Original article

Acetic Acid Fermentability with *Clostridium thermoaceticum* and *Clostridium thermocellum* of Standard Compounds found in Beech Wood as Produced in Hot-Compressed Water

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Abstract

Acetic acid fermentability of various compounds from beech wood as produced in a two-step hot-compressed water treatment was evaluated by fermentation tests using standard compounds with *Clostridium thermoaceticum* and *Clostridium thermocellum.* For cellulose- and hemicellulose-derived products, the former microorganism was found to ferment compounds with low molecular weights such as monosaccharides, decomposed products and organic acids to acetic acid, while the latter was found to ferment compounds with high molecular weights such as polysaccharides and oligosaccharides to acetic acid. Lignin-derived products were, on the other hand, fermented by both microorganisms to acetic acid. Based on these lines of evidence, co-culture with *C. thermoaceticum* and *C. thermocellum* was evaluated and proven to increase acetic acid fermentability. Consequently, almost all compounds produced from beech wood in hot-compressed water were found to be converted to acetic acid when using these microorganisms in combination. Thus, hot-compressed water treatment coupled with acetic acid fermentation would be a powerful method to produce acetic acid from lignocellulosics.

Keywords

Acetic acid fermentation, *Clostridium thermoaceticum*, *Clostridium thermocellum*, Hot-compressed water, Beech wood

Introduction

Global warming due to the exhaustion of fossil fuels is an increasingly serious problem. Under such circumstances, bioethanol has been much studied as an alternative to fossil fuels because of its low emission of greenhouse gas and overall environmental friendliness. For example, molasses from sugarcane and starch from corn are readily converted to ethanol by yeast. However, it is preferable to use inedible raw materials for bioethanol production. Therefore, the focus has recently been on bioethanol from inedible biomass resources such as lignocellulosics, and numerous studies have been performed on their hydrolysis. However, lignocellulosics do not saccharize into simple sugars as easily as starch or molasses and must be pretreated with an acid catalyst, steam explosion, supercritical water, or enzyme for saccharification to obtain fermentable sugars. The obtained sugars are then fermented with yeast such as *Saccharomyces cerevisiae,* as shown in the following equation:

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$$

Overall, 1 mole of glucose is converted to 2 moles of ethanol and CO_2 by microorganisms under anaerobic conditions; thus, indicating low utilization efficiency of carbon to ethanol. Because of this, some researchers are skeptical as to whether the use of fermentative ethanol can contribute to overall CO_2 reduction. Against this background, our research group has proposed a new process for ethanol production from lignocellulosics by acetic acid fermentation. This new process involves 3 stages: 1) decomposition of lignocellulosics by hot-compressed water, 2) the conversion of decomposed products such as sugars to acetic acid microbiologically under anaerobic condition, and 3) the hydrogenolysis of acetic acid to ethanol by a metal catalyst. From these stages, the following reaction can be derived.

$$C_6H_{12}O_6 + 6H_2 \rightarrow 3CH_3CH_2OH + 3H_2O$$

Here, it should be noted that all carbon atoms that make up the glucose are converted efficiently via acetic acid to bioethanol without releasing any CO_2, reducing 50% more ethanol when compared with the above-mentioned fermentative ethanol[1].

In our previous report on two-step hot-compressed water treatment, it was clarified that Japanese beech (*Fagus crenata*) was converted to various compounds such as oligosaccharides and monosaccharides, some saccharides decomposed products, organic acids and lignin-derived products, and thus failed to saccharize into simple sugars[2]. Therefore, to explore the potential for efficient use of these various compounds, their acetic acid fermentability was evaluated in this study under anaerobic fermentation conditions with *Clostridium thermoaceticum* and *Clostridium thermocellum*. The aim was to establish a new process coupled with hot-compressed water treatment for ethanol production from lignocellulosics by acetic acid fermentation.

