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BIOENERGY/BIOFUELS/BIOCHEMICALS

Lactobacillus casei as a biocatalyst for biofuel production

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Abstract Microbial fermentation of sugars from plant biomass to alcohols represents an alternative to petroleum-based fuels. The optimal biocatalyst for such fermentations needs to overcome hurdles such as high concentrations of alcohols and toxic compounds. Lactic acid bacteria, especially lactobacilli, have high innate alcohol tolerance and are remarkably adaptive to harsh environments. This study assessed the potential of five *Lactobacillus casei* strains as biocatalysts for alcohol production. *L. casei* 12A was selected based upon its innate alcohol tolerance, high transformation efficiency and ability to utilize plant-derived carbohydrates. A 12A derivative engineered to produce ethanol (*L. casei* E1) was compared to two other bacterial biocatalysts. Maximal growth rate, maximal optical density and ethanol production were determined under conditions similar to those present during alcohol production from lignocellulosic feedstocks. *L. casei* E1 exhibited higher innate alcohol tolerance, better growth in the presence of corn stover hydrolysate stressors, and resulted in higher ethanol yields.

Keywords *Lactobacillus casei* · Alcohol · Tolerance · Biofuels

Introduction

Worldwide demand for renewable transportation fuels is growing. Alcohols, which can be produce by microbial fermentation of sugars [12], can serve as direct substitutes for petroleum-based fuels. However, one of the challenges faced during microbial production of alcohols is their toxicity to the biocatalytic host, causing low titers and the need to integrate additional removal and recovery processes during fermentation [30]. Therefore, there is interest in screening microorganisms for high innate alcohol tolerance, as these organisms could have potential as biocatalysts for the production of alcohols.

Lignocellulosic biomass is inexpensive, abundant, and renewable making it an ideal substrate for the production of biofuels. However, the production of lignocellulosic hydrolysates often results in the production of microbial inhibitors such as hydroxymethyl furfural (HMF), acetate and phenolic compounds [20, 33]. The conversion of hemicellulose into hexose and pentose sugars requires the biocatalyst be capable of metabolizing both types of sugars. Additionally, the high osmotic pressure created by the enzymatic release of these sugars can be a significant bacterial hurdle [41]. Last, the low pH of the hydrolysate (~4.8) is a hurdle to many biocatalysts [18, 24]. Therefore, an industrial biocatalyst for the production biofuels must be robust enough to grow and produce the desire product under these conditions.

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Saccharomyces has been the biocatalyst of preference for starch-based ethanol production, due largely to its historic applications as an ethanologen. However, this organism typically lacks the ability to utilize pentose sugars [18], which is important in lignocellulosic-based alcohol production. Additionally, *S. cerevisiae* only secretes enzymes at low levels [42] and is sensitive to compounds generated during biomass pretreatment and hydrolysis [32]. As a result, bacteria such as *Zymomonas mobilis* and *Escherichia coli* have been extensively studied as potential biocatalysts for alcohol production [18]. *Z. mobilis* garnered attention as a potential biocatalyst due to its natural ability to produce ethanol and its high tolerance to ethanol [10]. Like *Saccharomyces*, however, native strains of *Z. mobilis* are unable to utilize pentose sugars [10, 25]. The Gram-negative model bacterium *E. coli* has also been used for optimization as biocatalysts for the production of ethanol and advanced biofuels [1, 2, 36]. However, these efforts have only been successful at lab scale [35]. In particular, the sensitivity of *E. coli* to alcohol, low pH, and osmotic stress makes it a less attractive biocatalyst for production of alcohols. All of the biocatalyst evaluated, to date, for the production of biofuels from lignocellulosic biomass have limitations.

Lactic acid bacteria (LAB) are well known for their ability to survive and grow at low pH and in the presence of high concentrations of alcohols [14, 19, 26]. LAB are the primary spoilage agents in alcoholic beverages such as wine and beer [4], as well as bioethanol plants [37]. Among LAB, lactobacilli display the highest alcohol tolerance with some species able to grow in up to 18 % ethanol or 3 % butanol [23, 29]. LAB can be isolated from a variety of environments, including plant mass (i.e., silage), and many strains collected from plant material have the ability to ferment lignocellulose-derived carbohydrates such as glucose, xylose, cellobiose and arabinose [5, 6, 8, 22]. Additionally, LAB are commonly exposed to osmotic stress from salts or sugars during manufacture of fermented foods. In fact, some LAB have been already studied for the production of ethanol with promising results [15, 27, 28, 38]. As a whole, these attributes suggest lactobacilli may be useful as biocatalytic platforms for the production of alcohols.

Lactobacillus casei is an aciduric, facultatively heterofermentative lactic acid bacterium that produces lactic acid as the major end-product of carbohydrate fermentation. The species is remarkably adaptive and can be isolated from a variety of environments including plants [21]. Strains of *L. casei* are used industrially in the preparation of fermented foods, as probiotics, and for L-lactic acid production [9, 11, 13]. This industrial history has shown that *L. casei* is safe and led to its GRAS designation. More importantly, *L. casei* boasts several physiological (i.e., a relatively simple metabolism; [31]) and genetic advantages (availability of genome sequences, genome-scale metabolic models and methods for integration of foreign DNA; [39, 43, 44]) that make it an appealing candidate for metabolic engineering toward production of biofuels.

