Impact of Heavy Metal Ions on the Simultaneous Saccharification and Fermentation of Formosan Alder Biomass to Form Lactic Acid

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Formosan alder (*Alnus formosana*) is a fast-growing, adaptable, pioneer native tree species in Taiwan, and it is particularly suitable for reforestation. In this study, steam-exploded Formosan alder biomass was employed to investigate lactic acid production by simultaneous saccharification and fermentation (SSF) in the presence of different heavy metals. Impacts of added heavy metals on saccharification processing were investigated. In the presence of 1410 mg Cr^{6+}/L, negative impacts were observed for SSF. The same level of Cr^{6+} adversely affected fermentation by *Lactobacillus casei* and *L. acidophilus* compared to the blank controls. Positive impacts for SSF by Cd^{2+} were demonstrated with 108 mg Cd^{2+}/L, and the same conditions favored fermentation by *L. casei* and *L. acidophilus*. No impacts for SSF by Pb^{2+} up to 6830 mg Pb^{2+}/L were found for both *Lactobacillus* strains. This study demonstrates that SSF for production of lactic acid from Formosan alder biomass was able to tolerate a wide range of heavy metal concentration regimes. Hence, this study provides an alternative use for biomass harvested from phytoremediation sites. Such biomass can be used as sustainable regenerative biomaterial, and thereby it can further enhance the benefits of environmental remediation.

Keywords: Alnus formosana; Heavy metals; Lactobacillus; Enzyme hydrolysis; Lactic acid

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INTRODUCTION

Some heavy metals are naturally present in soils at trace levels (*e.g.*, Cd, Cr, Cu, Ni, Pb, and Zn), but higher levels can be reached due to industrial-related activities. The release of an enormous amount of potentially toxic compounds has resulted from improper management and disposal of heavy metals (Yu *et al.* 2019). Soil contamination by heavy metals has raised concerns over public health and the ecosystem because of their carcinogenic and mutagenic effects. Phytoremediation is highly regarded as an alternative method for soil and groundwater pollution remediation globally. However, there is a need to explore its application for biomass harvested from polluted sites after remediation.

Formosan alder (*Alnus formosana*) is a medium-sized deciduous tree endemic to Taiwan, where it grows fast and often forms pure stands in eroded or denuded areas. It is also a shade-intolerant pioneer tree species that grows along river banks and in other open habitats (Wang and Chien 2016). Formosan alder is a nitrogen fixing species, and it is also an ideal tree species for afforestation of eroded areas and for soil and water conservation (Wang and Chien 2016).

Lactic acid is a naturally occurring organic acid that can be used in a wide variety of industries, such as the cosmetic, pharmaceutical, chemical, food, and most recently, the medical industries (Komesu *et al.* 2017). *Lactobacillus plantarum* (MF042018) exhibits a high degree of resistance to both nickel (500 ppm) and chromium (100 ppm) with multiple antibiotic resistance index above 0.5, and so it can be applied as a promising biosorbent for the removal of heavy metals from industrial wastewaters (Ameen *et al.* 2020). About 350,000 tons of lactic acid are produced globally every year. It also serves as a monomer in the synthesizing of polylactic acid, which is an increasingly important biodegradable and biocompatible polymer (Djukić-Vuković *et al.* 2019; Le Guenic *et al.* 2019). Various types of biomass, like cassava bagasse, wheat straw, rice straw, and sugarcane bagasse, have been used to produce lactic acid by simultaneous saccharification and fermentation processes (Unrean 2018; Aulitto *et al.* 2019; Tu *et al.* 2019; Chen *et al.* 2020; Rawoof *et al.* 2020). It has generally been observed that pH, nutrient source, substrate, and temperature significantly affect the lactic acid production (Abdel-Rahman *et al.* 2011). Pretreatment, hydrolysis, and fermentation are major steps for producing ethanol and lactic acid from lignocellulosic materials (Tu *et al.* 2019; Ajala *et al.* 2020; Cubas-Cano *et al.* 2020; Ko *et al.* 2020). Among the pretreatment methods, steam explosion is a physico-chemical method used for the pretreatment process that is cost effective and requires relatively low energy (Kumar *et al.* 2020). Steam explosion has been regarded as an effective process to increase accessible surface area to assist further enzyme hydrolysis (Kumar *et al.* 2020).