Since the characteristics of *C. thermoaceticum* were reported by Fontaine et al.[3]. In 1942, its metabolism in terms of some saccharides[3-7] and aromatic compounds[8-13] have been studied. In addition, *C. thermocellum* is known to ferment cellulose to various compounds such as acetic acid, ethanol, lactic acid and hydrogen[14-19]. Although many studies have been conducted on the fermentability of cellulose and glucose, the fermentabilty of the compounds produced from lignocellulosics in hot-compressed water has not clarified yet. Therefore, in this study, the fermentability of these compounds to acetic acid was evaluated under the same fermentation conditions with *C. thermoaceticum* and *C. thermocellum.*

Materials and methods

Reviving a Freeze-Dried Culture

Freeze-dried cultures of *Clostridium thermoaceticum* (ATCC39073) and *Clostridium thermocellum* (ATCC27405) were obtained from American Type Culture Collection and revived by the following method: first, solutions 1 through 7 were prepared, and the obtained solution was sterilized by an autoclave at 121°C for 20min followed by cooling.

Solution 1:

10g glucose, 100ml distilled water

Solution 2:

5g yeast extract, 0.25g cysteine·HCl·H_2O, 1g $(NH_4)_2SO_4$, 0.25g $MgSO_4 \cdot 7H_2O$, 0.04g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.00024g $NiCl_2 \cdot 6H_2O$, 0.00029g $ZnSO_4 \cdot 7H_2O$, 0.000017g Na_2SeO_3, 0.2ml resazurin (1% solution), 500ml distilled water

Solution 3:

0.415g NaOH, 5g $NaHCO_3$, 4.4g K_2HPO_4, 7.5g KH_2PO_4, 300ml distilled water

Solution 4 :

5g cellobiose, 100ml distilled water

Solution 5:

4.5g yeast extract, 0.25g glutathione, 1.3g $(NH_4)_2SO_4$, 0.13g $MgCl_2 \cdot 6H_2O$, 0.0011g $FeSO_4 \cdot 7H_2O$, 0.13g $CaCl_2 \cdot 2H_2O$, 0.2ml resazurin (1% solution), 250ml distilled water

Solution 6:

1.43g KH_2PO_4, 7.2g $K_2HPO_4 \cdot 3H_2O$, 300ml distilled water

Solution 7:

6g sodium glycerophosphate, 100ml distilled water

On cooling, as soon as the temperature inside of the autoclave reached 100°C

or lower, the solution was moved to a glove box. The inside of the glove box was then flushed with CO_2 gas for *C. thermoaceticum* and N_2 gas for *C. thermocellum*. Subsequently, 10ml of solution 1, 25ml of solution 2 and 15ml of solution 3 were mixed and freeze-dried culture of *C. thermoaceticum* was added, in addition, 10ml of solution 4, 25ml of solution 5, 10ml of solution 6 and 5ml of solution 7 were mixed together with *C. thermocellum*. After dehydration of the freeze-dried culture, 2ml of these solutions containing *C. thermoaceticum* or *C. thermocellum* was poured into a vial, which was then capped with butyl rubber and aluminum seal to keep in the CO_2 gas for *C. thermoaceticum* and N_2 gas for *C. thermocellum.* These vials were then taken out from glove box and placed in an incubator set at 60°C for 4 days. After incubation, the vial was stored at 4°C for use in the subsequent experiments.

Preparation of Inoculum

To prepare the inoculums of *C. thermoaceticum*, solutions 1, 2 and 3 were sterilized and kept in a glove box filled with CO_2 gas, as described above. Subsequently, 5ml of solution 1, 25ml of solution 2 and 15ml of solution 3 were mixed in a 100ml vial, to which 2ml of the solution containing *C. thermoaceticum* revived as described above was added. After being capped with butyl rubber and sealed with aluminum sealer, the vial was incubated with media at 60°C for 80h with magnetic stirring to prepare the inoculum. For *C. thermocellum*, 5ml of solutions 4, 25ml of solution 5, 10ml of solution 6 and 5ml of solution 7 were used instead of solutions 1-3. Using the same procedure as mentioned above, the inoculum of *C. thermocellum* was prepared under N_2 gas.