To explore this possibility, we examined the impact of different alcohols on two important growth parameters, growth rate (μ_{max}) and maximal optical density achieved (OD_{max}), on five different *L. casei* strains. One strain, *L. casei* 12A, was selected for further study and engineered to produce ethanol instead of lactate as its major end-product from carbohydrates (Phrommao et al., manuscript in preparation). The growth of this ethanologen, designated *L. casei* E1, was compared to two other bacterial ethanologens, *E. coli* GLBRC E1 and *Z. mobilis* ATCC 31821, in a medium that simulates the composition of a lignocellulosic hydrolysate, synthetic corn stover hydrolysate (mSynH) [36].

Materials and methods

Bacterial strains and growth conditions

The bacterial strains utilized in this study are described in Table 1. Working cultures were prepared from frozen stocks by two sequential transfers (0.1 % inoculum) in 5 ml of an

Table 1 Bacterial strains utilized in this study

Organism	Strain description	Reference/source
Lactobacillus casei ATCC 334	Wild-type	[37]
L. casei 12A	Wild-type	[21, 29]
L. casei A2-362	Wild-type	[21]
L. casei CRF28	Wild-type	[21]
L. casei UW-1	Wild-type	[21]
L. casei E1	12A Δ*ldh1*::Ppgm-PET[a]	This study
Escherichia coli GLBRCE1	K-12 *pflB*::PET[a], Δ*ldhA*, Δ*ack*	[13]
Zymomonas mobilis ATTC 31821	Wild-type	ATCC[b]

[a] Production of ethanol cassette

[b] American type culture collection

appropriate broth medium, with incubations conducted statically at 37 °C for 24 and 18 h, respectively. When comparing *L. casei* strains, working cultures were prepared by sequential transfers in all-purpose tween (APT) broth (Difco™ BD). When comparing three bacterial ethanologens, *L. casei* 12A Δ*ldh1*::Ppgm-PET, *E. coli* GLBRC E1 [36] and *Z. mobilis* (ATCC® 31821™), the first transfers were made in de Man, Rogosa and Sharpe (MRS), Luria–Bertani (LB), or Nutrient Broth (NB) medium (Difco™ BD), respectively. For all three cultures, the second transfer was performed in modified synthetic corn stover hydrolysate (mSynH). The ingredient list for mSynH is provided in Online Resource 1.

Construction of *L. casei* 12A Δldh1::Ppgm-PET (E1)

To construct the ethanologen *L. casei* E1, a 12A derivative lacking *ldh*1 was constructed using the procedure described by Broadbent et al. [7]. Briefly, a synthetic production of ethanol (PET) cassette encoding the *Z. mobilis* pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADHII) genes under the control of the native *L. casei* 12A phosphoglycerate mutase (*pgm)* promoter (*L. casei* 12A Δldh1::Ppgm-PET) was assembled and codon optimized using Java Codon Adaptation Tool and inserted into the 12A *ldh*1 locus. Chromosomal integration of PET cassette was confirmed by PCR using primers that annealed to flanking sequences upstream (forward: CGGGTTGTCGCGGTAGCTGC) and downstream (reverse: CAGCAAAGACTGATGTCGTCCATTGCC) of the *L-ldh*1 gene.

Alcohol tolerance of *L. casei* strains

To evaluate tolerance of *L. casei* strains to different alcohols, working cultures were inoculated (initial $OD_{600} = 0.15$) into APT broth containing (v/v): 5 or 10 % ethanol; 3 or 5 % propanol; 1 or 1.7 % butanol; 0.5 or 1 % 2-methyl-butanol. Next, 1 ml of each solution was transferred into 2 ml crimp-top vials (Thermo Fisher Scientific), and then the vials were sealed to minimize evaporation and incubated statically at 37 °C. OD_{600} was determined every hour during the first 14 h, then at 24, 32 and 48 h, using a spectrophotometer (Bio Rad SmartSpec Plus).

Alcohol tolerance of different ethanologens

The three bacterial ethanologens were inoculated at 0.1 % in mSynH medium containing either no alcohol, 3 % (v/v) ethanol, or 0.7 % (v/v) isobutanol. One ml of inoculated mSynH medium was transferred into 2 ml crimp-top vials, and then the vials were sealed to minimize evaporation and incubated statically at 37 °C. The OD_{600} was determined at time 0, 6, 16, 24, 30, 40 and 48 h, as described above.

Resistance of ethanologens to common stressors in corn stover hydrolysate

The three bacterial ethanologens were inoculated at 0.1 % in mSynH medium containing one of the following stresses: no stressors (control), 626 mM glucose (osmotic stress), acetate (acetamine 95 mM and sodium acetate 33 mM), a cocktail of known lignotoxins (Online Resource 2), or a combination of all three stressors [36]. The OD_{600} was determined every 10 min using a plate reader (Varioscan, Thermo Scientific), during static incubation at 37 °C for 72 h. Maximal growth (OD_{max}) and growth rate (μ_{max}) were calculated for each condition.