Previous studies have shown that the biomass harvested after phytoremediation could be utilized for bioethanol production (Ko *et al.* 2017; Geiger *et al.* 2019; Wu *et al.* 2020). Ko *et al.* (2017) found that the enzymatic hydrolysis efficiency for Zn-polluted biomass was 90% of the unpolluted biomass, while it was 77% for Cd, and approximately the same for Cr. The fermentation efficiency of the heavy metal containing biomass was higher than the control biomass. Geiger *et al.* (2019) indicated that hydrolysates produced from Cu exposed biomass achieved a significantly greater ethanol yield and volumetric productivity compared to those of the control biomass. Wu *et al.* (2020) also showed that the Cd-accumulated biomass showed greatly enhanced enzymatic saccharification and bioethanol production by significantly increasing cellulose accessibility and lignocellulose porosity. Therefore, both the phytoremediation of heavy metal contaminated soil and value-added cellulosic production in biomass have the potential to fulfill the goal of green remediation and to contribute to sustainability.

There are three ways in which heavy metals can influence cellulase activity in biochemical reactions: (1) complexation; (2) combination with active groups; and (3) reaction with the enzyme (Karaca *et al.* 2010). This study considered lactic acid production from Formosan alder biomass after the growth of the trees had been employed for phytoremediation of heavy metal contaminated soil. The ions of Cu, Cd, and Cr were added to simulate the schemes for the utilization of Formosan alder biomass harvested after phytoremediation. Then, steam-exploded Formosan alder biomass was subjected to SSF for lactic acid production in the presence or absence of the metal ions. *Lactobacillus casei* and *L. acidophilus* were used in the SSF processes. The impact of heavy metal presence on saccharification and SSF processes were assessed.

EXPERIMENTAL

Lactobacilli Strains

The lactobacilli strains *Lactobacillus casei* (ATCC 334) and *Lactobacillus acidophilus* (ATCC 4356) were bought from Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). Both strains are facultative anaerobic with an optimal growth temperature of 37 °C. *Lactobacillus casei* and *Lactobacillus acidophilus* need distinctive periods of time until the glucose production is exhausted. *Lactobacillus casei* is known to require 72 h, and *Lactobacillus acidophilus* needs 144 h. Other characteristics of two *Lactobacillus* species are as reported (Hammes and Hertel 2006).

The seed culture of bacteria was cultivated in de Man, Rogosa, and Sharpe (MRS) medium, which contained 20 g/L glucose, 10 g/L beef extract, 10 g/L proteose peptone, 5 g/L yeast extract, 2 g/L ammonium citrate, 5 g/L sodium acetate, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L $MnSO_4 \cdot H_2O$, 2 g/L K_2HPO_4, and 1 g/L polysorbate 80 (Tween 80). For inoculum preparation, a separate colony was transferred to a 100-mL Schoot bottle containing 100 mL MRS medium supplemented with 2 % (v/v) glucose at 37 °C for 24 h.

Formosa Alders Biomass

Fifteen-year-old *A. formosana* was collected from NTU Experimental Forest of central Taiwan. The bark was removed, wood chips were prepared (width 3 cm, length 4 cm, and thickness 0.5 cm), and the chips were milled using a RT-08 grinder. The 60- to 40-mesh samples were collected to determine the chemical compositions content by the following standard methodology: moisture content as per TAPPI T258 om-06 (2006), solvent extractive of wood and pulp as per TAPPI T204 cm-07 (2007), holocellulose as per TAPPI T249 cm-09 (2009), Klason lignin as per CNS 14907 (2005), pentosans as per TAPPI T223 cm-01 (2001), and ash content as per TAPPI T211 om-02 (2002).