Acetic Acid Fermentation

Various standard compounds listed in Table 1 which are commercially available were used as substrates for acetic acid fermentation. By changing the loading weight of each substrate in solution 8, the initial concentration of each substrate in the fermentation media was varied as in Table 1.

Solution 8:

Various compounds listed in Table 1, 100ml distilled water

For acetic acid fermentation with *C. thermoaceticum*, 5ml of inoculum, 25ml of solution 2, 15ml of solution 3 and 5ml of solution 8 were mixed in a 100ml vial under CO_2 gas, while for acetic acid fermentation with *C. thermocellum*, 5ml of inoculum, 25ml of solution 5, 10ml of solution 6, 5ml of solution 7 and 5ml of solution 8 were mixed in a similar manner under N_2 gas. For acetic acid fermentation with co-culture of *C. thermoaceticum* and *C. thermocellum*, 2.5ml of inoculum of *C. thermoaceticum*, 2.5ml of inoculum of *C. thermocellum*, 25ml of solution 2, 15ml of solution 3 and 5ml of solution 8 were mixed in a 100ml vial under N_2 gas. Subsequently, after capping and sealing the vial, fermentation was carried out at 60°C for 240h under magnetic stirring.

Analyses

The fermentation medium was filtered through a 0.45μm filter to separate the microorganisms. The obtained filtrate was then analyzed by high performance liquid chromatography (HPLC) with the conditions described below.

To determine the concentrations of oligosaccharides and monosaccharides, an Aminex HPX-87P column (Bio-Rad) was used with a refractive index detector (Shimadzu, RID-10A). The mobile phase was 0.005mol/l H_2SO_4 at a flow-rate of 0.6ml/min. The column oven temperature was set at 45°C.

To measure the concentrations of organic acids and decomposed products of saccharides, an Aminex HPX-87H column (Bio-Rad) was used with a refractive index detector and the column oven temperature set at 85°C. The distilled water was used as mobile phase at a flow-rate of 0.6ml/min.

To measure the concentrations of lignin-derived products, fermentation monitoring column (Bio-Rad) was used with UV detector; the mobile phase 0.01mol/l H_2SO_4 at a flow-rate of 0.8ml/min. The column oven temperature was set at 60°C.

To evaluate the fermentability of the substrates used in this study, their conversion efficiency to acetic acid was estimated using the following equation.

Conversion efficiency (%) = Maximum weight of acetic acid produced (g) / Initial weight of substrate in the fermentation medium (g) × 100

Results and discussion

Fermentation with *C. thermoaceticum*

Figure 1 shows the concentration changes in monosaccharides such as xylose, mannose and glucuronic acid as fermented with *C. thermoaceticum* to yield acetic acid. In the case of xylose, the concentration decreased and became zero after 60h of fermentation time, while acetic acid levels increased and reached a maximum of 7.7g/l. For mannose, the concentration decreased slowly and 7.3g/l of mannose still remained even after 240h of fermentation with a resultant 1.8g/l of acetic acid produced, indicating that the fermentability of mannose is lower than xylose. The fermentability of glucuronic acid was much greater than that of mannose, and it was found that 6.7g/l of glucuronic acid was consumed with 5.6g/l of acetic acid produced. Thus, compared with xylose, a much longer time is necessary for

complete fermentation of mannose and glucuronic acid.

Based on the same test for other relevant compounds at 60°C for 240h fermentation, the conversion efficiency to acetic acid with *C. thermoaceticum* were estimated as shown in Table 1. The results showed that polysaccharides and oligosacchanides were not fermented, but glucose, fructose, and xylose were consumed completely within 72h with high conversion efficiency. Compared to these monosaccharides, glucuronic acid showed lower conversion efficiency. Mannose, galactose, rhamnose and arabinose were unconsumed even after 240h of fermentation, with low conversion efficiencies between 4.2 and 12.6%. However, it is worth mentioning that all the monosaccharides obtained from wood in hot-compressed water are fermentable with *C. thermoaceticum*. On the other hand, polysaccharides such as cellulose and xylan, and oligosaccharides such as cellohexaose, cellobiose and xylotriose, were not fermentable with *C. thermoaceticum*, and only xylobiose was converted to acetic acid.

