J Ind Microbiol Biotechnol (2016) 43:13–23
DOI 10.1007/s10295-015-1700-z

BIOENERGY/BIOFUELS/BIOCHEMICALS

Degradation of phenolic compounds by the lignocellulose deconstructing thermoacidophilic bacterium *Alicyclobacillus Acidocaldarius*

John E. Aston[1] · William A. Apel[1] · Brady D. Lee[2] · David N. Thompson[1] · Jeffrey A. Lacey[1] · Deborah T. Newby[1] · David. W. Reed[1] · Vicki S. Thompson[1]

Received: 5 March 2015 / Accepted: 1 October 2015 / Published online: 5 November 2015

Abstract *Alicyclobacillus acidocaldarius*, a thermoacidophilic bacterium, has a repertoire of thermo- and acid-stable enzymes that deconstruct lignocellulosic compounds. The work presented here describes the ability of *A. acidocaldarius* to reduce the concentration of the phenolic compounds: phenol, ferulic acid, ρ-coumaric acid and sinapinic acid during growth conditions. The extent and rate of the removal of these compounds were significantly increased by the presence of micro-molar copper concentrations, suggesting activity by copper oxidases that have been identified in the genome of *A. acidocaldarius*. Substrate removal kinetics was first order for phenol, ferulic acid, ρ-coumaric acid and sinapinic acid in the presence of 50 μM copper sulfate. In addition, laccase enzyme assays of cellular protein fractions suggested significant activity on a lignin analog between the temperatures of 45 and 90 °C. This work shows the potential for *A. acidocaldarius* to degrade phenolic compounds, demonstrating potential relevance to biofuel production and other industrial processes.

Keywords *Alicyclobacillus acidocaldarius* · Thermophiles · Phenolics · Bioremoval

Introduction

Alicyclobacillus acidocaldarius is a thermoacidophilic bacterium capable of deconstructing lignocelluloses, hemicelluloses and celluloses as documented in research [2, 17, 41, 42], reviews on the topic [4, 5, 15] and in components of several patent [33–35]. Based on the ability of *A. acidocaldarius* to deconstruct hemicellulose and cellulose, we were interested in investigating its ability to deconstruct or lower the concentration of lignin derivatives, specifically the phenolic compounds: phenol, ferulic acid, ρ-coumaric acid and sinapinic acid, as these compounds represent the base derivatives of lignin groups.

Phenol and its derivatives are aromatic compounds that occur naturally in the environment, most commonly within lignocellulosic polymers, which are estimated to cycle 200 billion tons of carbon annually [9]. The lignin polymer is the most significant component of lignocellulose by mass, and second only to cellulose in its contribution to living terrestrial biomass [11]. Lignin is an amorphous, polyphenolic material constructed from the enzyme-mediated polymerization of three phenylpropanoid monomers, coniferyl, ρ-coumaryl and sinapyl alcohols [12]. Lignin also serves to protect the more simple polymers, hemicellulose and cellulose, from being quickly degraded by bacteria and fungi. Due to the amount of fixed carbon bound in lignin matrices globally, it has received a significant attention as a compound important to biofuel applications [25]. To make biomass-to-fuel processes more economically feasible, it is important that technologies be developed that effectively remove lignin and its inhibitory derivatives, primarily phenolic compounds, from the energy rich, fermentable sugars that comprise the hemicellulose and cellulose.

Electronic supplementary material The online version of this article (doi:10.1007/s10295-015-1700-z) contains supplementary material, which is available to authorized users.

✉ John E. Aston
john.aston@inl.gov

[1] Biological and Chemical Processing Department, Idaho National Laboratory, Idaho Falls, ID, USA

[2] Energy and Environment Directorate, Pacific Northwest National Laboratory, Richland, WA, USA

In addition to being naturally ubiquitous, phenol and phenolic compounds are also produced during industrial processes, including petroleum refining, plastic manufacturing, resin production, pesticide production, steel manufacturing and the production of paints and varnish [3, 19]. Because phenolic compounds are water soluble, they are highly mobile in waste waters generated during these processes [10], and may be distributed to downstream river, lake, and soil sites, where they can accumulate to higher concentrations and cause ecological concerns [13]. Because of this, several techniques have been developed to remove phenol and its derivatives from industrial effluents and contaminated waters with bioremediation receiving the most attention due to its low environmental impact and relatively low cost [26]. To this end, the removal of phenol has been observed using bacterial and fungal species including *Candida tropicalis* [30], *Acinetobacter calcoaceticus* [24], *Alcaligenes eutrophus* [18], *Pseudomonas putida* [22] and *Burkholderia cepacia* G4 [31].

With respect to the degradation and removal of phenol and phenolic compounds, genes for five putative multi-copper oxidases (GenBank Accession Numbers: ACV 59461.1; ACV 59462.1; ACV 58146.1; ACV 58141.1 and ACV 59388.1) have been identified in the sequenced genome of *A. acidocaldarius* [21]. These genes and select properties are shown in the supplemental files. Multi-copper oxidase such as laccase and laccase-like multi-copper oxidases (LMCO) are copper-containing genes that have been shown to act as polyphenol or phenol oxidases [27]. The presence of the genes for these enzymes prompted the addition of copper to certain media sets to determine any effects on the removal of phenolic compounds by *A. acidocaldarius*.

The work presented here builds on these and other efforts by reporting the ability of *A. acidocaldarius*, a thermoacidophile, to degrade phenol and its common derivatives, ferulic acid, ρ-coumaric acid and sinapinic acid under aerobic growth conditions. The findings presented suggest that *A. acidocaldarius* and/or enzymes produced by *A. acidocaldarius* may have applications in removing phenolic compounds from processes or waste streams at high temperatures and low pHs, conditions common for many bioenergy and industrial processes, including biomass preconversions, lignin deconstruction/solubilization and the mediation of potentially inhibitory phenolic compounds that are produced during the conversion of lignocellulose to fermentable sugars during biofuel applications.

