 pK18MS- Δgcd - $\Delta gtsABCD$ is a superior biological detoxification strain, which can effectively eliminate the toxic compounds in

depolymerized lignocellulose streams while retaining the hexose and pentose sugars in the hydrolysate for subsequent lactic acid fermentation.

The feasibility of detoxified hydrolysate for lactic acid fermentation by *B. coagulans* was investigated. As shown in Fig. 7, the hydrolysate treated by *P. putida* KT2440 pK18MS- $\Delta gcd\Delta gtsABCD$ containing glucose (6.1 g/L), xylose (about 35 g/L) as well as arabinose (4.5 g/L) could be effectively consumed by fermentation of strain *B. coagulans* within 96 h, resulting in a lactic acid production of 35.8 g/L and a corresponding yield of 90%. However, the hydrolysate of the identical concentration that has not been detoxified by *P. putida* KT2440 pK18MS- $\Delta gcd\Delta gtsABCD$ has neither the consumption of sugar nor the production of lactic acid was observed (Fig. 2). As anticipated, the degradation and transformation of diverse inhibitors by *P. putida* in hydrolysate greatly decrease the inhibitors concentration and contribute to the broth less toxic for fermentation. The results suggested that the detoxification of *P. putida* was extremely beneficial for improving the utilization of sugar and lactic acid fermentation by *B. coagulans* in the hydrolysate with high content of complex inhibitors. Additionally, which also suggested that the co-culture system was more advantageous than mono-culture fermentation for the production of biochemicals from highly toxic lignocellulosic hydrolysate.

Comparison of lactic acid production from lignocellulosic biomass with other studies

To date, extensive investigations have been conducted to realize the production of lactic acid from lignocellulosic biomass (Table 2). The production of lactic acid from the pretreated wheat straw hydrolysate by an adapted *B. coagulans* was reported. 35.5 g of lactic acid was obtained from 80 g wheat straw with a yield of 70.9% [34]. Besides, to reduce processing costs as well as simplification of the process, one genetically engineered *Bacillus subtilis* capable of both degrading the cellulose and producing lactic acid was developed. Nevertheless, the recombinant cellulolytic *B. subtilis* suffered from low titers of lactic acid [35]. Low efficiency was main barriers for lactic acid production from lignocellulosic biomass. Currently, most of studies on lactic acid production from lignocellulosic biomass commonly use the simultaneous saccharification and fermentation (SSF) method. Cui et al. reported a mixed cultures of *Lactobacillus rhamnosus* and *Lactobacillus brevis* for bioconversion corn stover into lactic acid through SSF process [36]. Although relatively high productivity of lactic acid was obtained, which require the addition of high-cost cellulase. Recently, consolidated bioprocesses (CBP) targeting lactic acid production was exploited by using a synthetic microbial consortia of *Trichoderma reesei* and *Lactobacillus pentosus*. Ultimately, 34.7 g/L and 19.8 g/L of lactic acid can be obtained from 5% (w/w) microcrystalline cellulose and pretreated beech wood, respectively [37]. Despite intensive research efforts spanning years, as an immature but promising technology, there are limit reports on the conversion of lignocellulose by microbial co-cultivation especially in lignocellulosic hydrolysates made up of numerous inhibitors and high concentration. In this work, we developed synthetic microbial consortia of *P. putida* and *B. coagulans* could achieve effective production of lactic acid from lignocellulosic hydrolysate with high inhibitors contents. The titer and yield of lactic acid attained by the coculture system were comparable to those previously reported. Although the results obtained by this approach are encouraging, the process regulation and optimization of microbial cocultivation are still essential in the future work, which probably promote the concentration and productivity of lactic acid to satisfy the requirement of practical lignocellulose biorefining.

Table 2
Comparison of lactic acid production from lignocellulosic biomass with other studies.