Alcohol tolerance of ethanologens

Working cultures of the three ethanologens were inoculated (0.1 %) in mSynH containing different concentrations (v/v) of ethanol or isobutanol depending on the strain. *L. casei* E1 was inoculated into a 50 ml master mix of mSynH containing 0, 3, 5, 7, or 10 % ethanol or 0, 0.7, 1.2, 1.7 and 2.2 % isobutanol. *E. coli* GLBRC E1 and *Z. mobilis* 31821 were inoculated into a 50 ml master mix of mSynH containing 0, 1, 2, 3 or 4 % ethanol or 0, 0.3, 0.5, 0.7 or 1.0 % isobutanol. Inoculated mSynH medium was transferred into crimp-top vials and growth monitored as described in "Alcohol tolerance of *L. casei* strains". Alcohol concentrations needed to reduce maximal growth (OD_{max}) and growth rate (μ_{max}) by 50 % were calculated using JMP® Version 8 (SAS Institute Inc., Cary, NC, USA) software.

Statistical analysis

For growth experiments, biological duplicates with assay replicates were conducted. To calculate maximal growth (OD_{max}), maximum growth rate (μ_{max}), and lag time, the spectrophotometric data were log transformed and nonlinear regression was applied using a modified Gompertz equation [3, 45]. Tukey's test was conducted to analyze differences among strains. Statistical analysis and model fitting were done using JMP® Version 8 (SAS Institute Inc., Cary, NC, USA) software.

Results and discussion

Alcohol tolerance of *L. casei*

One of the features that confer an advantage to lactobacilli over other organisms for the production of higher alcohols includes their high innate tolerance to these alcohols [4, 23]. Here, five different *L. casei* strains were screened for their tolerance to ethanol, propanol, butanol, isobutanol and

2-methyl-1-butanol. Results are expressed in concentration of alcohol needed to reduce maximal growth rate (μ_{max}) and maximal optical density (OD_{max}) by 50 % (Fig. 1).

In terms of μ_{max} reduction, *L. casei* UW-1 exhibited the highest tolerance to ethanol, propanol and 2-methyl-1-butanol, with 7, 4.19 and 0.89 % needed to reduce μ_{max} by 50 %, respectively. *L. casei* CRF28 presented the highest tolerance to butanol with 1.97 % needed to reduce μ_{max} by 50 %. This strain exhibited a slightly lower tolerance to 2-methyl-1-butanol when compared to *L. casei* UW-1 (with 0.86 versus 0.89 %), but the difference was not statistically significant. *L. casei* ATCC 334 and 12A exhibited the highest isobutanol tolerance, with 1.96 and 1.89 %, respectively. These results demonstrate that even in a species already known for alcohol tolerance, there is considerable strain-to-strain variation in alcohol tolerance.

Strain differences in OD_{max} were not as pronounced as were observed with μ_{max}. *L. casei* 12A achieved highest optical densities in the presence of ethanol, propanol, butanol, and 2-methyl-1-butanol, with 9.21, 3.45, 1.31, and 0.64 % needed to reduce OD_{max} by 50 %, respectively. *L. casei* UW-1 exhibited the highest tolerance to isobutanol, in terms of OD_{max} reduction, with 1.62 %.

Alcohol tolerance decreased as the alcohol chain length increased for all *L. casei* strains. This finding was expected, because alcohols become more hydrophobic as the number of carbons increase and so can cause more damage to the bacterial cytosolic membrane [17]. Interestingly, isobutanol appeared to be less toxic than butanol on growth rate of two strains, *L. casei* 12A and ATCC 334, and in reducing optical density for all five strains.

Strains UW-1 and 12A exhibited the best overall performance for reduction of growth rate and optical density, respectively, and, therefore, showed particular promise as biocatalysts for alcohol production. However, such biocatalysts should ideally consume a variety of plant-based carbon sources and *L. casei* UW-1 utilizes a relatively restricted number of carbohydrates [6]. *L. casei* 12A, on the other hand, is able to ferment a far more diverse set of carbohydrates, utilizing 26 different carbon sources associated with plant, gut or dairy niches [6]. The availability of methods for genetic modification is another key trait for biocatalyst development and Welker et al. recently showed *L. casei* 12A can be transformed at high frequency (5.4×10^6 cfu/μg DNA) [43]. Additionally, a genome-scale metabolic model was constructed for this strain [39]. For these reasons, *L. casei* 12A was selected as the biocatalytic platform to explore the potential of *L. casei* to produce alcohols.