The decomposed products, 5-hydroxymethylfurfural (5-HMF), furfural, erythrose and glycolaldehyde were found to be fermented to acetic acid with conversion efficiencies ranging from 28.0 to 60.0%, whereas levoglucosan and methylglyoxal could not be converted. Among the three organic acids listed in Table 1, formic acid and lactic acid were fermented to acetic acid.

The lignin-derived products listed in Table 1 could also be utilized as substrates for *C. thermoaceticum* to produce acetic acid. The conversion efficiency, however, varied to a great extent from 3.0 to 58.7%. Figure 2 shows examples of the obtained results in the fermentation of guaiacol and syringaldehyde to acetic acid and by-products. The concentration of acetic acid increased and that of guaiacol decrease as the fermentation proceeded. An increase in the concentration of catechol was simultaneously found with the increase in acetic acid. Meanwhile, in the fermentation of syringaldehyde, acetic acid was produced with a simultaneous production of 3,4,5-trihydroxybenzaldehyde. In addition, 3,4

dihydroxy-5-methoxybenzaldehyde was produced.

Lux et. al. reported that vanillin can be fermented to acetic acid with conversion of its methoxyl group to hydroxyl group[11]. In this study, catechol was produced as in Figure 2 during the fermentation of guaiacol. Therefore, it seems reasonable to assume that a similar reaction would take place in producing acetic acid from lignin-derived products. A possible fermentation pathway for guaiacol and syringaldehyde was, therefore, proposed as shown in Figure 3. Although acetic acid is a target product in this research, these aromatic compounds with hydroxyl groups are valuable as useful chemicals derived from lignocellulosics.

Fermentation with *C. thermocellum*

Table 1 also shows the conversion efficiency of various compounds fermented with *C. thermocellum*. The conversion efficiency for the fermentation of cellulose was the same as that for glucose. Although acetic acid was not produced from xylan, xylotriose and xylobiose with *C. thermocellum*, these compounds were found to be hydrolyzed to xylooligosaccharides, xylobiose and xylose. Since *C. thermocellum* is reported to show xylanase activity[20,21], xylan must be hydrolyzed to xylooligosaccharides and xylose.

Oligosaccharides from cellulose such as cellohexaose and cellobiose were consumed completely, but the conversion efficiency of cellohexaose was lower than that of glucose. However, in the fermentation of cellobiose, the conversion efficiency for all fermentation products were higher than those of glucose. Monosaccharides such as glucose, mannose, fructose, and glucuronic acid were fermented and acetic acid, lactic acid, formic acid and ethanol were produced. For fructose, the conversion efficiency to lactic acid was lower, while that for ethanol was higher, compared to glucose, mannose and glucuronic acid. However, a significant difference among these monosaccharides could not be found in their

conversion efficiency to acetic acid, which was around 18.0%.

Among various decomposed products, methylglyoxal, 5-HMF, furfural and erythrose were found to be fermented to acetic acid with various conversion efficiencies ranging from 3.3 to 25.0%. However, levoglucosan and glycolaldehyde could not be converted to acetic acid. Additionally, none of the organic acids tested were fermented with *C. thermocellum*.

It was also found that, although the conversion efficiency varied, *C. thermocellum* could convert lignin-derived products. In the fermentation of syringaldehyde, for example, the same intermediate and fermentation products were found as in the fermentation with *C. thermoaceticum*. Thus, lignin-derived products are thought to be possibly converted to acetic acid with *C. thermocellum* by the similar fermentation pathway to that shown in Figure 3.

Fermentation as Co-Cultured with *C. thermoaceticum* and *C. thermocellum*

From the fermentation with *C. thermoaceticum* or *C. thermocellum* as described above, *C. thermoaceticum* was found to be preferable for fermenting low molecular weight compounds such as monosaccharides, decomposed products, and organic acids. *C. thermocellum*, on the other hand, was found to be suitable for fermenting high molecular weight compounds such as polysaccharides and oligosaccharides. Therefore, various compounds in Table 1 were fermented in a co-culture with *C. thermoaceticum* and *C. thermocellum*. The obtained conversion efficiency is also shown in Table 1. All compounds except for levoglucosan and glycolic acid were found to be converted to acetic acid.

