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Effect of Lignocellulose-Derived Inhibitors on Growth of and Ethanol Production by Growth-Arrested *Corynebacterium glutamicum* R▽

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In cellulosic ethanol production, pretreatment of a biomass to facilitate enzymatic hydrolysis inevitably yields fermentation inhibitors such as organic acids, furans, and phenols. With representative inhibitors included in the medium at various concentrations, individually or in various combinations, ethanol production by *Corynebacterium glutamicum* R under growth-arrested conditions was investigated. In the presence of various inhibitors, the 62 to 100% ethanol productivity retained by the *C. glutamicum* R-dependent method far exceeded that retained by previously reported methods.

Worldwide attention has recently turned to bioethanol production as a strategy to combat global warming and to improve global energy security (15, 24). However, feedstocks of current bioethanol production methods are currently derived from edible parts of food crops such as sugarcane and corn. This leads to an undesirable direct competition between bioethanol production and the food supply (6, 25). A switch to a more abundant lignocellulosic biomass, some of which may be obtained from inedible parts of food crops, should help to reduce pressure on the food crops and possibly generate increased demand for bioethanol (6, 15, 25).

In a previous study of biomass pretreatment with dilute acid and hot water, the major degradation by-products released included organic acids such as acetate, furans such as furfural and 5-hydroxymethylfurfural (5-HMF), and phenols such as 4-hydroxybenzaldehyde (4-HB), vanillin, and syringaldehyde (10). Although the pretreatment of lignocellulosic biomasses is a necessity for efficient saccharification and ethanol production (10, 15), ethanol production by microorganisms is inhibited in the presence of small concentrations of some of these by-products of pretreatment (20, 27–29). In order to avoid such inhibition, various treatments for the detoxification of fermentation inhibitors have been investigated (10). For industrial ethanol production, however, a method that eschews the detoxification steps is desirable to keep costs down and reduce method complexity (26).

The aerobic bacterium *Corynebacterium glutamicum* has widely been used in the industrial biological production of amino acids and nucleic acids (9, 23). *C. glutamicum* R can metabolize biomass-derived sugars such as glucose and mannose (13). Additionally, we previously isolated adaptive mutants capable of not only metabolizing cellobiose but also simultaneously metabolizing glucose and cellobiose (14). More recently, we also reported recombinant *C. glutamicum* R strains capable of efficient xylose utilization (8), xylose being one of the most abundant pentose sugars found in lignocellulosic hydrolysates. For ethanol production, we previously constructed ethanologenic *C. glutamicum* R to demonstrate ethanol production under growth-arrested conditions (7). Growth-arrested conditions were enabled by oxygen deprivation of cells in a reactor, leading to high volumetric ethanol productivity. In this study, we investigated the effect of fermentation inhibitors found in lignocellulosic hydrolysates on ethanol production by ethanologenic *C. glutamicum* R under growth-arrested conditions.

The microorganism used in this study was a *C. glutamicum* R marker-less *ldhA*-deficient mutant bearing pCRA723 (strain R-*ldhA*-pCRA723) which expressed *Zymomonas mobilis* genes coding for pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adhB*) (7). Growth experiments were initiated using cells at an optical density at 610 nm (OD_{610}) of 0.1 and performed aerobically in a test tube containing 20 ml of A medium (7) with 200 mM glucose and various concentrations of inhibitors. The tubes were shaken (200 rpm) at 33°C for 12 h. Relative growth was the difference between the OD_{610} of a culture with an inhibitor and that of a reference culture without the inhibitor after 12 h of cultivation. For ethanol production under growth-arrested conditions, *C. glutamicum* strain R-*ldhA*-pCRA723 cells grown in aerobic-phase cultures were harvested by centrifugation. Cell pellets were subsequently washed twice with mineral salts medium (7). The cells were then resuspended to a final dry cell concentration of 10 g $liter^{-1}$ in mineral salts medium containing 200 mM glucose and incubated at 33°C. Dissolved oxygen in medium was maintained at less than 0.01 ppm. The relative ethanol productivity (percentage) was the difference between the initial volumetric production rate in the experimental phase with inhibitors and that of a reference culture during the first 3 h of the reaction. Detailed conditions for growth and ethanol production and analytical methods for our study have been described previously (7).