Materials and methods

Culture medium and inoculum preparation

Chemicals were obtained from Fisher Chemical (Fairlawn, NJ, USA). *A. acidocaldarius* (ATCC 27009) was cultured in a basal mineral medium that contained the following (in g L^{-1}): $CaCl_2{\cdot}2H_2O$, 0.25; $MgSO_4{\cdot}7H_2O$, 0.5; $(NH_4)_2SO_4$, 1.3; Fe(III) ·EDTA, 0.047; KH_2PO_4, 3.0 and xylose, 4.17 (30 mM). The xylose was withheld from substrate-free controls. In addition, 1 mL L^{-1} each of trace mineral stock solutions A, B, and C was added. Stock solution A contained (in g L^{-1}): $MgCl_2$, 25.0; $CaCl_2{\cdot}2H_2O$, 6.6; H_3BO_3, 0.58; $Fe(III)Cl_3{\cdot}6H_2O$, 5.0; $CoNO_3{\cdot}6H_2O$, 0.05; and $NiCl_2{\cdot}6H_2O$, 0.02. Stock solution B contained (in g L^{-1}): $MnSO_4{\cdot}H_2O$, 2.0; $ZnSO_4{\cdot}7H_2O$, 0.5; and Na_2MoO_4, 0.025. Stock solution C was a vitamin solution that contained (in g L^{-1}): pyridoxine hydrochloride, 0.08; folic acid, 0.012; thiamine hydrochloride, 0.13; riboflavin, 0.042; nicotinamide, 0.084; ρ-aminobenzoate, 0.088; biotin, 0.01; cyanocobalamin, 0.0004; D-pantothenic acid calcium, 0.086; myo-inositol, 0.021; choline bromide, 0.053; orotic acid sodium salt, 0.021; and spermidine, 0.1. After the basal salts medium and stock solutions were mixed, the medium pH was adjusted to 4.0 using 6 N HCl and the medium was filtered (0.2 μm) under sterile conditions. Phenolic compounds and copper sulfate (as a potential enzyme co-factor) were added prior to filtration. The medium was then incubated at 60 °C for up to 1 h to solublilize the phenolic compounds.

Prior to each experiment, *A. acidocaldarius* cells preserved at −80 °C in 1-mL vials at 10 % dimethyl sulfoxide (DMSO) were introduced as the initial inoculum. Cultures were grown in 125-mL Erlenmeyer flasks (40 mL medium volume), fitted with foam stoppers, and shaken at 120 rotations per minute (rpm) in an Innova 4230 temperature controlled incubator (New Brunswick, Edison, NJ, USA) at 60 °C. These initial cultures were allowed to grow into the late-exponential phase, when 0.4 mL aliquots were collected, concentrated via centrifugation (7000×*g*), and washed in substrate-free medium, before being inoculated into fresh medium. This growth and re-inoculation process was repeated two times to allow the cells to adapt to the experimental conditions. Cell growth was monitored by measuring optical density at 595 nm using an UV–Vis V160u spectrophotometer (Shimadzu, Columbia, MD, USA). All experiments were performed in triplicate and average values and 95 % confidence intervals were calculated.

Measurement of phenolic compounds

An Agilent 7100 Capillary Electrophoresis (Foster City, CA, USA) instrument equipped with a diode-array detector was used to measure the concentrations of phenol, ferulic acid, ρ-coumaric acid and sinapinic acid. Data acquisition and analysis were accomplished using Agilent ChemStation software. Methods developed by Bonoli et al. [6] were used, with the exception that the conditioning steps using

HPCE-grade (Fisher Optima) and buffer solution were done for 5 min, rather than 2 min. This change resulted in more defined peaks when several consecutive samples were run. The running buffer used was 45 mM sodium tetraborate (pH 9.6). Samples were injected at the anodic end in a low pressure mode (3.4 kPa) for 3 s. Separation was carried out using a voltage of 27 kV for 10 min with a capillary temperature of 30 °C. The resulting current was 110 μA. Before each injection, the capillary was rinsed in high pressure mode (137.9 kPa) with 0.1 N HCl for 2 min, HPCE-grade water for 2 min, and then re-equilibrated with the running buffer for 2 min. After each cycle, the capillary was rinsed with HPCE-grade water for 2 min. All rinses were conducted at 30 °C. Phenol, ferulic acid, ρ-coumaric acid and sinapinic acid eluted at approximately 7.0, 5.2, 5.9 and 3.8 min, respectively. Sample concentrations were determined from correlations calculated using analytical grade standards. Using these methods, the detection limits for phenol, ferulic acid, ρ-coumaric acid and sinapinic acid were approximately 10 μM.

Collection of protein fractions

To isolate protein fractions, cells grown under conditions identical to those used in the phenolic removal experiments were harvested by centrifuging 40 mL-culture aliquots at 7000×*g* for 15 min. The protein isolation method described here was adopted from Zambrano et al. [40]. The supernatant was decanted and the pellet was washed with 5 mL of a 25 mM phosphate buffered saline (PBS) solution. This process was repeated twice before 5 μL of DNase/RNase and 20 μL of a protease inhibitor cocktail were added to the samples, which were then frozen using liquid nitrogen, and allowed to thaw. The freeze–thaw application was repeated twice. Samples were then transferred to a 15 mL conical-bottom tube and sonicated twice for 1 min. Visual inspection using a light microscope at 1000× suggested significant cell lysis. The samples were allowed to incubate at room temperature for 15 min, and then centrifuged at 15,000×*g* for 35 min at 4 °C. The supernatant was collected and, if necessary, saved at −80 °C. The pellet was then re-suspended in a 0.5 M NaCl solution with sodium acetate added to a concentration of 20 mM. DNase/RNase and protein inhibitor cocktails were added at 5 and 20 μL, respectively. This solution was centrifuged at 15,000×*g* for 25 min and the supernatant was added to the fraction collected previously. Collectively, this fraction was considered to predominantly contain soluble proteins, such as those often concentrated in the cytoplasm. The pellet was again re-suspended in 200 μL of 0.4 % triton X-100 in 25 mM PBS and centrifuged for 25 min at 15,000×*g*. The resulting supernatant was collected and considered to predominantly contain membrane-associated proteins. The remaining pellet was re-suspended in 200 μL of PBS and collected as the insoluble, membrane-bound, fraction. The protein concentrations in these fractions were measured using a Bradford assay kit (Bio-Rad, Hercules, CA, USA), and diluted to approximately 10 μg μL^{-1} in the reaction buffers. It should be noted that this method was used to affect some degree of fractionation between cytoplasmic/soluble proteins and membrane-bound proteins. The fractions are denoted as such, but this was a qualitative effort and the original cellular location of the separated proteins was not confirmed.