To conduct this assessment, a synthetic PET cassette was integrated into an *L. casei* 12A derivative strain lacking *ldh*1 to yield an ethanologen, *L. casei* E1 (Table 1). The ability of *L. casei* E1 to grow under conditions designed to mimic those which would be encountered

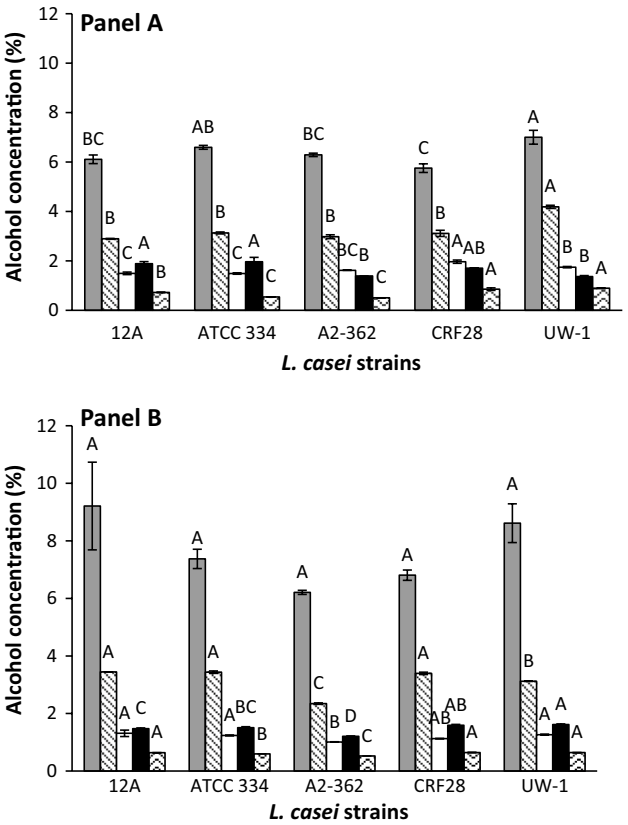

Fig. 1 Alcohol concentration needed to reduce maximum growth rate (**a**) and maximal optical density (**b**) by 50 % for five *Lactobacillus casei* strains. Alcohols tested: ethanol (▩), propanol (▨), butanol (□), isobutanol (■) and 2-methyl-1-butanol (▧). *Error bars* represent standard error with $n = 4$. Strains that share at least one *letter* within the same condition are not statistically significant from each other

during the production of alcohols from lignocellulosic feedstocks was then tested and compared to the ethanologens *E. coli* GLBRC E1 [36] and *Z. mobilis* ATCC 31821 (Table 1).

The three bacteria were grown in mSynH, a medium that simulates the composition of corn stover hydrolysate except that it does not contain any stressors. In mSynH, *L. casei* E1, *E. coli* GLBRC E1 and *Z. mobilis* ATCC 31821 achieved OD_{max} values of 1.8, 2.4 and 2.5, respectively (Fig. 2, panel a). *L. casei* is a nutritionally fastidious organism and mSynH is not a rich media, which might explain the lower OD_{max} achieved by this strain. However, *L. casei* E1 exhibited a higher maximal growth rate ($\mu_{max} = 0.14\ h^{-1}$) when compared to *E. coli* GLBRC E1 ($\mu_{max} = 0.09\ h^{-1}$) or *Z. mobilis* ATCC 31821 ($\mu_{max} = 0.08\ h^{-1}$). Additionally, *L. casei* E1 exhibited a higher OD_{max}, and faster growth rate in comparison to *E. coli* GLBRC E1 and *Z. mobilis* ATCC 31821 when the strains were grown in mSynH supplemented with either 3 % ethanol or 0.7 % isobutanol (Fig. 2, panel b and c).