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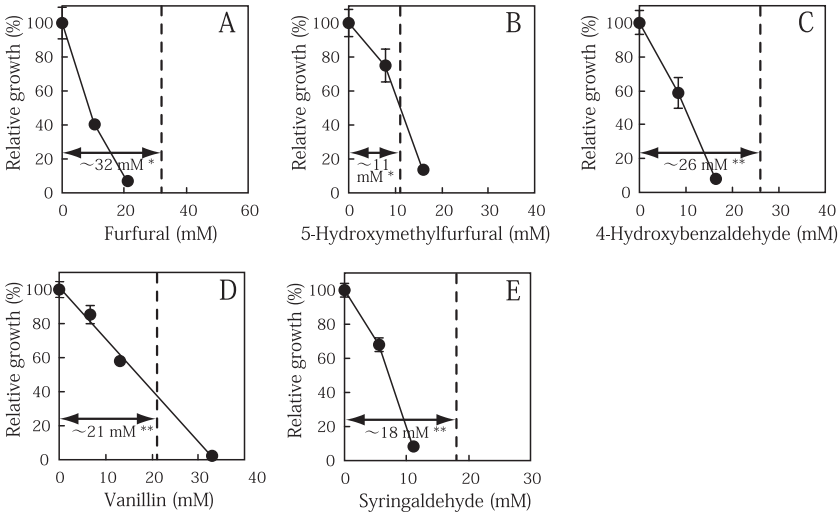

FIG. 1. Relative levels of growth of *Corynebacterium glutamicum* strain R-*ldhA*-pCRA723 under aerobic conditions with glucose in the presence of furfural (A), 5-hydroxymethylfurfural (B), 4-hydroxybenzaldehyde (C), vanillin (D), and syringaldehyde (E). Relative growth was the difference between the OD_{610} of a culture with the indicated inhibitor and that of the reference culture without the inhibitor after 12 h of cultivation. Values are averages from at least triplicate experiments. Standard deviations are represented by error bars. *, a typical concentration range of the inhibitor in prehydrolysate derived from the lignocellulosic biomass is represented by the arrow and dotted line (17); **, a typical concentration range of the inhibitor in prehydrolysate derived from the lignocellulosic biomass is represented by the arrow and dotted line, which is equal to a range of 0 to 3.2 g liter^{-1} of total phenols (17). The average final OD_{610} ± standard deviation from all reference experiments was 15 ± 1 after 12 h.

Effect of individual inhibitors. Xylose and other pentose sugars are liberated during the degradation of hemicellulose, and further degradation releases furfural, while 5-HMF is the result of hexose degradation. These furans are known to be highly toxic for the growth and fermentation of ethanologenic microorganisms. In *C. glutamicum*, the cell growth of both strain R-*ldhA*-pCRA723 (Fig. 1A and B) and type strain ATCC 13032 (data not shown) significantly decreased with the increase in concentration of furfural and 5-HMF. The relative growth of strain R-*ldhA*-pCRA723 with 21 mM furfural and 16 mM 5-HMF decreased to 7% and 14% of the growth of the reference culture, respectively (Fig. 1A and B). *C. glutamicum* displayed sensitivities to furfural and 5-HMF similar to those of yeasts such as *Saccharomyces cerevisiae* CBS 1200, *Candida shehatae* ATCC 22984, and *Pichia stipitis* NRRL Y 7124 but was more sensitive to furfural than bacteria such as *Z. mobilis* ATCC 10988, *Escherichia coli* ATCC 1175, and *E. coli* LY01 (2, 3, 27). We previously reported that growth-arrested conditions allowed the *C. glutamicum* R wild type and recombinants to metabolize sugar without growth (7). This metabolic activity under growth-arrested conditions may be advantageous in so far as they avoid inhibitory effects on growth. Therefore, we determined ethanol production by *C. glutamicum* strain R-*ldhA*-pCRA723 under growth-arrested conditions with furfural and 5-HMF. Consequently, strain R-*ldhA*-pCRA723 in our growth arrest test retained 82% of its relative ethanol productivity with 52 mM furfural and 62% with 79 mM 5-HMF (Fig. 2A and B). In contrast, Martín and Jönsson (16) and Talebnia et al. (22) reported that the relative ethanol productivity of *S. cerevisiae* is drastically reduced to 47% with 52 mM furfural or to 18% with 60 mM 5-HMF (Table 1). This indicates that our method employing growth-arrested *C. glutamicum* strain R-*ldhA*-pCRA723 showed greater tolerance to furfural and 5-HMF than other methods.