Enzyme assays

Each of the enzyme reactions was conducted at 60 °C and stirred at 200 rpm in a heated, temperature controlled cell within an Evolution 600 UV–vis spectrophotometer (Thermo Scientific, Waltham, MA, USA). The absorbance of the various assay-dependent oxidation products was measured with time, and data were collected and analyzed using VisionPro software (Visionpro, Calgary, Canada). The following assays were used:

Laccase activity

The enzyme solution (0.1 mL) was added to 0.1 mL sodium acetate (0.1 M, pH 5.0) and 0.8 mL of 0.3 % (w/v) 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). The oxidation product of ABTS was monitored with time at 420 nm.

Manganese peroxidase

0.5 mL of the enzyme solution was added to 0.1 mL of 0.1 % (w/v) phenol red, 0.1 mL of 0.25 M sodium lactate, 0.2 mL of 0.5 % (w/v) bovine serum albumin, 0.05 mL of 2 mM $MnSO_4$ and 0.05 mL of 100 mM H_2O_2 in 20 mM sodium succinate (pH 4.5). The oxidation of phenol red was monitored at 610 nm.

Lignin peroxidase

0.5 mL of the enzyme solution was added to 0.5 mL H_2O_2, 0.5 mL of 10 mM veratryl alcohol and 1.0 mL of 125 mM sodium tartrate (pH 3.0). The reaction mixture was monitored at 310 nm to measure H_2O_2 oxidation.

Modeling copper solubility and speciation

Visual MINTEQ (version 2.53) was used to predict the speciation of copper sulfate that was added to sets of growth media as a potential enzyme co-factor. This modeling was done to approximate the amount of soluble and free-form

copper that might be biologically available to the cells during growth. To determine the likely speciation of the media components, MINTEQ solved the thermodynamic equilibrium solutions using activities from the Debye–Huckel equation and the default MINTEQA2 thermodynamic database. The temperature was set to 60 °C and the free-proton concentration was calculated from the pH, which was fixed at 4.0. This method resulted in a prediction that over 99 % of the copper added would exist in the free-divalent form with respect to abiotic considerations.

Statistics and calculations

For each experiment, tests were performed in triplicate and average values and 95 % confidence intervals were calculated. The 95 % confidence intervals were calculated using a t-distribution assumption, which resulted in somewhat larger error bars than what would have been observed had a normal distribution of data variance been assumed. This is a more conservative estimate, and likely more accurate assumption, based on the limited number of replicates.

Results

Effect of copper concentration on cell growth and phenolic compound removal

When *A. acidocaldarius* was grown with xylose as the carbon and energy source, copper in the growth medium appeared to extend the lag phase of growth from between three and six hours without copper, to over nine hours when 100 μM copper was added (Fig. 1). In addition, the rate of cell growth and total cell yield decreased by approximately 20–30 % (calculations not shown). However, when copper was added to a concentration of 10 μM, there appeared to be no adverse effect on the total cell yield or rate of growth. Adding copper to 25 or 50 μM decreased total cell yields by less than 10 %, but did not have an appreciable effect on the rate of cell growth. The time required for the cultures to reach stationary phase ranged from 24 h at copper concentrations of 0 and 10 μM to approximately 30 h at copper concentrations of 25, 50 and 100 μM.

The amount of copper in the culture media affected the rates and extent of phenolic compound removal by *A. acidocaldarius* (Fig. 2). In the absence of copper, phenol was degraded from a concentration of 0.52 ± 0.01 mM to 0.18 ± 0.03 mM in 24 h (Fig. 2a). However, when copper was added to an initial concentration of 100 μM, phenol was reduced to a concentration of 0.10 ± 0.02 mM in 6 h, and was completely removed (to below the detection limit) within 24 h. Copper had similar effects on the removal of ferulic, ρ-coumaric and sinapinic acids. For example, no statistically significant removal was measured in cultures containing ferulic acid when copper was absent; however, when copper was added to a concentration of 100 μM, ferulic acid concentrations decreased from 0.50 ± 0.03 mM to 0.29 ± 0.01 mM over 48 h (Fig. 2b). Similar observations were made of cultures containing ρ-coumaric and sinapinic acid (Fig. 2c, d), although some removal of sinapinic acid was observed in the absence of copper, from 0.51 ± 0.01 mM to 0.43 ± 0.01 mM over 48 h. Experiments using substrate-free (live cells) and heat-killed cells with and without copper showed no statistically significant decrease in phenolic compound concentrations (data not shown), suggesting that the decreases in phenolic concentrations are not due to cellular sorption.