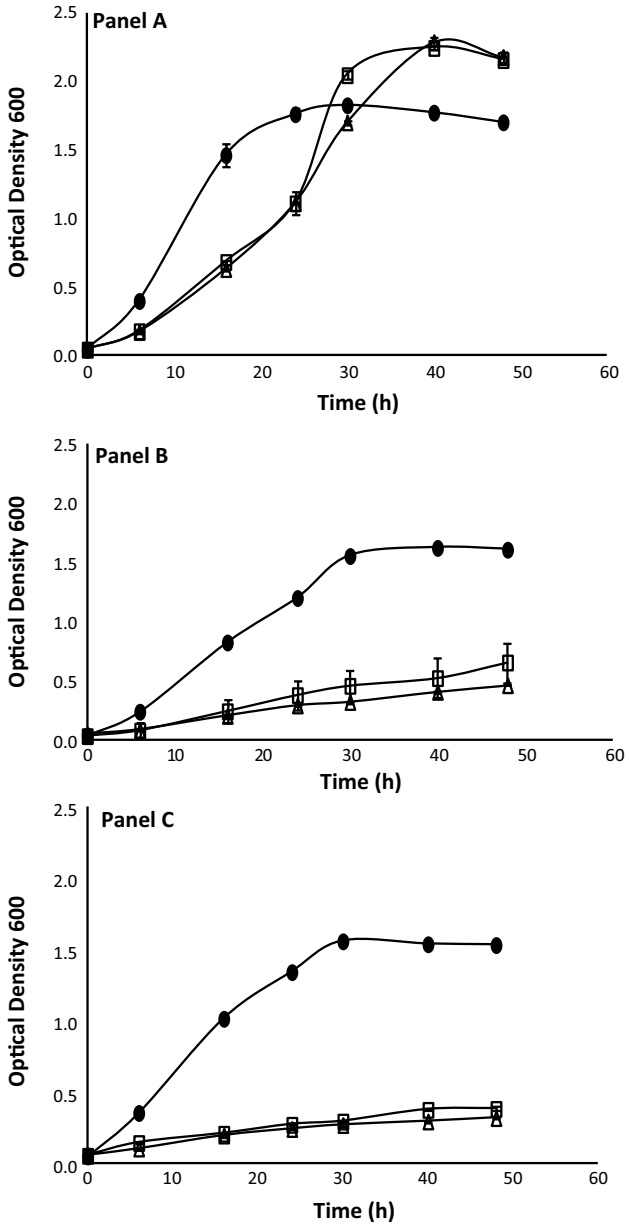

Fig. 2 Growth curves of *Lactobacillus casei* E1 (—●—), *Escherichia coli* GLBRC E1 (—□—) and *Zymomonas mobilis* ATCC 31821 (—△—). **a** Growth of the strains in modified synthetic corn stover hydrolysate base medium (mSynH). **b** Growth of the strains in mSynH supplemented with 3 % ethanol. **c** Growth of the strains in mSynH supplemented with 0.7 % isobutanol. *Error bars* represent standard error with $n = 4$

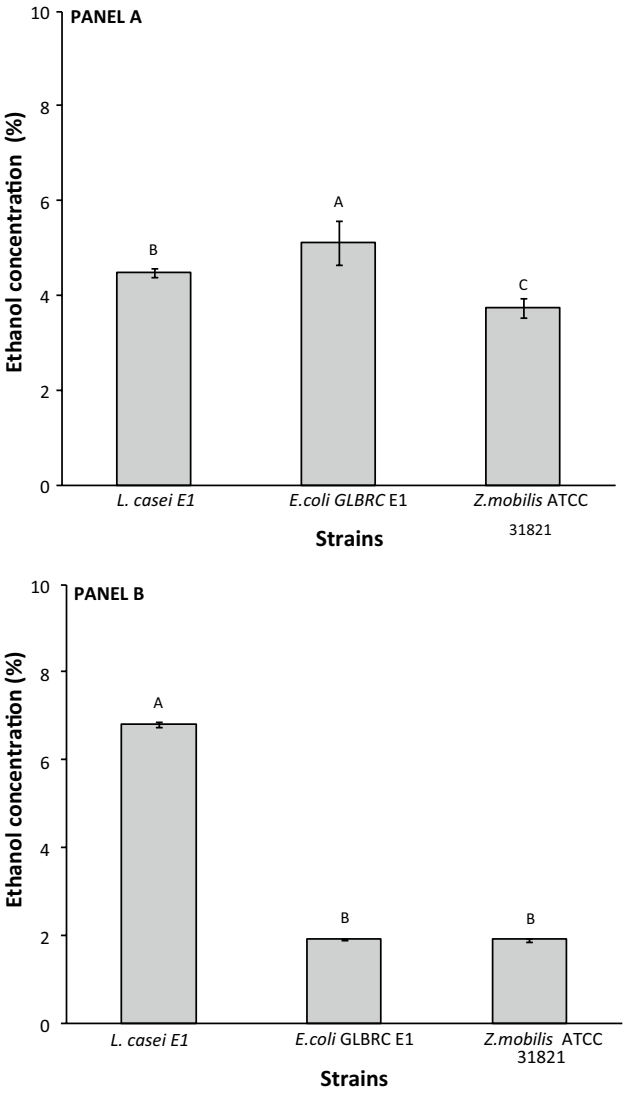

Fig. 3 Ethanol concentration to reduce maximum growth rate (**a**) and maximal optical density (**b**) by 50 % of *Lactobacillus casei* E1, *Escherichia coli* GLBRC E1 and *Zymomonas mobilis* ATCC 31821 during growth in modified synthetic corn stover hydrolysate. *Error bars* are standard errors with $n = 4$. Strains that share *letters* are not statistically significant from each other

To calculate the concentration of ethanol and isobutanol needed to reduce μ_{max} and OD_{max} by 50 % for *L. casei* E1, *E. coli* GLBRC E1 and *Z. mobilis* ATCC 31821, the strains were grown in mSynH supplemented with different alcohol concentrations (Figs. 3, 4; Online Resource 3). The ethanol concentrations which resulted in a 50 % decrease in μ_{max} for *L. casei* E1, *E. coli* GLBRC E1 and *Z. mobilis* ATCC 31821 were 4.5, 5.1 and 3.8 %, respectively, and the differences were statistically significant among all ethanologens ($p < 0.05$). The ethanol concentrations which resulted in a 50 % decrease in OD_{max} for *L. casei* E1, *E. coli* GLBRC E1 and *Z. mobilis* ATCC 31821 were 6.8, 1.9 and 1.9 %, respectively (*L. casei* E1 was statistically different from the other two ethanologens; $p < 0.05$). These results indicate that *L. casei* E1 has a higher tolerance to ethanol than either *E. coli* GLBRC E1 or *Z. mobilis* ATCC 31821. *L. casei* E1 also exhibited