4-HB, vanillin, and syringaldehyde are generated by the partial breakdown of lignin through the *p*-hydroxyphenyl residue, guaiacyl residue, and syringyl residue, respectively, and exert inhibitory effects on microbial ethanol production. In growth experiments with 4-HB, vanillin, and syringaldehyde, the phenols significantly inhibited the cell growth of *C. glutamicum* strain R-*ldhA*-pCRA723 (Fig. 1C to E) and wild-type strain ATCC 13032 (data not shown) at lower concentrations than those in actual syrup. As shown in Fig. 1C to E, the relative growth of strain R-*ldhA*-pCRA723 with 16 mM 4-HB, 33 mM vanillin, and 11 mM syringaldehyde decreased to 8%, 2%, and 8% of that of the reference culture, respectively. *C. glutamicum* showed sensitivity to phenols similar to that of yeasts such as *S. cerevisiae* CBS 1200, *C. shehatae* ATCC 22984, and *P. stipitis* NRRL Y 7124 and of bacteria such as *Z. mobilis* ATCC 10988, *E. coli* ATCC 1175, and *E. coli* LY01 (2, 3, 27). Meanwhile, under growth-arrested conditions, *C. glutamicum* strain R-*ldhA*-pCRA723 yielded ethanol productivity not less than 93% of that of the reference culture, with 16 mM 4-HB, 13 mM vanillin, or 11 mM syringaldehyde (Fig. 2C to E). With 33 mM vanillin and 28 mM syringaldehyde, relative productivity was 78 to 85% for the process employing growth-arrested *C. glutamicum* strain R-*ldhA*-pCRA723, while 41 mM 4-HB reduced the ethanol productivity to 49% (Fig. 2C to E). The difference may be due to the molecular structures of these inhibitors, with the ability of each inhibitor to penetrate the cell membrane being dependent on the number of methyl groups that the inhibitor possesses (3, 12). In contrast, *S. cerevisiae* ATCC 96581 showed ethanol productivities that were 36, 58, and 69% of that of the reference culture with 10 mM concentrations of the phenols 4-HB, vanillin, and syringaldehyde, respectively (Table 1). Compared to methods employing other microorganisms (12), our method employing growth-arrested *C. glutami-*

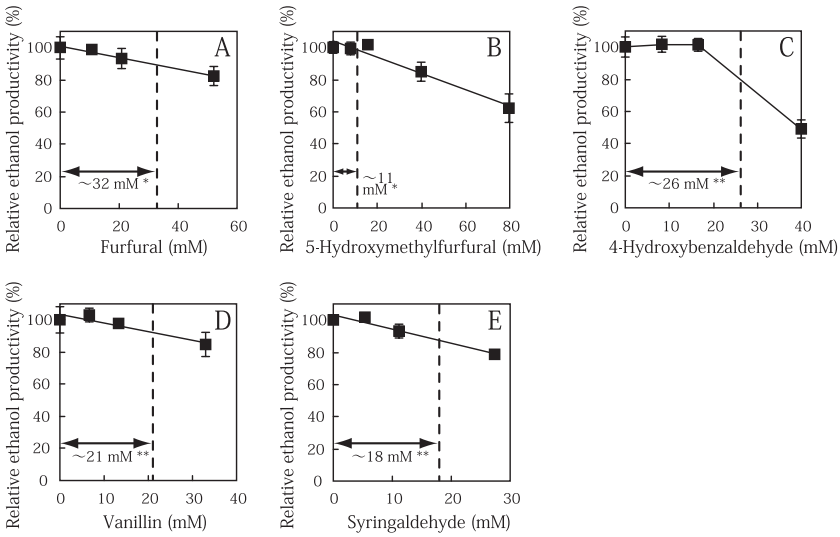

FIG. 2. Relative ethanol productivity of *Corynebacterium glutamicum* strain R-*ldhA*-pCRA723 under growth-arrested conditions with glucose in the presence of furfural (A), 5-hydroxymethylfurfural (B), 4-hydroxybenzaldehyde (C), vanillin (D), and syringaldehyde (E). The relative ethanol productivity (percentage) was the difference between the initial volumetric production rate at the experimental phase with inhibitors and that of the reference culture during the first 3 h of a reaction. Values are averages from at least triplicate experiments. Standard deviations are represented by error bars. The arrow and dotted line shown here are the same as those shown in Fig. 1. The average initial volumetric ethanol productivity of all reference experiments was 73 ± 3 mmol liter^{-1} h^{-1}.

cum strain R-*ldhA*-pCRA723 showed high tolerance to 4-HB, vanillin, and syringaldehyde.