Initial removal rates were calculated from mass balance equations and plotted against the copper concentrations used in each experiment (Fig. 3). The relationships

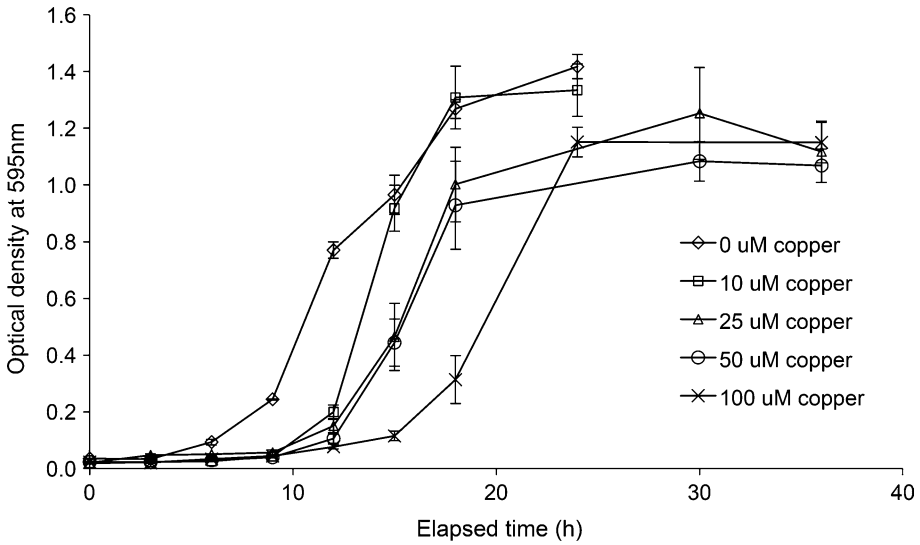

Fig. 1 Growth of *Alicyclobacillus acidocaldarius* in the presence of copper (0–100 μM). The values shown represent the averages of triplicate experiments ±95 % confidence intervals

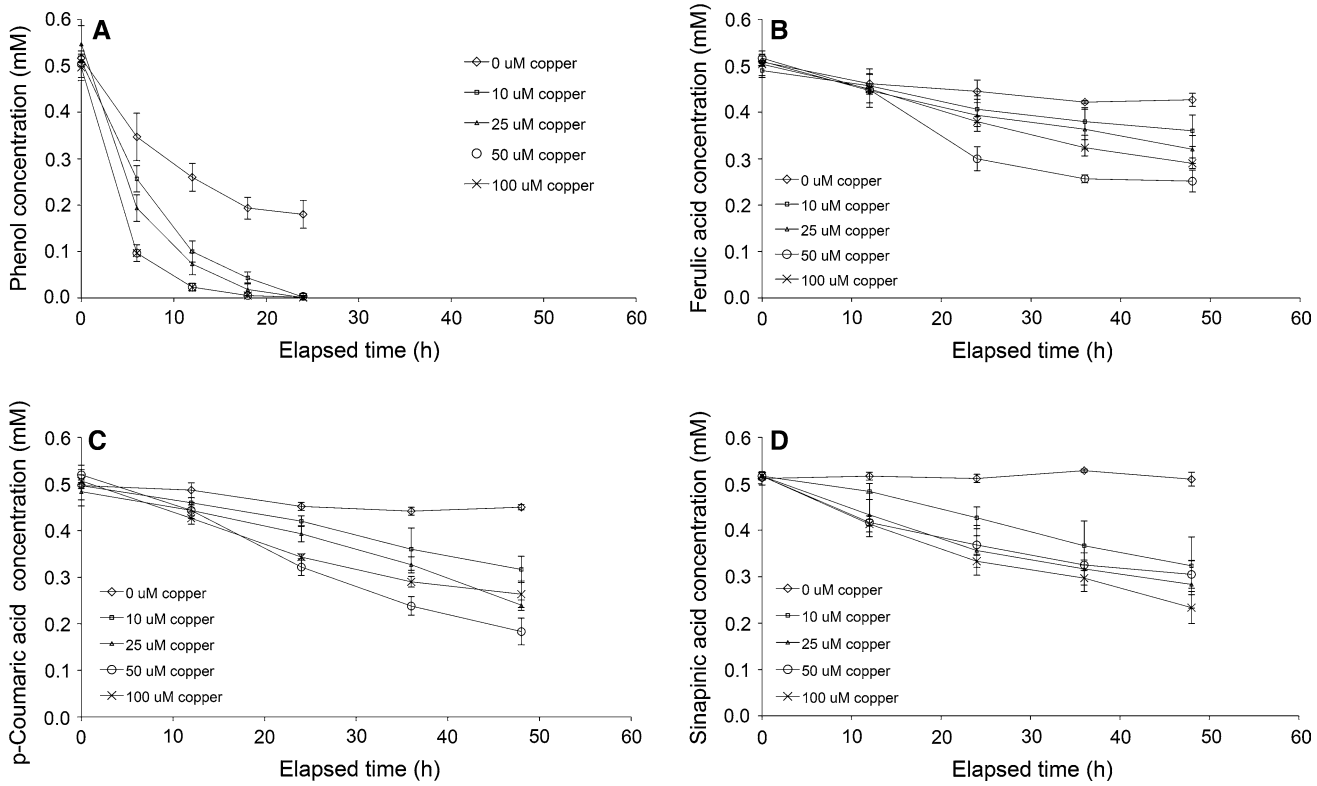

Fig. 2 Removal of **a** phenol, **b** ferulic acid, **c** ρ-coumaric acid and **d** sinapinic acid by a growing culture of *Alicyclobacillus acidocaldarius* in the presence of copper (0, 10, 25, 50 or 100 μM). The phenolic compounds were each added at an initial concentration of 0.5 mM. The values shown represent the averages of triplicate experiments ±95 % confidence intervals

resembled first-order Michaelis–Menten substrate kinetics, with the exception that relatively low reaction rates were observed in the absence of copper. The V_{max} and K_m values for each phenolic compound (phenol, ferulic acid, ρ-coumaric acid and sinapinic) were determined graphically with respect to medium copper concentration using Lineweaver–Burk plots, and are shown in Table 1. While K_m and V_{max} were significantly greater for phenol than the phenolic acids, they were statistically equivalent among ferulic, ρ-coumaric and sinapinic acid. Of note, these data show that the removal rates of phenolic compounds increase with copper concentration up to approximately 50 μM. Using higher copper concentrations did not result in statistically higher rates of removal.