Neither *C. thermoaceticum* nor *C. thermocellum* could ferment xylan or xylotriose to acetic acid. However, as mentioned above, *C. thermocellum* hydrolyzes these to xylobiose and xylose, and *C. thermoaceticum* can ferment these products to acetic acid. This is why acetic acid could be obtained from xylan

and xylotiose by applying co-culture fermentation with *C. thermoaceticum* and *C. thermocellum*.

During fermentation with *C. thermocellum* as shown in Table 1, not only acetic acid but also some organic acids such as lactic acid and formic acid were produced as by-products. However, the obtained organic acids could be converted to acetic acid by *C. thermoaceticum* in the co-culture fermentation. Therefore, for example, in the co-culture fermentation of cellulose, the conversion efficiency became higher than that with *C. thermocellum* alone.

Figure 4 shows the conversion pathway of various compounds from beech wood extracted in a two-step hot-compressed water treatment in co-culture with *C. thermoaceticum* and *C. thermocellum.* In this way, almost all compounds can be converted to acetic acid by exploiting the different characteristics of *C. thermoaceticum* and *C. thermocellum*. Therefore, the co-culture fermentation system is effective in producing acetic acid from various compounds produced in the two-step hot-compressed water treatment of beech wood.

In the conventional method of producing bioethanol from lignocellulosics with yeasts, substrates are limited to hexoses such as glucose, mannose, galactose only. Xylose and glucuronic acid, which are abundant in hardwoods and herbaceous plants are not generally fermentable. However, in this study, all monosaccharides were found to be convertible to acetic acid. In addition, oligosaccharides, some decomposed compounds, and lignin-derived compounds are also convertible to acetic acid.

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Table 1. Conversion efficiency for various compounds as fermented by *Clostridium thermoaceticum*, *Clostridium thermocellum,* and their co-culture.