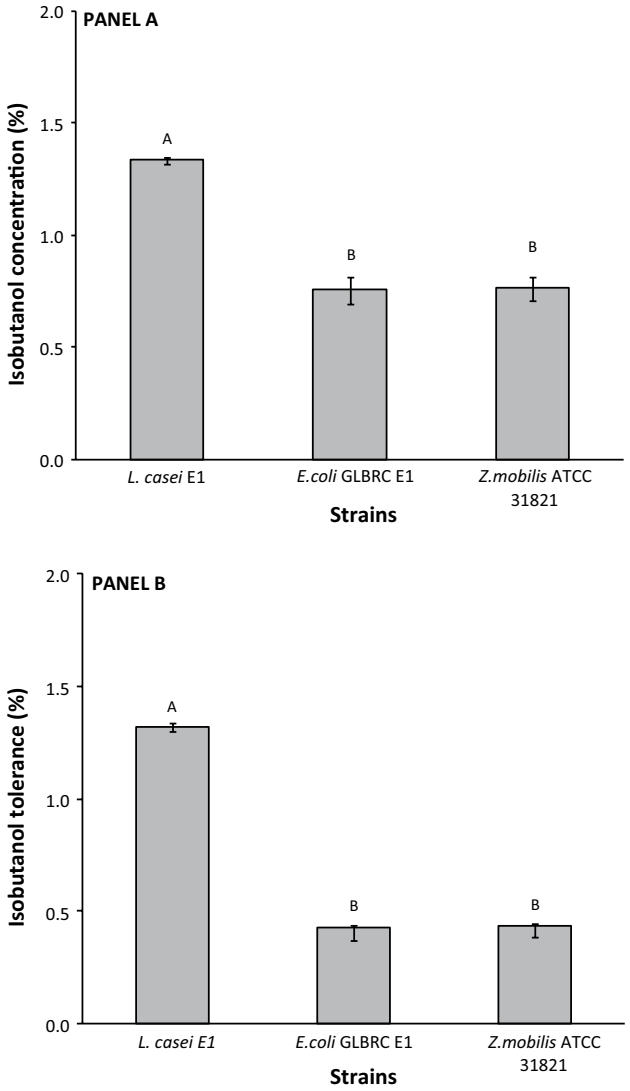

Fig. 4 Isobutanol concentration to reduce maximum growth rate (**a**) and maximal optical density (**b**) by 50 % of *Lactobacillus casei* E1, *Escherichia coli* GLBRC E1and *Zymomonas mobilis* ATCC 31821 during growth in modified synthetic corn stover hydrolysate. *Error bars* are standard errors with $n = 4$. Strains that share *letters* are not statistically significant from each other

significantly ($p < 0.05$) higher isobutanol tolerance, with 1.3 % of this alcohol needed to reduce μ_{max} and OD_{max} by 50 versus 0.8 % to reduced μ_{max} and 0.4 % to reduce OD_{max} by 50 % in both *E. coli* GLBRC E1 and *Z. mobilis* ATCC 31821.

For *E. coli* GLBRC E1 and *Z. mobilis* ATCC 31821, optical density was more sensitive to ethanol or isobutanol than growth rate. *L. casei* E1 exhibited the opposite trend, where μ_{max} showed more sensitivity to ethanol; for isobutanol, both growth parameters showed the same level of sensitivity. This observation may indicate that *L. casei* 12A has different mechanisms for tolerance to ethanol and isobutanol.

Resistance to common stressors in corn stover hydrolysate

Production of corn stover hydrolysate results in the release of microbial inhibitors into the medium. Examples include high concentrations of hexose and pentose sugars, which increase the osmotic pressure, the formation of lignotoxins, and acetate. It was important, therefore, to compare the impact of these stressors on the growth of *L. casei* E1, *E. coli* GLBRC E1 and *Z. mobilis* ATCC 31821 in mSynH.

As is summarized in Fig. 5 and Online Resource 4, incubation in mSynH with either a single stressor or a combination of stressors showed lignotoxin compounds were the most significant ($p < 0.05$) inhibitory single stress for *L. casei* E1, with 36 % reduction in μ_{max}, and the least significant ($p < 0.05$) inhibitory stress was acetate with 7 % reduction in μ_{max}. Since *L. casei* is a member of the lactic acid bacteria group, which commonly produces a mixture of lactic and acetic acids, its tolerance to acetate and lower pH was expected. The combination of osmotic pressure and lignotoxins were the most significant ($p < 0.05$) toxic to *L. casei* E1, but no further inhibition was observed when all three stressors were present (reducing μ_{max} of *L. casei* E1 by 48 %).

In contrast, the most significant ($p < 0.05$) inhibitory single stress for *E. coli* GLBRC E1 was acetate followed by lignotoxin compounds and the addition of both of these stressors was the most toxic combination, with a 70 % reduction in μ_{max} (Fig. 5; Online Resource 4). The combination of all three stressors resulted in 80 % growth reduction. Interestingly, it appears that the inhibitory effect of acetate is reduced when combined with osmotic pressure, suggesting that cross protection may exist between osmotic and pH stress in *E. coli* [40]. One possible explanation for this finding is that osmotic stress might enhance the production of compounds (i.e., amino acids) that alleviate acetate stress [16].