Effect of initial concentrations of phenolic compounds on cell growth and on the removal of phenolic compounds

The extent and Michaelis–Menten relationships of phenolic compound removal were observed for *A.acidocaldarius* with respect to the initial concentrations of phenolic compounds (Figs. 4, 5). In addition, the inhibitory effects on the growth of *A. acidocaldarius* cultured in the presence of varying concentrations of phenol, ferulic acid, ρ-coumaric acid and sinapinic acid (Fig. 4) are described here. As with copper, the data show that phenol increases the time of lag-phase growth, from less than 6 h in the absence of phenol to between 6 and 12 h when phenol is present in concentrations up to 5 mM. In addition, there is a statistically significant effect on the total cell yield and growth rate when phenol is added to a concentration of 5 mM, but little or no effect when added to lower concentrations. Ferulic acid, ρ-coumaric acid and sinapinic acid also increase the duration of lag-phase growth from under 6 h in the absence of these compounds to over 18 h when these compounds are added to a concentration of 750 μM. Although there is little effect of these compounds on the total cell yield, the rate of growth decreased steadily with increasing initial concentrations.

The rate and extent of removal for each of the compounds tested increased with initial concentration. In each of these experiments, the copper concentration was set at 50 μM and, like copper, the relationship between phenolic compound removal and initial concentration appears to be first order in nature. When added to concentrations of 0.5, 1.0 or 2.0 mM, phenol was completely degraded within 24 h. When added to an initial concentration of 5.0 mM, it was degraded to 0.95 ± 0.20 mM after 24 h. Between 25 and 50 % of the

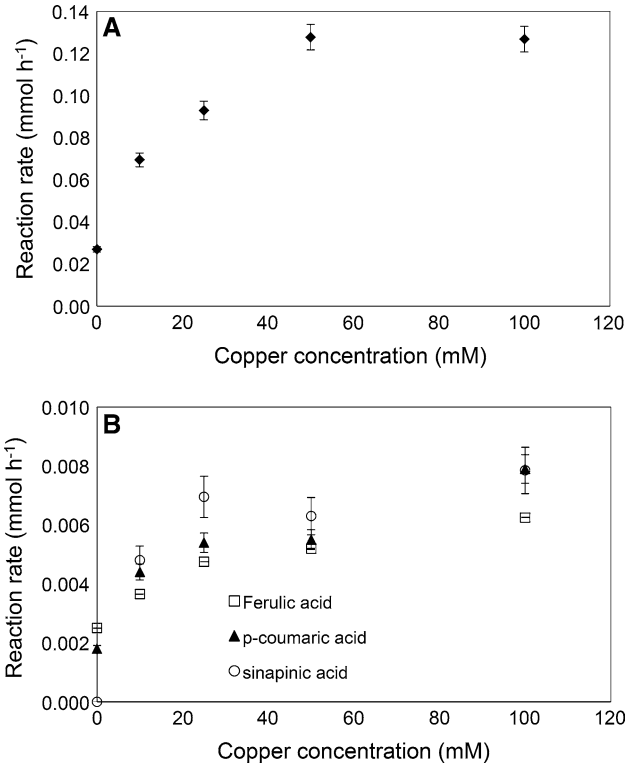

Fig. 3 Removal of **a** phenol and **b** ferulic acid, ρ-coumaric acid and sinapinic acid by *Alicyclobacillus acidocaldarius* in the presence of copper. The initial concentration for each of the phenolic compounds was 0.5 mM. The values shown represent the averages of triplicate experiments ±95 % confidence intervals

Table 1 K_m (half-saturation constant) and V_{max} (maximum reaction rate) as determined by Michaelis–Menten kinetics in a Linewever Burk plot, with respect to the initial copper concentration

	K_m (mM)	V_{max} (mmol h^{-1})
Phenol	11.2 ± 2.8	0.145 ± 0.306
Ferulic acid	6.03 ± 2.04	0.006 ± 0.002
ρ-Coumaric acid	3.60 ± 1.87	0.006 ± 0.002
Sinapinic acid	5.51 ± 1.80	0.008 ± 0.002

These data describe the dependency of enzyme activity on the copper concentration (half-saturation constant) and the maximum potential reaction rate of the enzyme (maximum reaction rate) with increasing copper concentration. These parameters are one way to describe the importance of copper concentrations in the reactions that remove each of the compounds tested here (see Fig. 3). The initial concentration of each compound was 0.5 mM. The values shown represent the averages of triplicate experiments ± 95 % confidence intervals. The confidence intervals suggest that the K_m and V_{max} data for phenol are clearly significantly higher than those for the phenolic acids. However, there is no statistically significant difference in the K_m or V_{max} data among the phenolic acids

initial ferulic acid, ρ-coumaric acid and sinapinic acid were degraded over a 36 to 72 h period when added to initial concentrations between 250 and 750 μM. However, when added to an initial concentration of 100 μM, each of these compounds was nearly completely degraded within 36 h. Phenol, ferulic acid, ρ-coumaric acid and sinapinic acid show first-order relationships with respect to their initial concentration (Fig. 5). The K_m and V_{max} values for phenolic removal were calculated with respect to the phenolic concentrations, as described previously, and are shown in Table 2. Again, the K_m and V_{max} values for phenol removal were significantly higher than those for ferulic, ρ-coumaric and sinapinic acid, which were not statistically different from each other within 95 % confidence.

Enzyme assay activity

The enzyme activities of collected protein fractions were calculated from assays designed to measure substrate oxidation by laccase-like, lignin peroxidase-like and manganese peroxidase-like enzymes (Table 3). These fractions were collected using methods designed to isolate the cytoplasmic/soluble, membrane-associated, and membrane-bound proteins. However, as the proteins in these fractions were not characterized, their true origin should be considered unknown, but with likely affinities towards either cytoplasmic origins, membrane-associated origins or membrane-bound origins. In each case, the addition of copper to the laccase assay increased the enzyme activity several fold. This activity was largely relegated to the membrane-associated and membrane-bound fractions.

Because of the fundamental mechanisms attributed to lignin and manganese peroxidases, these assays were carried out in the absence of copper. Some activity was measured in the soluble fraction, and a very small, nearly statistically insignificant amount was observed from the membrane-associated fraction. No activity was observed in the membrane-bound fraction for either lignin or manganese peroxidase.