A. formosana wood chips were pretreated by steam explosion using 1.5% sulfuric acid. Biomass was air-dried for 24 h and then soaked in 1.5% sulfuric acid. The sulfuric acid steam explosion was conducted with a dried solid to liquid ratio of 1:7 at 180 °C for 10 min.

Enzyme Assays and Lactic Acid Fermentation

First, 1.5% acid steam-exploded *Alnus formosana* was hydrolyzed with cellulase complex CTec (Novozyme, Bagsværd, Denmark). The enzyme loadings were equivalent to 1, 5, 10 IU endoglucanase (CMCase)/mL. Enzyme suspension volumes of 0.4, 1, and 2 mL were used to deliver 1, 5, and 10 IU/g α-cellulose. Hydrolysis was conducted in a total volume of 100 mL MRS medium, and 2 g samples in a 100-mL conical flask. The flasks were water bathed at 37 °C, shaken at 100 rpm, and the samples were analyzed by high-performance liquid chromatography (HPLC; Jasco RI-930, Tokyo, Japan).

Next, 5 IU/g α-cellulose CTec, 1.5% (w/w) dried steam exploded *Alnus formosana* biomass and 2.5 mL inoculum solution were added to fermentation broth with designated heavy metal concentration. Lactic acid fermentation was conducted at 37 °C.

The heavy metal ions investigated were chromium (Cr^{6+}), copper (Cu^{2+}), and cadmium (Cd^{2+}), which were prepared as aqueous solutions of $K_2Cr_2O_7$, $CuSO_4 \cdot 5H_2O$, and $CdSO_4$ for respective concentrations for enzymatic hydrolysis and fermentation. All reagents used were analytical grade or equivalent.

Analytical Assay

Lactic acid was chromatographed on a HPLC system equipped with a reflective index detector (Jasco RI-810), column oven (Col Box), and auto-sampler (JASCO AS-950). Before injection, the samples were filtered using a 0.22-µm membrane filter. Filtered samples (20 µL) were injected into the HPLC system. Lactic acid samples were eluted using 0.009 M H_2SO_4 and double distilled water as the mobile phase, separately. The 300 mm × 7.8 mm column (9 µm ReproGel H, Dr. Maisch GmbH, Germany) was used at 70 °C with a flow rate of 0.7 mL/min. The results are the averages of triplicate testing.

RESULTS AND DISCUSSION

Impacts of Cr^{6+}

The presence of Cr^{6+} was found to have different impacts on the cellulase activities, enzyme hydrolysis of biomass, and glucose fermentation and SSF of both lactobacilli strains, as shown in Fig. 1. The top panel (Fig. 1a) shows that 14.14 and 141.4 mg/L of Cr^{6+} positively impacted cellulase activities, with 116.0 and 106.1% relative cellulase activities demonstrated. However, the presence of increasing Cr^{6+} showed a decreasing trend, with 92.2% relative cellulase activities demonstrated at 1414 mg Cr^{6+}/L. The presence of Cr^{6+} did not noticeably impact the extent of enzyme hydrolysis of pretreated biomass. Figure 1(a) shows that relative efficiencies of enzyme hydrolysis of biomass were more than 100% for all three concentrations of Cr^{6+}. The presence of heavy metal ions can provide additional sites for enzyme-substrate binding or block the catalytic sites to deter enzyme hydrolysis. The results show that the promoting effect was more dominant than the inhibiting effect at a lower Cr^{6+}concentration.

The middle panel of Fig. 1b shows the relative efficiencies of lactic acid conversions from glucose and biomass SSF by *L. casei*. Trends for the impact of the presence of Cr^{6+} on relative lactic acid conversions from glucose and biomass SSF by *L. casei* were similar for the range of Cr^{6+}employed in this study. Below 100 mg Cr^{6+} /L, the presence $Cr^{6+}$of did not adversely impact the relative efficiencies of lactic acid conversions from glucose and biomass SSF by *L. casei*. At 1414 mg Cr^{6+}/L, relative lactic acid conversions from glucose and biomass SSF by *L. casei* dropped below 20%. Relative efficiencies of enzyme activity and the extent of enzyme hydrolysis of pretreated biomass at 1414 mg Cr^{6+}/L were still around 100%, as shown in Fig. 1(a). Hence, the above observation suggested that the stress of Cr^{6+} might adversely impact the physiology of *L. casei*. Consistent drops of relative lactic acid conversions from both glucose and biomass SSF by *L. casei* suggested that the physiological role *L. casei* played was the deciding factor of the aforementioned trend.