Zymomonas mobilis ATCC 31821 growth was most significantly ($p < 0.05$) affected by the lignotoxin cocktail, followed by the combination of lignoxotin compounds and acetate, and the combination of all three stressors (Fig. 5; Online Resource 4). As was observed with *E. coli* GLBRC E1, the increased osmotic stress from glucose addition helped ameliorate cell inhibition by acetate.

The combination of the three stressors represents a more realistic scenario of what a selected biocatalyst will face during biofuel production. Under these conditions, the growth of *L. casei* E1 was the least significantly ($p < 0.05$)

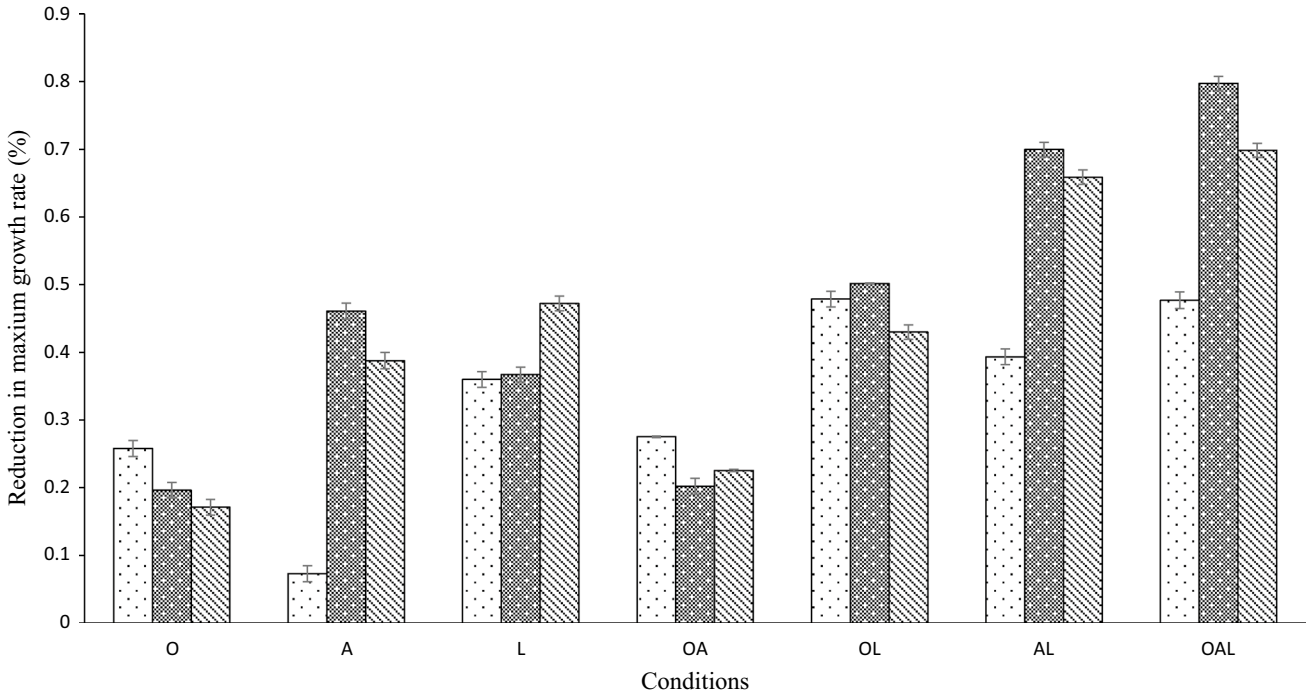

Fig. 5 Percent reduction in maximum growth rate of *Lactobacillus casei* E1 (□), *Escherichia coli* GLBRC E1 (▩), and *Zymomonas mobilis* ATCC 31821 (▧). Strains were grown in modified synthetic corn stover hydrolysate (mSynH) supplemented with one or more of the following stressors: osmotic stress (O), acetate (A), lignotoxic compounds (L), osmotic stress and acetate (OA), osmotic stress and lignotoxic compounds (OL), acetate and lignotoxic compounds (AL) and osmotic stress, acetate and lignotoxic compounds (OAL). Growth was compared to that of mSynH with no supplementation, (see Online Resource 4 for statistical analysis)

affected, which confirmed this strain is a promising biocatalyst for biofuel production (Online Resource 4).

To further evaluate the effects of lignotoxin compounds on the growth of *L. casei* E1, the strain was propagated in growth in mSynH supplemented with individual lignotoxin compounds (Table 2). The most significant ($p < 0.05$) phenolic compounds in mSynH that reduced μ_{max} the most was ρ-coumaric acid and feruloyl amide reduces significantly ($p < 0.05$) OD_{max} the most (Table 2). As described by Pisithkul et al. [34], these compounds act as inhibitors for de novo biosynthesis of nucleotides. In particular, feruloyl amide was found to be a competitive inhibitor of glutamine 5-phosphoribosyl-1-pyrophosphate amidotransferase, the enzyme that catalyzes the first step in de novo purine biosynthesis.