Laccase activity from the membrane-associated fraction was the most significant activity measured, and as this was a copper-dependent effect, it may be relevant to the removal of phenolics observed in the whole-cell experiments. Because of this, the laccase assays were repeated with the membrane-associated fraction between 45 and 90 °C to measure the effect of temperature on this reaction. Activities (normalized to the maximum observed activity) of 0.32 ± 0.15, 1.00 ± 0.07, 0.97 ± 0.04, and 0.23 ± 0.08 at 45, 60, 75 and 90 °C, respectively were measured (data shown in the supplemental material).

Discussion

A. acidocaldarius is able to reduce the concentration of phenolic compounds as a function of both initial copper

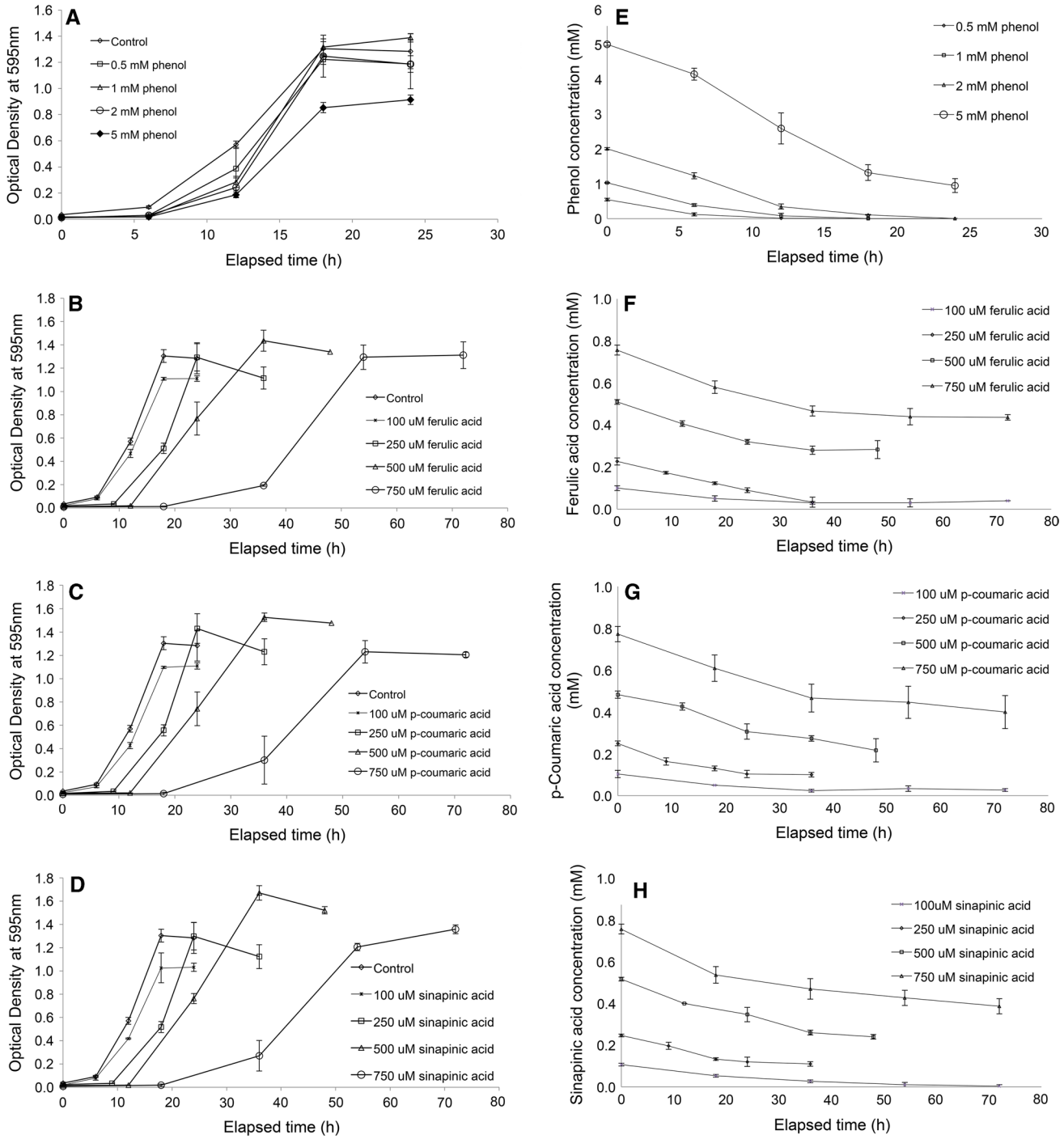

Fig. 4 *Alicyclobacillus acidocaldarius* growth and removal of phenol (**a**, **e**), ferulic acid (**b**, **f**), ρ-coumaric acid (**c**, **g**) and sinapinic acid (**d**, **h**) in the presence of 50 μM copper. The values shown represent the averages of triplicate experiments ±95 % confidence intervals

and initial phenolic compound concentrations. Relevant to these findings, and the potential applications, the data show that although some growth inhibition results from the applicable concentrations of these compounds, this inhibition does not appear to interfere significantly with the removal of phenol, ferulic acid, ρ-coumaric acid or sinapinic acid. In addition, the majority of the substrate is metabolized before the cultures reached the late-exponential growth phase. These two findings suggest that the enzymes needed to carry out the removal of phenolic compounds were not limited by the relatively low cell concentrations and slowed growth during the initial conditions tested here. The