The bottom panel of Fig. 1c shows the relative efficiencies of lactic acid conversions from glucose and biomass SSF by *L. acidophilus*. Trends for the impact of the presence of Cr^{6+} on relative lactic acid conversions from glucose and biomass SSF by *L. acidophilus* were quite different for the range of Cr^{6+}emoplyed in this study. Below 707.01 mg Cr^{6+}/L, the presence $Cr^{6+}$of did not adversely impact the relative efficiencies of lactic acid conversions from glucose by *L. acidophilus*. At 141.4 mg Cr^{6+}/L, relative lactic acid conversion from biomass SSF by *L. acidophilus* dropped below 35.8 %. Because relative enzyme activities and lactic acid conversions from glucose at this Cr^{6+}concentration regime remained, the above finding suggested that the Cr^{6+} might form a complex with byproducts from biomass SSF blocking lactic acid conversion from biomass SSF by *L. acidophilus*.

The above finding demonstrates that *L. casei* can be applied to perform SSF lactic acid conversion from pretreated biomass from presence of less than 100 mg/L Cr^{6+} from a practical perspective.

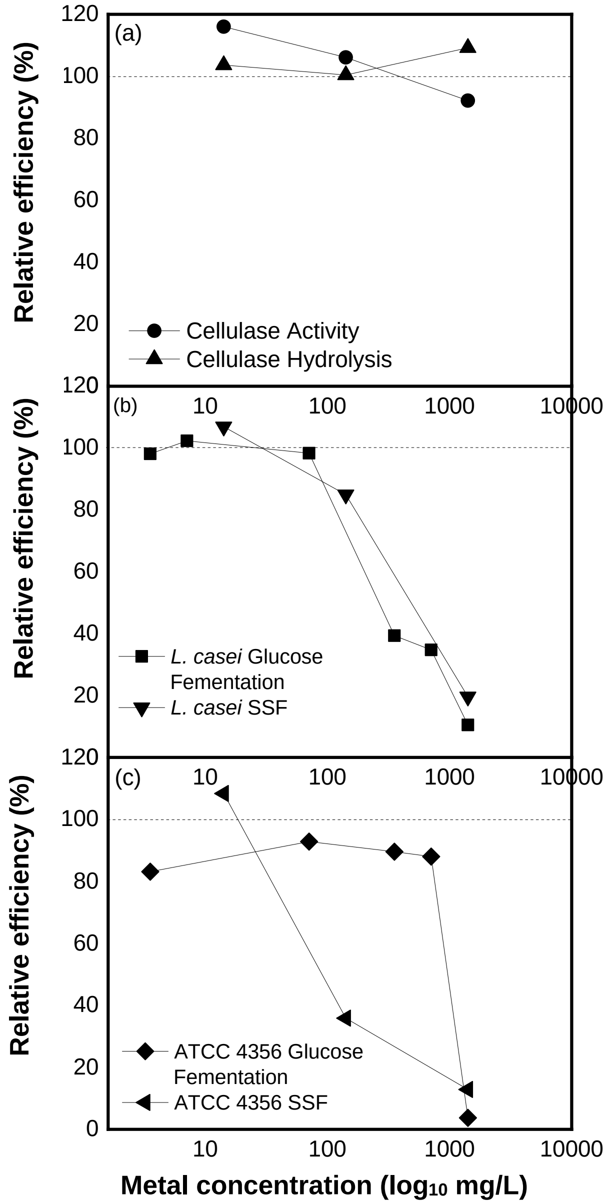

Fig. 1. Impacts of Cr^{6+} on (a) cellulase activities and enzyme hydrolysis of biomass, (b) glucose fermentation, and (c) SSF of both lactobacilli strains