Strain	Strategy	Substrate	C _{LA} , max (g L ⁻¹)	Yield (%)/(g/g)	Productivity (g L ⁻¹ h ⁻¹)	References
<i>B. coagulans</i> CC17A	adaptive evolution, SSF	wheat straw	35.5	70.9	n.a.	34
<i>B. subtilis</i>	over-expression of endoglucanase and knocked out alpha-acetolactate synthase	cellulose	4.1	63	0.03	35
<i>L. rhamnosus</i> / <i>L. brevis</i>	co-culture, SSF	Corn stover	20.95	0.70	0.58	36
<i>T. reesei</i> / <i>L. pentosus</i>	CBP	microcrystalline cellulose	34.7	62.4	0.16	37
<i>T. reesei</i> / <i>L. pentosus</i>	CBP	Beech wood	19.8	85.2	0.10	37
<i>P. putida</i> / <i>B. coagulans</i>	Sequential co-culture	corn stover hydrolysate	35.8	0.90	0.37	this study

Conclusions

In this study, a synthetic consortium was constructed for converting high-toxic lignocellulosic hydrolysate into lactic acid. The consortium consisted of engineered *P. putida* capable of consuming and transforming inhibitors while leaving the lignocellulosic sugars intact for subsequent fermentation and *B. coagulans* capable of utilizing fermentable sugars to produce lactic acid. Compared with fermentation of undetoxified hydrolysate by monoculture without lactic acid production, a microbial co-culture can generate 35.8 g/L lactic acid with a yield of 90% from the equal titer of hydrolysate. Results herein affirmed the potential of using synthetic consortia to compartmentalize biological functions into proper strains for accomplishing complicated tasks that are difficult to achieve with monocultures.

Methods

Materials

Corn steep liquor was kindly donated by Shandong Xipu Biotechnology Co., Ltd (Shandong, China). Concentrated undetoxified corn stover dilute-acid pretreatment hydrolysate was kindly provided by Shanghai Yigao Biotechnology Co., Ltd (Shanghai, China). *P. putida* KT2440 and *B. coagulans* NL01 were

obtained from the Biochemical Engineering Research Institute of Nanjing Forestry University. Furfural (FAL) (99%) and 5-Hydroxymethylfurfural (HMF) (99%) were purchased from Sigma-Aldrich. Levulinic acid (> 97%) was purchased from TCI (Japan).

Plasmid and strain constructions

The construction of *gcd* and *gtsABCD* mutants were conducted as described in our previous study [38]. Concisely, Genomic DNA of *P. putida* KT2440 was isolated using Bacteria Genomic DNA Extraction Kit (Takara, Japan). The upstream and downstream homologous arms of *gcd* and *gtsABCD* gene were separately PCR-amplified using genomic DNA from *P. putida* KT2440 as the template and the respective primers (Table S3). After gel purification, overlap extension PCR of *gcd* and *gtsABCD* homologous arms were executed using the primers *gcd*-up.f and *gcd*-down.r or *gtsABCD*-up.f and *gtsABCD*-down.r. The resulting PCR products were recovered by Gel and PCR Clean-up system (Promega, USA) and ligated into pEASY-Blunt cloning vector to form new plasmids. The plasmids were digested with *EcoR* I and *Bam*H I and subsequently cloned into the corresponding restriction sites of the suicide vector pK18mobsacB to yield the recombinant plasmids pK18MS- Δ *gcd* and pK18MS- Δ *gtsABCD*. Followed by electroporation plasmid pK18MS- Δ *gcd* into electrocompetent *P. putida* KT2440. Single crossover and double-crossover transformants of *P. putida* KT2440 pK18MS- Δ *gcd* were selected separately on LB plate with 50 μ g/mL kanamycin and LB agar plate supplemented with 15% (w/v) sucrose incubated at 30 °C. The correct double-crossover recombinant of *P. putida* KT2440 pK18MS- Δ *gcd* as starting strain was used for further construction of *gtsABCD* knockout bacteria. The knockout process is the same as described above.