Ethanol production under stress normally found in corn stover hydrolysate

While growth parameters are important to assess, alcohol production is the ultimate critical parameter for selecting a biocatalyst. As is shown in Fig. 6, *L. casei* E1 consumed significantly ($p < 0.05$) more glucose and produced more ethanol (113 and 77 mM, respectively) than *E. coli* GLBRC E1 (34 and 61 mM) or *Z. mobilis* 31821 (30 and 57 mM) in mSynH with all three stressors. Ethanol was, of course, the primary end-product in *E. coli* GLBRC E1 and *Z. mobilis* ATCC 31821 fermentation, but not in *L. casei* E1. Instead, we were surprised to observe that 84 mM of lactate accumulated at the end of fermentation.

Lactic acid is the primary metabolic end-product of lactic acid bacteria and at least six enzymes may catalyze the conversion of pyruvate to lactic acid in *L. casei* 12A: four L-lactate dehydrogenases, as well as D-lactate dehydrogenase, and D-hydroxyisocaproate dehydrogenase (D-*hic*). In the absence of L-*ldh1* activity, L-*ldh2* or D-*hic* is likely the key enzyme driving flux from pyruvate to lactate (Phrommao et al., manuscript in preparation). Deletion of these lactate dehydrogenases will likely be required to eliminate conversion from pyruvate to lactate and increase ethanol yield.

Conclusions

The work presented within this manuscript suggests *L. casei* is superior to *E. coli* or *Z. mobilis* as a bacterial biocatalyst for alcohol production. *L. casei* E1 exhibited better

Table 2 Maximum growth rate (μ_{max}) and optical density (OD_{max}) exhibited by *Lactobacillus casei* E1 growing in modified synthetic corn stover hydrolysate (mSynH) supplemented with individual lignotoxin compounds

Condition	μ_{max}	OD_{max}
mSynH	0.18 ± 0.01^{a}	1.84 ± 0.01^{a}
Furan derivatives		
HMF	0.17 ± 0.02^{b}	1.73 ± 0.00^{e}
Phenolic compounds		
Feruloyl amide	0.14 ± 0.00^{de}	1.69 ± 0.00^{g}
Coumaroyl amide	0.14 ± 0.01^{e}	1.73 ± 0.01^{e}
p-Coumaric acid	0.13 ± 0.01^{f}	1.71 ± 0.01^{f}
Ferulic acid	0.15 ± 0.00^{d}	1.73 ± 0.01^{e}
Benzoic acid	0.18 ± 0.01^{a}	1.83 ± 0.01^{a}
Syringic acid	0.17 ± 0.01^{b}	1.73 ± 0.01^{de}
Cinnamic acid	0.16 ± 0.01^{c}	1.80 ± 0.01^{b}
Vanillic acid	0.17 ± 0.01^{ab}	1.83 ± 0.00^{a}
Caffeic acid	0.14 ± 0.00^{e}	1.81 ± 0.01^{b}
Vanillin	0.17 ± 0.01^{b}	1.78 ± 0.01^{c}
Syringaldehyde	0.17 ± 0.01^{b}	1.78 ± 0.02^{c}
4-Hydroxybenzaldehyde	0.15 ± 0.01^{d}	1.76 ± 0.01^{d}
4-Hydroxyacetophenone	0.16 ± 0.00^{cd}	1.77 ± 0.01^{d}
All lignotoxins	0.12 ± 0.01^{g}	1.69 ± 0.01^{g}

Letters for each value that is shared by same condition are not statistically different. Letters are to be compares within columns, treating the mSynH condition as control

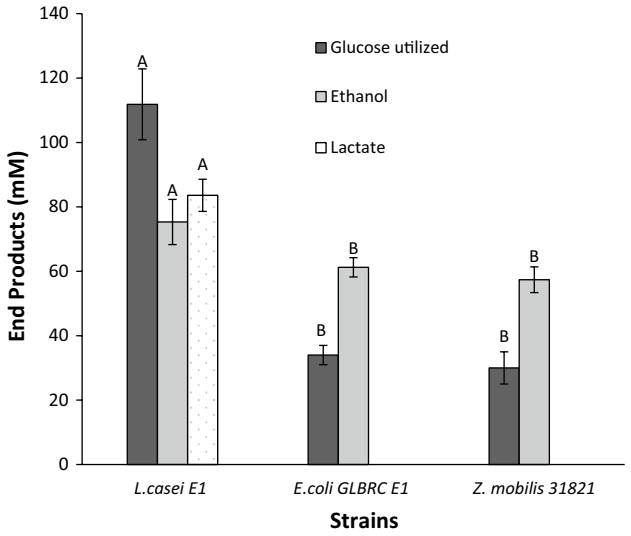

Fig. 6 Glucose consumption and ethanol production of *L. casei* E1, *E. coli* GLBRC E1 and *Z. mobilis* ATCC 31821. Fermentations were done in modified synthetic corn stover hydrolysate supplemented with osmotic stress (626 mM glucose), acetate (95 mM acetamine plus 33 mM acetate) and lignotoxic compounds (Online Resource 2). Strains that share *letters* are not statistically significant from each other. *Error bars* are standard errors with $n = 3$

growth and higher innate tolerance to alcohol and other stressors commonly found in corn stover hydrolyzate. It also produced higher levels of ethanol in mSynH with added stressors. Further strain optimization would enable even higher ethanol yield and help reduce unwanted end-products in the fermentation.

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