Acetate, one of the most abundant organic acids generated through pretreatments, is known to result from the hydrolysis of acetylxylan in hemicellulose. The inhibitory effect of acetate on the ethanol production of various microorganisms is well known (10). In our study of using growth-arrested *C. glutamicum* strain R-*ldhA*-pCRA723 with acetate applied as a sole inhibitor at concentrations up to 244 mM, the cells retained 88% of the productivity of the reference culture, with pH controlled at 7.5 (data not shown).

Effect of inhibitors comprising pretreatment hydrolysates. In previous studies, the concentrations and combinations of inhibitors in the actual syrup were diversified, due to the vari-

TABLE 1. Ethanol productivity of *Corynebacterium glutamicum* strain R-*ldhA*-pCRA723 and other organisms in the presence of inhibitors

Inhibitor	Concn (mM)	Organism	Ethanol productivity		Substrate (mM)[e]	Reference
			Relative rate (%)	Concn [mmol liter^{-1} h^{-1} (g liter^{-1} h^{-1})]		
Furfural	52	*Corynebacterium glutamicum* strain R-*ldhA*-pCRA723	82	58 (2.7)	Glu (200)	This study
	52	*Saccharomyces cerevisiae* CBS 8066[a]	47	35 (1.6)	Glu (167)	22
	47	*Escherichia coli* ATCC 11303[b]	6	1.1 (0.05)	Xyl (533)	1
5-HMF	79	*C. glutamicum* strain R-*ldhA*-pCRA723	62	43 (2.0)	Glu (200)	This study
	60	*S. cerevisiae* CEN.PK[c]	36	5.6 (0.26)	Glu (139)	16
	60	*S. cerevisiae* TMB 3400[c]	18	3.0 (0.14)	Glu (139)	16
4-HB	16	*C. glutamicum* strain R-*ldhA*-pCRA723	101	77 (3.6)	Glu (200)	This study
	10	*S. cerevisiae* ATCC 96581[d]	36	30 (1.4)	Glu (167)	12
Vanillin	13	*C. glutamicum* strain R-*ldhA*-pCRA723	98	69 (3.2)	Glu (200)	This study
	10	*S. cerevisiae* ATCC 96581[d]	58	43 (2.0)	Glu (167)	12
Syringaldehyde	11	*C. glutamicum* strain R-*ldhA*-pCRA723	93	71 (3.3)	Glu (200)	This study
	10	*S. cerevisiae* ATCC 96581[d]	69	56 (2.6)	Glu (167)	12

[a] First batch operation under anaerobic conditions in a conical flask containing 100 ml mineral salts medium and calcium alginate beads. The initial dry cell concentration was about 5 g liter^{-1} (30°C, pH 5.0).
[b] Batch operation under oxygen-limited conditions in a fermentor containing 350 ml complex medium. The initial dry cell concentration was 0.33 g liter^{-1} (30°C, pH 6.8).
[c] Batch operation under oxygen-limited conditions in glass vessels containing 22 ml mineral salts medium. The initial dry cell concentration was about 0.2 g liter^{-1} (30°C, pH 5.5).
[d] Batch operation in a stirred flask containing 40 ml mineral salts medium. The initial dry cell concentration was 2 g liter^{-1} (30°C, pH 5.0 to 5.5).
[e] Glu, glucose; Xyl, xylose.

TABLE 2. Relative ethanol productivity of *Corynebacterium glutamicum* strain R-*ldhA*-pCRA723 under growth-arrested conditions with model inhibitors of various methods

Method	Relative ethanol productivity (%)[f]
Dilute-acid prehydrolysate derived from corn stover[a,b]	98 ± 1
Supercritical water treatment prehydrolysate derived from cedar[a,c]	101 ± 4
Alkaline water oxidation prehydrolysate derived from wheat straw[a,d]	97 ± 4
Steam explosion prehydrolysate derived from sugarcane bagasse[a,e]	104 ± 2

[a] Compounds that were less than 0.05 g liter^{-1} were omitted.
[b] The composition was 43 mM acetate, 13.1 mM furfural, and 2.4 mM 5-HMF (19).
[c] The composition was 5.4 mM vanillin, 3 mM 5-HMF, 2.5 mM furfural, and 0.5 mM coniferylaldehyde (18).
[d] The composition was 90.6 mM formate, 28.1 mM acetate, 17.2 mM glycolic acid, 4.9 mM lactic acid, 2.5 mM succinate, and 1.2 mM malate (11).
[e] The composition was 70 mM acetate, 22 mM formate, 11 mM furfural, 3.2 mM 5-HMF, 2.9 mM *p*-coumaric acid, 1.1 mM ferulic acid, and 0.9 mM 4-HB (17).
[f] Values are averages and standard deviations from at least triplicate determinations. The average initial volume of ethanol for all reference experiments was 73 ± 1 mmol liter^{-1} h^{-1}.