The growth and transformation of sugars, organic acids and furan aldehydes by *P. putida*

P. putida KT2440 and its knockout strains were routinely pre-cultured overnight in 5 mL of Luria Broth (LB) medium at 30 °C and 200 rpm. Then, the overnight cultures were collected by centrifuging at 6000 *g* for 5 min and supernatants were removed. The recovered cells were washed for twice with 0.85(w/v) NaCl. Followed by cells were inoculated at OD₆₀₀ of 0.1-0.2 in 250-ml shake-flask with 25 mL of M9 minimal medium [28] containing different substrates. The concentration of added glucose and xylose was depicted in the text or respective figure caption. In addition, acetate, levulinic acid, FAL, and HMF were added to the medium at specified concentrations, respectively. After addition of the inhibitors, the initial pH of the medium was adjusted to 7.0 using NaOH solution.

Biodetoxification of lignocellulosic hydrolysate by engineered *P. putida*

The engineered *P. putida* KT2440 pK18MS- Δ *gcd*- Δ *gtsABCD* was pre-activated overnight. Then, 2% seed cultures were inoculated into 200 mL LB medium in 1 L shake flask incubated for around 12 h, the cells were centrifuged (6000 *g*, 5 min) and pellets were washed with 0.85(w/v) NaCl solution twice. Subsequent cells harvested are used for biodetoxification. The biodetoxification was performed in an Erlenmeyer flask with 50 mL pretreated hydrolysate-containing fermentation medium (pH 7.0) without nitrogen source supply, and 1 g/L inoculum of *P. putida* KT2440 pK18MS- Δ *gcd*- Δ *gtsABCD* cells at 30 °C, 200 rpm under aerobic conditions.

Fermentation experiments of *B. coagulans* strain

B. coagulans NL01 was used for lactic acid fermentation. Typically, the preculture of *B. coagulans* NL01 was conducted at 50 °C and 150 rpm for 12 h in seed medium, which containing (g/L): Xylose 20, Yeast extract 1, Corn steep powder 2.5, NH₄Cl 1, MgSO₄ 0.2, CaCO₃ 10, and then transferred into corresponding hydrolysate-containing fermentation medium with a 10% (v/v) inoculum for lactic acid production. Fermentation experiments were performed in 150-mL shake-flask containing 50 mL of fermentation medium. The samples were withdrawn at regular intervals for determining the amount of residual sugars and lactic acid produced during the reaction. The hydrolysate-containing medium used for *B. coagulans* fermentation was prepared as follows: concentrated undetoxified corn stover dilute-acid pretreatment hydrolysate was neutralized (pH 6.5), then the hydrolysate was added to the fermentation medium (Ouyang et al. [34]) of *B. coagulans* deprived of carbon source.

The process for production of lactic acid from lignocellulosic hydrolysate by bacteria-bacteria co-culture

A two-stage biological process for the detoxification of hydrolysate and the generation of lactic acid was performed as follows. The detoxification using *P. putida* KT2440 pK18MS- Δgcd - $\Delta gtsABCD$ was conducted at 30 °C with 200 rpm for 24 h under aerobic conditions to degrade and convert inhibitors of hydrolysate, the pH was monitored and adjusted to 7.2 with 5 M HCl. Followed by *B. coagulans* cells at 10% of the inoculum, organic nutrients (yeast extract (2.5 g/L) and corn steep power (1.2 g/L)) as well as CaCO₃ (about half of the total sugars concentration) were added to the hydrolysate for lactic acid fermentation. The reaction was shaken for further hours in the incubator at 50 °C, 150 rpm.

Analytic methods

All the concentrations of the monomeric sugars, organic acids, furan aldehydes and lactic acid were detected by Agilent 1260 HPLC system with reflective index detector. The analysis was conducted using a column of Aminex HPX-87H (Bio-Rad, USA) operated at 55 °C and the eluent flow rate was 0.6 mL/min with 5 mM H₂SO₄, unless otherwise stated. The concentrations of phenolic inhibitors were determined as previously reported by Ouyang et al [34]. The cell growth was monitored turbidimetrically at optical density of 600 nm (OD₆₀₀). When sugar and sugar acid are concomitantly presented in medium, the concentration of monosaccharide was detected according to the procedures described by Han et al. [39].