Substrate		Initial concentration of substrate (g/l)	*C. thermoaceticum*		*C. thermocellum*					Co-culture	
			Substrate consumed (g/l)	Conversion efficiency (%)	Substrate consumed (g/l)	Conversion efficiency (%)				Substrate consumed (g/l)	Conversion efficiency (%)
						Acetic acid	Lactic acid	Formic acid	Ethanol		
Poly-saccharides	Cellulose	10.0	-	0.0	-	18.7	9.3	2.1	9.9	-	60.5
	Xylan	10.0	-	0.0	-	0.0	0.0	0.0	0.0	-	6.8
Oligo-saccharides	Cellohexaose	10.0	0.0	0.0	10.0	14.2	1.4	0.8	7.4	10.0	35.7
	Xylotriose	10.0	0.0	0.0	3.1	0.0	0.0	0.0	0.0	7.7	8.2
	Cellobiose	10.0	0.0	0.0	10.0	20.8	23.9	2.9	15.6	10.0	48.9
	Xylobiose	10.0	5.8	13.3	3.0	0.0	0.0	0.0	0.0	7.0	9.5
Mono-saccharides	Glucose	10.0	10.0	76.5	6.7	18.2	8.6	1.5	10.3	10.0	75.0
	Mannose	10.0	2.7	12.6	4.5	18.0	7.5	0.0	7.2	4.0	18.2
	Galactose	10.0	2.3	10.9	0.0	0.0	0.0	0.0	0.0	2.3	17.9
	Fructose	10.0	10.0	77.3	8.0	19.0	4.3	0.0	16.9	10.0	76.8
	Rhamnose	10.0	0.9	4.2	0.0	0.0	0.0	0.0	0.0	1.2	2.3
	Arabinose	10.0	3.0	8.4	0.0	0.0	0.0	0.0	0.0	3.9	12.8
	Xylose	10.0	10.0	76.5	0.0	0.0	0.0	0.0	0.0	10.0	67.6
	Glucuronic acid	10.0	6.7	48.0	5.1	18.0	9.4	0.0	6.7	9.4	49.3
Decomposed products	Levoglucosan	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Methylglyoxal	1.0	0.0	0.0	1.0	3.3	7.2	0.0	13.6	1.0	1.8
	5-HMF	0.1	0.1	60.0	0.1	15.0	0.0	0.0	20.0	0.1	41.0
	Furfural	0.1	0.1	30.0	0.1	25.0	10.0	10.0	0.0	0.1	8.1
	Erythrose	1.0	1.0	42.0	1.0	6.6	9.6	0.0	19.5	1.0	29.6
	Glycolaldehyde	1.0	1.0	28.0	0.0	0.0	0.0	0.0	0.0	1.0	5.5
Organic acids	Glycolic acid	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Lactic acid	1.0	0.6	65.0	0.0	0.0	0.0	0.0	0.0	0.7	84.9
	Formic acid	1.0	1.0	40.0	0.0	0.0	0.0	0.0	0.0	1.0	51.5
Lignin-derived products	Coniferylalcohol	0.1	0.1	5.0	0.1	3.0	0.0	0.0	0.0	0.1	21.0
	Sinapylalcohol	0.1	0.1	3.0	0.1	65.0	0.0	0.0	0.0	0.1	65.9
	Coniferylaldehyde	0.1	0.1	15.0	0.1	37.8	0.0	0.0	0.0	0.1	59.0
	Sinapylaldehyde	0.1	0.1	5.0	0.1	50.0	0.0	0.0	0.0	0.1	81.6
	Vanillin	1.0	1.0	39.0	0.06	6.7	0.0	0.0	11.4	1.0	28.2
	Syringaldehyde	1.0	1.0	47.6	0.47	28.5	0.0	0.0	8.7	1.0	16.7
	Guaiacol	1.0	1.0	58.2	0.15	31.4	0.0	0.0	0.0	1.0	16.6
	Syringol	1.0	1.0	58.7	0.08	4.0	0.0	0.0	11.4	1.0	45.1