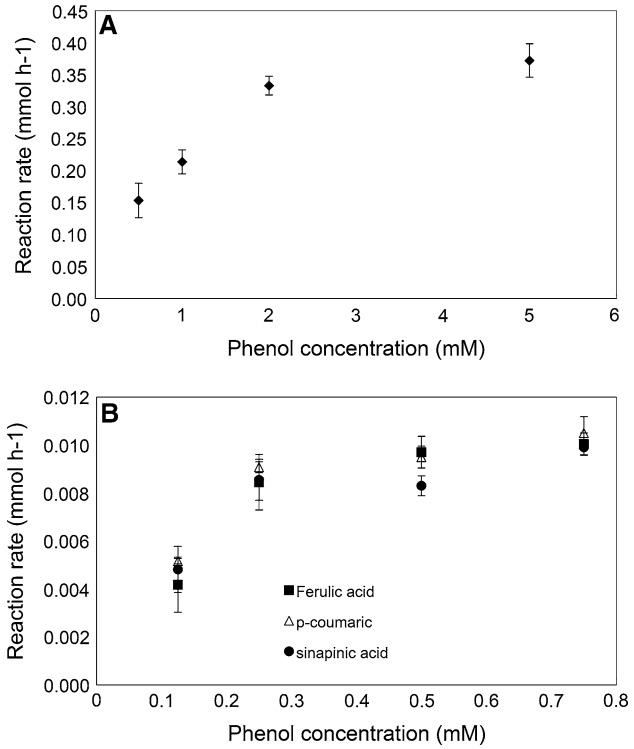

Fig. 5 Reaction rates for the removal of **a** phenol and **b** ferulic acid, ρ-coumaric acid and sinapinic acid by *Alicyclobacillus acidocaldarius* in the presence of copper at 50 μM. The values shown represent the averages of triplicate experiments ±95 % confidence intervals

Table 2 K_m (half-saturation constant) and V_{max} (maximum reaction rate) as determined by Michaelis–Menten kinetics in a Linewever Burk plot, with respect to initial substrate concentration

	K_m (mM)	V_{max} (mmol h^{-1})
Phenol	1.01 ± 0.13	0.460 ± 0.031
Ferulic acid	0.37 ± 0.09	0.017 ± 0.004
ρ-Coumaric acid	0.21 ± 0.06	0.014 ± 0.003
Sinapinic acid	0.20 ± 0.05	0.013 ± 0.003

These data describe the dependency of enzyme activity on the substrate concentration (half-saturation constant) and the maximum potential reaction rate of the enzyme (maximum reaction rate) with increasing substrate concentrations. These parameters are one way to describe the effects of substrate concentration on the reactions that remove the compounds tested here (see Fig. 5). The initial copper concentration in each test was 50 μM. The values shown represent the averages of triplicate experiments ± 95 % confidence intervals. The confidence intervals suggest that the K_m and V_{max} data for phenol are clearly significantly higher than those for the phenolic acids. The K_m value for ferulic acid also appears to be higher than the K_m values for ρ-Coumaric acid and Sinapinic acid. However, there is no statistically significant difference in the V_{max} data among the phenolic acids

first-order dependence on phenolic concentrations (Fig. 4) supports the suggestion that, under the conditions tested here, the removal rate of phenolic compounds is not limited by enzyme concentrations, or factors affecting enzyme concentrations, such as cellular growth rates and total cell numbers.

The removal rates and kinetic parameters measured here are greater to, or comparable to, several previous studies of phenolic compound removal [7] and are also comparable to values observed during the oxidation of BTEX compounds [37]. Combined with the thermos- and acidic-tolerance of *A. acidocaldarius* and its enzymes, these results are relevant to several biotechnological applications, specifically those related to the feedstock preconversions and biochemical conversions of biomass feedstocks where lignin-derived phenolics may inhibit downstream microbial activities. Within this study, the greater kinetic parameters and higher removal rates observed for phenol, compared to ferulic acid, ρ-coumaric acid and sinapinic acid, are not surprising, as phenol offers less steric hindrance to direct enzyme oxidation. Likewise, the similarity in parameters and removal rates observed for ferulic acid, ρ-coumaric acid and sinapinic acid are not surprising, as these are structurally similar compounds. However, these comparisons are relevant nonetheless, as there is a lack of literature directly comparing the removal, and removal kinetics, of these compounds.

The observed first-order dependence on copper (Fig. 3) is interesting, and may suggest that *A. acidocaldarius* does not accumulate copper to a significant extent; in the manner that other microorganisms have been observed to concentrate necessary co-factors, sometimes by several orders of magnitude [28]. Conversely, this observation suggests that at least some copper-dependent oxidases in *A. acidocaldarius* simply replace copper after it oxidizes the substrate, in the case that the enzyme transfers electrons directly to the substrate. Another possibility is that the genetic translation of the relevant enzymes has pseudo-first-order dependencies on copper concentrations. Such dependencies on protein translation have been reported previously for copper oxidases, although no kinetic data were reported [39].

In addition to the whole-cell experiments, enzyme assays were carried out using various protein fractions and substrate analogs. It is interesting to note that, in the cytoplasmic and membrane-associated fractions, some non-copper-dependent activity was observed for the lignin peroxidase assays, which measured the oxidation of the relatively simple 2,4-dichlorophenol compound. This activity could explain how phenol and, to a lesser extent, ferulic acid, ρ-coumaric acid and sinapinic acid, were degraded in the absence of copper. Conversely, the highest activities were observed in the membrane-associated and membrane-bound fractions in the laccase assays. These activities were highly copper-dependent, suggesting that most of the phenolic removal observed may have been due to LMCOs, such as those suggested by genes in the genome of *A. acidocaldarius* (shown as a table in supplemental

Table 3 Measured activities for the laccase, lignin peroxidase and manganese peroxidase assays ± 95 % confidence intervals using various protein fractions

Protein fraction	Laccase	Lignin peroxidase	Manganese peroxidase
Cytoplasmic w/o copper	7.69 ± 3.21	13.44 ± 4.21	8.65 ± 2.55
Cytoplasmic w/copper	23.94 ± 8.74	BDL	BDL
Membrane-associated w/o copper	15.30 ± 4.60	5.20 ± 3.21	4.61 ± 2.90
Membrane-associated w/copper	213.77 ± 6.21	BDL	BDL
Membrane-bound w/o copper	27.91 ± 3.56	BDL	BDL
Membrane-bound w/copper	121.64 ± 21.20	BDL	BDL
Total activity w/o copper	50.90 ± 11.37	26.06 ± 7.42	13.26 ± 5.45
Total activity w/copper	395.50 ± 36.15	Not available	Not available