Impacts of Cu^{2+}

The presence of Cu^{2+} exhibited different effects on cellulase activities, enzyme hydrolysis of biomass, glucose fermentation, and SSF of both lactobacilli strains, as shown in Fig. 2. The top panel of Fig. 2a shows that 1.02 and 10.18 mg/L of Cu^{2+} positively impacted cellulase activities, with 105.6 and 110.9% relative cellulase activities demonstrated. However, the presence of increasing Cu^{2+}inhibited enzyme activity with 92.2% relative cellulase activities demonstrated at 101.8 mg Cu^{2+}/L. The presence of Cu^{2+}

did not remarkably impact the extent of enzyme hydrolysis of pretreated biomass. Figure 2a shows that the relative efficiencies of enzyme hydrolysis of biomass were more than 100% for all three concentrations of Cu^{2+}. Nonetheless, increasing Cu^{2+} increasingly inhibited enzyme hydrolysis of biomass, with 101.0% relative cellulase activities demonstrated at 101.8 mg Cu^{2+}/L. The presence of heavy metal ions can provide additional sites for enzyme-substrate binding or block the catalytic sites to deter enzyme hydrolysis. The results show that the promoting effect was more dominant than the inhibiting effect at a lower Cu^{2+}concentration. However, for higher Cu^{2+}concentration, inhibiting effects gradually dominated.

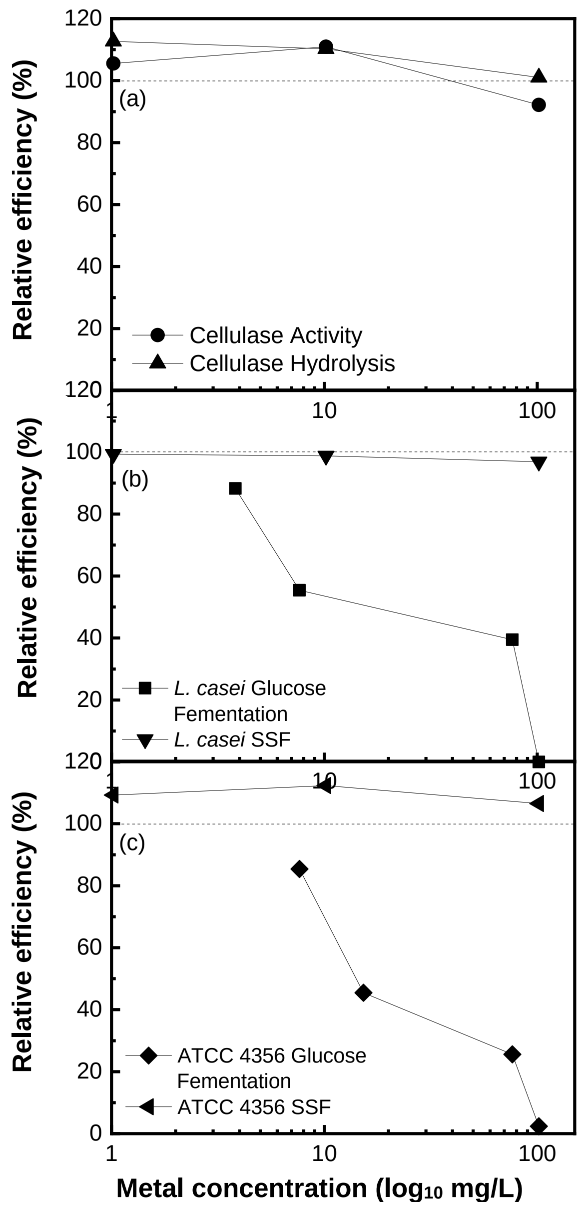

Fig. 2. Impacts of Cu^{2+} on (a) cellulase activities and enzyme hydrolysis of biomass, (b) glucose fermentation, and (c) SSF of both lactobacilli strains