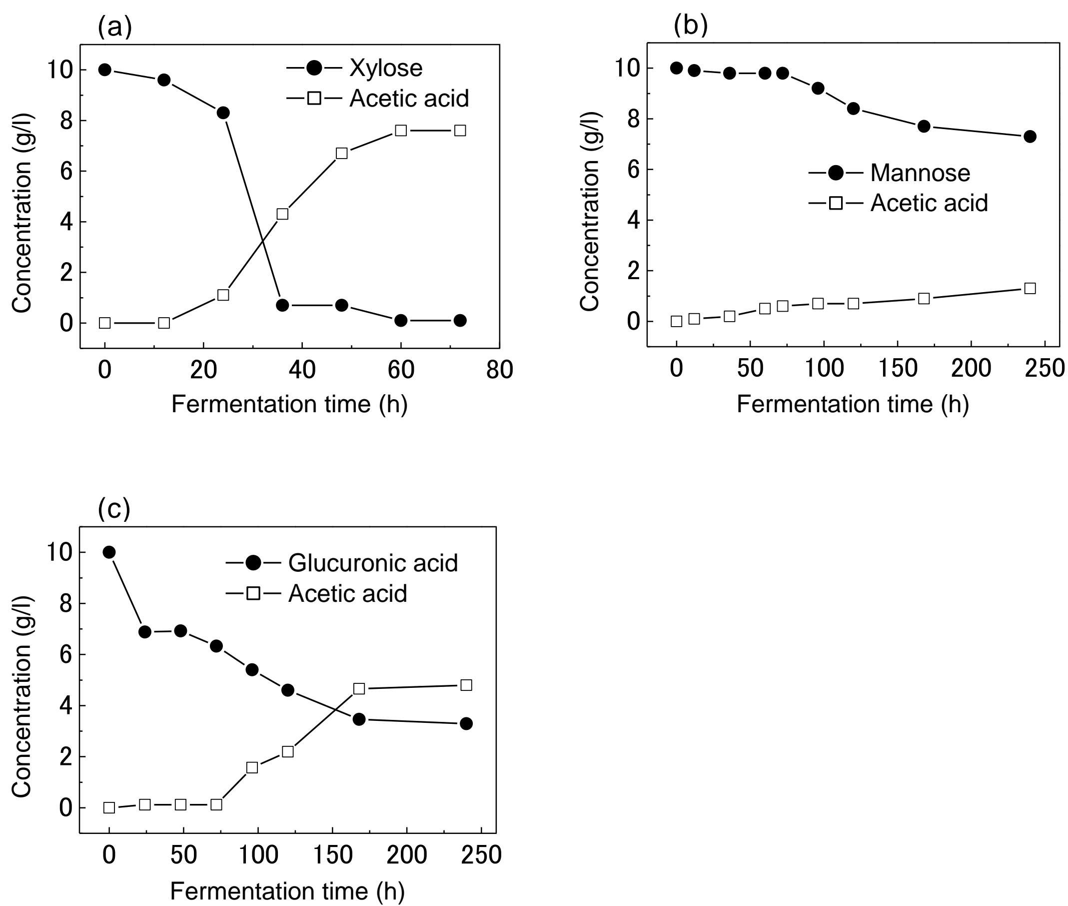


Fig. 1. Concentration changes on fermentation with *Clostridium thermoaceticum* of (a) xylose, (b) mannose, and (c) glucuronic acid to give acetic acid as a product.

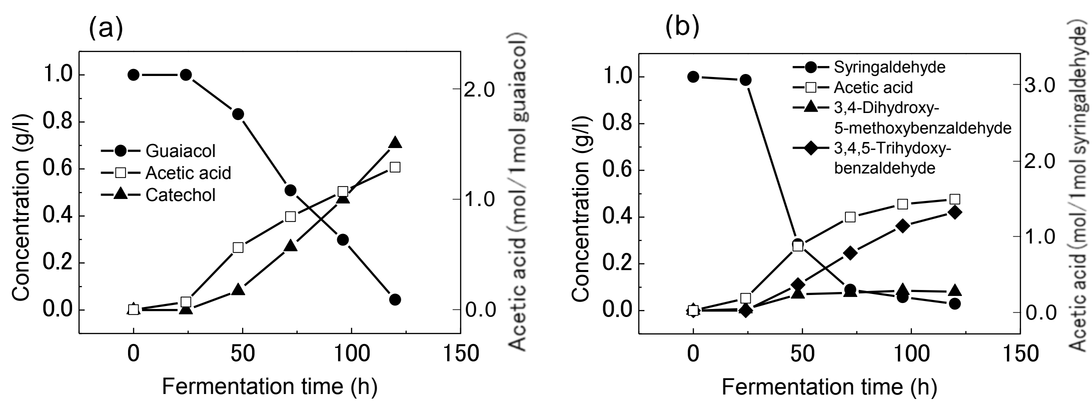
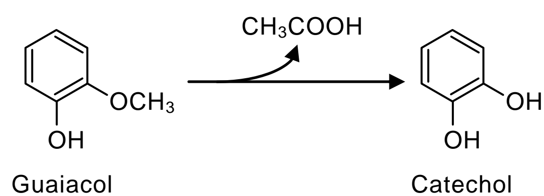


Fig. 2. Concentration changes on fermentation with *C. thermoaceticum* of (a) guaiacol and (b) syringaldehyde to give acetic acid and by-products.