The values reported represent units of activity, and are equivalent to 1 μmol μg^{-1} min^{-1}. The values shown represent the averages of triplicate experiments ± 95 % confidence intervals. Note that the total activity w/copper for the lignin peroxidase and manganese peroxidase tests is listed as 'Not Available,' as each of the fractions tested w/copper yielded activities below the detection limit

BDL below detection limit, which was approximately 1 unit of activity

Table 4 Genes proposed to be involved in phenol metabolism by *Alicyclobacillus acidocaldarius*

Genome locus	Putative function	Genbank accession
Aaci_1613	Hypothetical protein	ACV58625.1
Aaci_1614	Transcriptional regulator, GntR family	ACV58626.1
Aaci_1615	Catechol 2,3-dioxygenase	ACV58627.1
Aaci_1616	Amidohydrolase 2 (2-amino-3-carboxymuconate semialdehyde decarboxylase)	ACV58628.1
Aaci_1617	4-Oxalocrotonate tautomerase family enzyme	ACV58629.1
Aaci_1618	4-Oxalocrotonate decarboxylase	ACV58630.1
Aaci_1619	4-Hydroxy-2-oxovalerate aldolase	ACV58631.1
Aaci_1620	Acetaldehyde dehydrogenase	ACV58632.1
Aaci_1621	2-Oxopent-4-enoate hydratase	ACV58633.1
Aaci_1622	Betaine-aldehyde dehydrogenase (2-hydroxymuconic semialdehyde dehydrogenase)	ACV58634.1
Aaci_1623	Extradiol ring-cleavage dioxygenase class III protein subunit B	ACV58635.1

files). In silico analysis of the protein products from three of the five genes analyzed showed the presence of signal peptides indicating transfer of these proteins to the exterior of the cell. This analysis provides additional support for the higher aromatic monomer degradation activities for the membrane-bound and membrane-associated fractions.

Assimilation of phenol and lignin-associated aromatic monomers by *A. acidocaldarius* for growth is further supported by the presence of genes for the *meta*-fission pathway for phenol degradation (Table 4). In addition, Aaci_1623 encodes an enzyme involved in metabolism of aromatic compounds and may be involved in phenol metabolism. This metabolism proceeds through the 4-oxalocrotonate branch as opposed to the hydrolytic branch, a characteristic seen in other bacteria, such as *Comamonas testosteroni* strain TA441 [1]. In contrast to phenol hydrolase catalyzed oxidation of phenol to catechol in *C. testosteroni*, phenol oxidation by *A. acidocaldarius* is catalyzed by one or more of the putative multi-copper oxidases. Using this proposed mechanism, phenol is indirectly hydroxylated by the multi-copper oxidase [36], followed by ring cleavage and further breakdown into compounds that feed into the tricarboxylic acid cycle by other enzymes of the *meta*-fission pathway for phenol metabolism.

Laccase and LMCOs have great biotechnological potential for converting lignin in plant biomass to less structurally complicated polyphenols or aromatic monomers [14], as well as applications in the forest products industry [38]. In addition, like the lignin and manganese peroxidases, laccases are also thought to oxidize phenolic lignin residues [32], making such assays applicable in the remediation of certain processes, such as those in the textile industry. In line with such potential applications, the research presented here suggests that putative LMCOs of *A. acidocaldarius* do in fact degrade phenol and the phenolic compounds that are derivatives of compounds integral to lignocellulosic structures.

Previously, laccase genes and proteins have been primarily studied in mesophilic bacteria, including species of *Bacillus*

[29], *Pseudomonas* [16] and *Streptomyces* [23]. In addition, thermo- and hyperthermophilic laccases have been isolated from prokaryotes, including Eubacteria, such as *Thermus thermophilus*, *Thermobifida fusca* and *Thermobaculum terrenum* [8], and *Archaea* such as *Pyrobaculum aerophilum* [20]. The work presented here, notably the temperature range over which the studied enzyme fractions were active, builds on such results and implies a broad and industrially relevant temperature range for biotechnological applications for the enzymes catalyzing this activity. Specifically, the temperature range tested includes conditions that may be used in the preconversions of lignocellulosic feedstocks where organic extractives, including phenolics, may be liberated. Of note, previous efforts have identified glycosyl hydrolase enzymes from *A. acidocaldarius* that showed activity across a similar temperature range (unpublished data).

These findings contribute to an important research need to identify and characterize biomass degrading enzymes that are active under conditions often encountered in the biorefinery of lignocelluloses (i.e., high temperature, low pH). The use of these specialized enzymes in the processes may enable the mineralization of biomass to occur at higher temperatures (less cooling of substrate) and lower pH (less neutralization), saving money in both capital and operating costs. In addition to the possible deconstruction of biomass, the removal of extractable organics, such as phenolics, may benefit downstream conversion processes.

Conclusions

The present study shows the potential for the thermoacidophile *A. acidocaldarius* to degrade phenolic compounds found in lignin demonstrating potential relevance to biofuel production and other industrial processes. The majority of the observed removal was dependent on added copper, both in whole-cell and enzyme assay experiments. This suggests that putative multi-copper oxidases identified in the genome of *A. acidocaldarius* demonstrated laccase-like activity and may play an important role in the degradation of these lignin byproducts and constituents. Enzyme assays demonstrated that the majority of the activity was due to fractions associated with membrane-associated or membrane-bound proteins. Finally, observed enzyme activities from 45 to 90 °C show that *A. acidocaldarius* may possess laccase-like enzymes applicable to biotechnological processes over a wide range of temperatures.

Acknowledgments This work was supported by the Idaho National Laboratory Directed Research and Development program under Department of Energy Idaho Operations Office Contract DE-AC07-05ID14517.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human participants and/or animals This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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