Abbreviations

FAL: Furfural; HMF: 5-Hydroxymethylfurfural; FA: Furoic acid; HMFA: 5-Hydroxymethyl-2-furancarboxylic acid; *gcd*: glucose dehydrogenase; *gtsABCD*: glucose transporter; NaOH: Sodium hydroxide; SSF: Simultaneous saccharification and fermentation.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by the National Key Research and Development Program of China (2018YFA0902200), and the National Natural Science Foundation of China (22078163).

Authors' contributions

All authors contributed to the conception and planning of the study. LZ performed experiments, data analysis and prepared the manuscript. SOY and YH helped to perform the research. ZZ and JOY helped to guide the writing and revise the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Shandong Xinpu Biotechnology Co., Ltd (Shandong, China) for kindly providing corn steep liquor.

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Figures

Figure 1

Construction diagram of the microbial coculture system for lactic acid production from lignocellulosic hydrolysate. (Toxic degradation products are mainly divided into three categories: furan aldehydes, organic acids and phenolic compounds; The green dots represent hexoses; The black dots indicate pentoses.)

Figure 2

Undetoxified corn stover hydrolysate fermentation by *B. coagulans* NL01. Growth (a) and lactic acid fermentation (b) of *B. coagulans* NL01 with different initial concentrations of corn stover hydrolysate. Data shown as mean $\pm$ SD from at least two independent experiments.

Figure 3

Schematic representation of glucose metabolism and xylose transformation in *P. putida* KT2440. Glucose catabolism in *P. putida* occurs through the simultaneous operation of three pathways that converge at the

level of 6-phosphogluconate. D-xylose was oxidized to the dead-end product D-xylonate by the periplasmic glucose dehydrogenase Gcd instead of being used as a carbon source for growth. Abbreviations used for the metabolic intermediates and enzymes within the biochemical network are as follows: PQQ, pyrroloquinoline quinone; Gcd, glucose dehydrogenase; FAD, flavin adenine dinucleotide; Gad, gluconate dehydrogenase; Glk, glucokinase; GnuK, gluconate kinase; KguK, 2-ketogluconate kinase; KguD, 2-ketogluconate-6-phosphatereductase; Zwf, glucose-6-phosphate dehydrogenase; Edd, 6-phosphogluconate dehydratase; Eda, 2-keto-3-deoxy-6-phosphogluconate aldolase; TCA cycle, tricarboxylic acid cycle; OM, (outer membrane); IM (inner membrane).

Figure 4

Growth characterization of wild-type *P. putida* KT2440 and its knockout strains in M9 minimal medium. *P. putida* KT2440 (a) and *P. putida* KT2440 pK18MS- Δ gcd (b) with 5 g/L D-glucose; *P. putida* KT2440 (c) and *P. putida* KT2440 pK18MS- Δ gcd (d) with 5 g/L D-xylose; *P. putida* KT2440 pK18MS- Δ gcd- Δ gtsABCD with 5 g/L D-glucose (e) and 5 g/L D-xylose (f). Data shown as mean $\pm$ SD from at least two independent experiments.

Figure 5

Inhibitors tolerance analysis of engineered *P. putida* KT2440 pK18MS-Δgcd-ΔgtsABCD in M9 minimal medium. The cell growth (a) and depletion (b) with different concentrations of sodium acetate; The cell growth (a) and depletion (b) with different concentrations of levulinic acid; The conversion of HMF (e) and FAL (f). Data shown as mean ± SD from at least two independent experiments.

Figure 6

Comparison of the profiles of inhibitors in undetoxified 30% (v/v) hydrolysate by engineered *P. putida* KT2440 pK18MS-Δgcd-ΔgtsABCD and without detoxified strain (control). The measured parameters include the main inhibitors. The profiles of organic acids and HMF with engineered *P. putida* (a) and without engineered *P. putida* (b); The profiles of phenolic compounds with engineered *P. putida* (c) and without engineered *P. putida* (d).

Figure 7

Production of lactic acid from undetoxified 30% (v/v) lignocellulosic hydrolysate by using two-bacteria co-culture system.

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