(a) Guaiacol



(b) Syringaldehyde

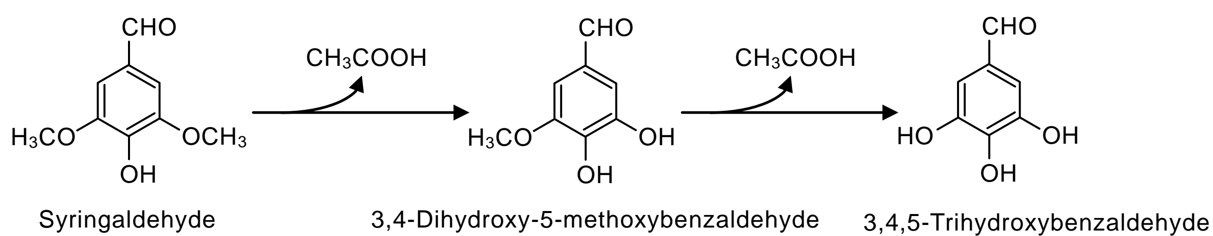


Fig. 3. Possible fermentation pathway to produce acetic acid from (a) guaiacol and (b) syringaldehyde.

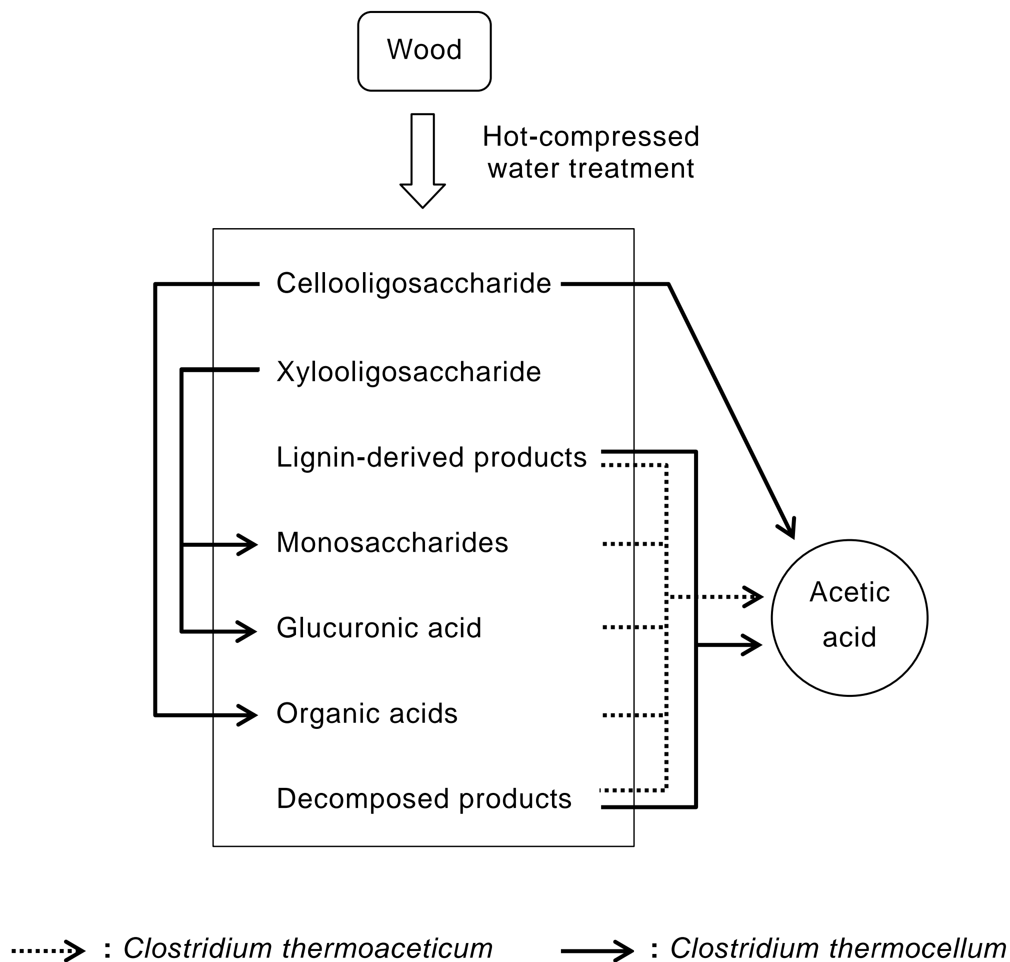


Fig. 4. Conversion pathway of various compounds obtained by hot-compressed water treatment of wood in the co-culture fermentation system.