The middle panel of Fig. 2b shows the relative efficiencies of lactic acid conversions from glucose and biomass SSF by *L. casei*. Trends for the impact of the presence of Cu^{2+} on relative lactic acid conversions from glucose and biomass SSF by *L. casei* differed greatly for the range of Cu^{2+} employed in this study. From 1.02 to 101.8 mg Cu^{2+}/L, the relative efficiencies of lactic acid conversions from biomass SSF by *L. casei* decreased from 99.3 to 96.9%. The presence of Cu^{2+} at the range employed in this study did not remarkably impact relative efficiencies of lactic acid conversions from biomass SSF by *L. casei*, although the relative efficiencies of lactic acid conversions from biomass SSF was reduced with increasing Cu^{2+}concentration.

However, the impact of the presence of Cu^{2+} on relative lactic acid conversions from glucose and *L. casei* was more remarkable than those from biomass SSF for the range of Cu^{2+} employed in this study (Fig. 2c). From 3.82 to 76.35 mg Cu^{2+}/L, the relative efficiencies of lactic acid conversions from biomass SSF by *L. casei* decreased from 88.2 to 39.4%. The presence of biomass surfaces might provide additional binding sites for Cu^{2+}, preventing Cu^{2+} from reaching the surface of *L. casei*. Relative efficiencies of lactic acid conversions from biomass SSF by *L. casei* were similar to those of enzyme activities and hydrolysis of biomass. The above findings suggested that the roles played by biomass surfaces might assist in protecting the relative efficiencies of lactic acid conversions from biomass SSF by *L. casei* from detrimental effects of Cu^{2+}. Performances of lactic acid conversions from biomass SSF by *L. acidophilus* were superior to those by *L. casei* in the presence of Cu^{2+}.

The above finding demonstrates that both *L. acidophilus* and *L. casei* can be applied to perform SSF lactic acid conversion from pretreated biomass in the presence of less than 100 mg/L of Cu^{2+} from a practical perspective.

Impacts of Cd^{2+}

The presence of Cd^{2+} exhibited different effects on cellulase activities, enzyme hydrolysis of biomass, glucose fermentation, and SSF of both lactobacilli strains, as shown in Fig. 3. The middle panel of Fig. 3a shows that 1.02, 10.18, and 107.8 mg/L of Cd^{2+} positively impacted cellulase activities, with 135.3, 140.4, and 124.2% relative cellulase activities demonstrated, respectively. The presence of Cd^{2+} did not significantly decrease the extent of enzyme hydrolysis of pretreated biomass. Figure 3a shows that 1.02, 10.18, and 107.8 mg/L amounts of Cd^{2+} affected the extents of enzyme hydrolysis of pretreated biomass, with 98.4, 117.0, and 117.7% relative values. The presence of heavy metal ions can provide additional sites for enzyme-substrate binding or block the catalytic sites to deter enzyme hydrolysis. The results showed that the promoting effect was more dominant than the inhibiting effect at a higher Cd^{2+} concentration.

The middle panel of Fig. 3b shows the relative efficiencies of lactic acid conversions from glucose and biomass SSF by *L. casei*. Trends for the impact of the presence of Cd^{2+} presence on relative lactic acid conversions from glucose and biomass SSF by *L. casei* differed for the range of Cd^{2+} employed in this study, although the difference was not as large as in the presence of Cu^{2+}. From the range between 1.08 to 107.85 mg Cu^{2+}/L, the relative efficiencies of lactic acid conversions from biomass SSF by *L. casei* changed from 103.9, 107.5 to 106.6%. The difference between the impacts of the presence of Cd^{2+} on the relative efficiencies of lactic acid conversions from biomass SSF was smaller than the those from glucose by *L. casei*. From 8.09 and 21.57 to 53.92 mg Cu^{2+}/L, the relative efficiencies of lactic acid conversions from glucose by *L. casei* changed from 103.9 and 87.7 to 86.5%, respectively.