ety and complexity of the pretreatments of biomass involved (10). Additive or synergistic inhibition by multiple kinds of inhibitors were reported for microorganisms (10). Therefore, ethanol production by *C. glutamicum* strain R-*ldhA*-pCRA723 was performed under growth-arrested conditions with inhibitor mixtures, the compositions of which matched those of previous reports of actual biomass pretreatments, while compounds that were less than 0.05 g liter^{-1} were omitted.

Dilute-acid hydrolysis has been investigated as one of the fast and easy operations, but this operation formed furans at high concentrations (10). Dilute-acid hydrolysis pretreatment of corn stover with inhibitors composed of organic acid and furans was employed as a model composition (19). Total furans were included at higher concentrations than other inhibitors tested in model compositions. With inhibitors of this model composition, growth-arrested *C. glutamicum* strain R-*ldhA*-pCRA723 retained 98% of its ethanol productivity, indicating a high tolerance to the combination of organic acids and furans (Table 2).

Supercritical water treatment of lignocellulose is a promising method because it is very rapid, occurring within several seconds, and requires no catalysts (18). A model composition of inhibitors consisting of furans and phenols derived from pretreatment of cedar with supercritical water (18) was employed. Of special note, total phenols were included at higher concentrations than other model inhibitors tested. In the present study, growth-arrested *C. glutamicum* strain R-*ldhA*-pCRA723 with inhibitors of this model composition retained all (101%) of its ethanol productivity (Table 2). This indicates that there was no inhibitory effect of the combination of furans and phenols on ethanol productivity by growth-arrested *C. glutamicum* strain R-*ldhA*-pCRA723.

Alkaline water oxidation pretreatment prevents the formation of furans and phenols (10). Hydroxycarboxylic acids such as glycolic acid and lactic acid are known as common degradation products from alkaline carbohydrate degradation (10). A model composition for alkaline water oxidation pretreated wheat straw was created (11) and was used in this study. This model composition of inhibitors consisted only of organic acids, with formate included at the highest concentration. With inhibitors of this model composition, growth-arrested *C. glutamicum* strain R-*ldhA*-pCRA723 retained 97% of its ethanol productivity, indicating a high tolerance to the combination of organic acids (Table 2).

Steam explosion pretreatment has been investigated as one of the attractive pretreatment methods owing to its low use of chemicals and energy consumption (5). The model composition of inhibitors derived from pretreatment of sugarcane bagasse with steam explosion was determined by reference to a previous report (17). This model composition of inhibitors consisted of organic acids, furans, and phenols. As shown in Table 2, growth-arrested strain R-*ldhA*-pCRA723 retained 104% of its ethanol productivity with the composition of inhibitors found with the steam explosion pretreatment. This indicates that despite the anticipated synergy of multiple inhibitors in an actual production method, the method employing growth-arrested *C. glutamicum* strain R-*ldhA*-pCRA723 should retain viable ethanol productivity.

Conclusion. We investigated the effects of inhibitors generated during the pretreatment of a lignocellulosic biomass on an ethanol production method employing growth-arrested *C. glutamicum* strain R-*ldhA*-pCRA723. The method showed high tolerance to all organic acid, furan, and phenolic inhibitors tested, mainly due to the growth-arrested conditions. Furthermore, combinations of inhibitors affected only slightly the relative ethanol productivity of the method employing *C. glutamicum* strain R-*ldhA*-pCRA723, despite the anticipated synergistic effects of multiple inhibitors. In our previous investigations of this method in the absence of inhibitors, a peak volumetric ethanol productivity of 0.62 mol liter^{-1} h^{-1} (7) and a final ethanol concentration of 1.7 M (21) were observed. This indicates that ethanol itself is not an inhibitor. In comparison to concentrations of other ethanol producers, the highest final ethanol concentrations of 2 M (from glucose), 1.1 M (from glucose), and 0.9 M (from xylose) have been reported for *S. cerevisiae* 27817, *E. coli* LY01, and *Z. mobilis* AX 101, respectively (4, 15). Through further optimization, the method employing *C. glutamicum* strain R-*ldhA*-pCRA723 may be developed into an efficient ethanol production method without detoxification steps.

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AUTHOR'S CORRECTION

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