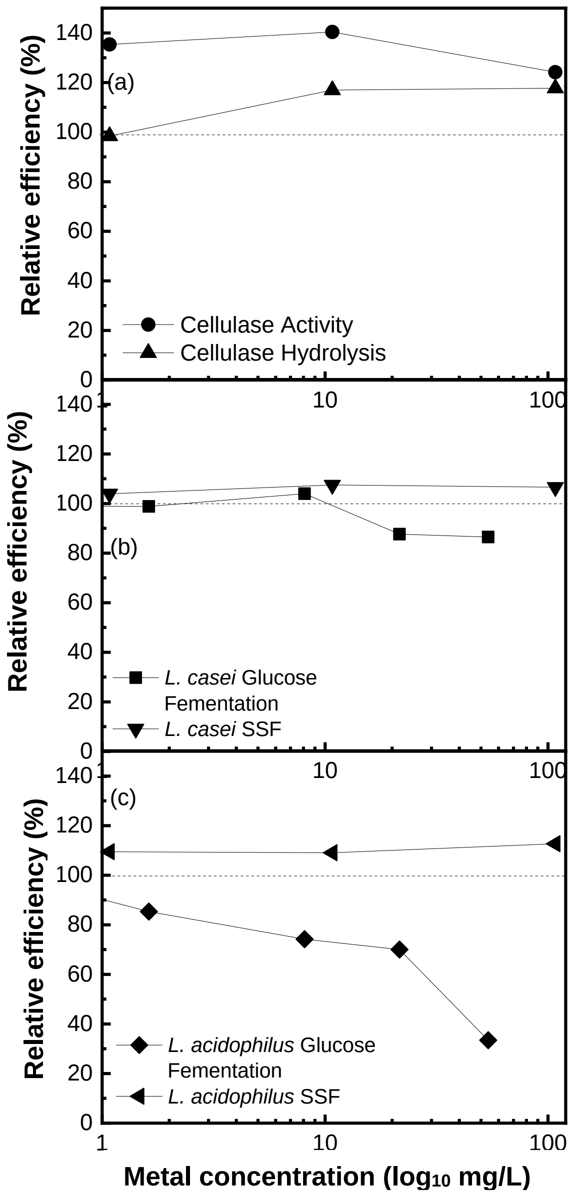

Fig. 3. Impacts of Cd^{2+} on (a) cellulase activities and enzyme hydrolysis of biomass, (b) glucose fermentation, and (c) SSF of both lactobacilli strains

The bottom panel of Fig. 3c shows the relative efficiencies of lactic acid conversions from glucose and biomass SSF by *L. acidophilus*. The differences in the impact of the presence of Cd^{2+} on relative lactic acid conversions from glucose and biomass SSF by *L. acidophilus* were much larger than those of *L. casei*. From 8.09 and 21.57 to 53.92 mg Cu^{2+}/L, the relative efficiencies of lactic acid conversions from glucose by *L. acidophilus* changed from 74.2% and 70.0% to 33.4%. The impacts of the presence of Cd^{2+} on relative lactic acid conversions from glucose by *L. acidophilus* were larger than those of *L. casei*. Differences of the impact of the presence of Cd^{2+} on relative lactic acid conversions from glucose and biomass SSF were much larger than those of Cu^{2+} for both *Lactobacillus* strains. The above findings can assist in finding optimal schemes for biomass conversions under the presence of different metals.

CONCLUSIONS

1. According to the dosage concentration for inhibition of lactic acid production, higher tolerance of cellulase to the presence of heavy metals was observed in the order Cd^{2+} > Cu^{2+} > Cr^{6+}.
2. The yield of lactic acid was the greatest at a Cd^{2+} concentration of 107.8 mg/L and the worst at a Cr^{6+} concentration of 1414.0 mg/L.
3. Different lactobacilli strains possess a variety of tolerance against each heavy metal. Fundamental data obtained from this study may assist designing optimal utilizing schemes for biomass harvested after phytoremediation.
4. The results of this study validate the viabilities of biochemical conversion processes for fermentable sugars and lactic acid from biomass harvested after phytoremediation, thereby further enhancing the benefits of environmental remediation.

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