International Biodeterioration & Biodegradation 62 (2008) 372–375

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Oxalate analysis methodology for decayed wood☆,☆☆,★

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ARTICLE INFO

Article history:
Received 19 December 2007
Received in revised form 31 March 2008
Accepted 3 April 2008
Available online 17 June 2008

Keywords:
Oxalate
Oxalic acid
HPLC
Colorimetric assay
Wood decay

ABSTRACT

Oxalate from partially decayed southern pine wood was analyzed by HPLC or colorimetric assay. Oxalate extraction efficiency, assessed by comparing analysis of whole wood cubes with ground wood, showed that both wood geometries could be extracted with comparable efficiency. To differentiate soluble oxalate from total oxalate, three extraction methods were assessed, using phosphate buffer, sulphuric acid or sodium hydroxide. Oxalate values from phosphate buffer extracts were two to three times higher than those from sulphuric acid extracts by both methods of analysis. Results for HPLC analysis were not significantly different from results for colorimetric analysis within each method of extraction. Results from sodium hydroxide extraction were inconsistent. The presence of oxalate in phosphate and sulphuric acid extracts was confirmed with oxalate decarboxylase but could not be confirmed for NaOH extracts. HPLC and the colorimetric assay remain valid methods of reporting soluble oxalic acid from decayed wood and are particularly useful for comparing relative determinations between treatments or fungal isolates.

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1. Introduction

The precise relevance of oxalic acid production by brown-rot decay fungi has eluded researchers for decades. Mechanistically, oxalic acid appears to play a critical, though possibly indirect role in initiation of the decay process. It is reported to reduce the viscosity of cellulose and hemicellulose (Bech-Anderson, 1987; Green et al., 1991; Shimada et al., 1991). Production and accumulation of extracellular oxalic acid ($pK_1 = 1.23$, $pK_2 = 4.26$) by brown-rot fungi causes a rapid drop in pH (Green et al., 1991). Furthermore, oxalic acid hydrolyzes the hemicellulose in wood, increasing accessibility of cellulose fibers to cellulases (Shimada et al., 1991). Schmidt et al. (1981) showed that the oxidation of oxalic acid to CO_2 can reduce Fe^{+3}, the form of iron naturally present in wood, to Fe^{+2} and generate hydroxyl radical (•OH). Because cellulose degradation by Fenton reagents mimics brown-rot, and ferrous iron is required for Fenton reactions to occur, it was proposed that oxalic acid plays an active role in the solubilization and reduction of iron in wood (Koenigs, 1974). This theory has often been reexamined and refined (Goodell, 2003).

Kaneko et al. (2005) demonstrated that rates of hydroxyl radical generation in cultures of four brown-rot fungi were directly proportional to the degradation rates of wood, and inversely proportional to the amount of oxalic acid in the cultures suggesting that •OH readily decomposes oxalic acid in culture. If some oxidants convert oxalic acid to formate anion radical and the formate radical reduces O_2 to superoxide anion, superoxide anion will reduce Fe^{+3} to Fe^{+2} and form H_2O_2 (Barr et al., 1992; Urzua et al., 1998). In this indirect manner, oxalic acid may produce Fe^{+2} and H_2O_2 to generate •OH. Munir et al. (2001) conducted biochemical analyses of glucose metabolism in relation to oxalate production and enzyme activities involved in the metabolic system. Their results indicate that glucose was not completely oxidized to CO_2 by the TCA cycle, but converted primarily to oxalate. They proposed that *Fomitopsis palustris*, and perhaps all wood rotting basidiomycetes, acquire biochemical energy by oxidizing glucose to oxalate.

The generally high copper tolerance of acid-forming brown-rot fungi was first theorized to be related to oxalic acid production by Rabanus (1933). The proposed role of oxalic acid production, discussed by Young (1961), included precipitation of copper by oxalate to form insoluble copper oxalate which would neutralize the copper in preservative solutions or by lowering the pH of the substratum. Crystals observed in decayed wood via SEM typify precipitated calcium oxalate or copper oxalate formed during the brown-rot process. Both theories have been examined more

☆ Scientific relevance: this paper clarifies methods for accurate extraction and analysis of soluble and insoluble oxalic acid from decayed wood.

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0964-8305/$ – see front matter Published by Elsevier Ltd.
doi:10.1016/j.ibiod.2008.04.001

thoroughly in solid medium (Woodward and De Groot, 1999), liquid medium (Jordan et al., 1996) and wood substrate (Clausen et al., 2000). It has been shown that simply lowering pH of the substrate does not initiate brown-rot decay, and although oxalate does tie up copper as copper oxalate, oxalic acid is not solely responsible for levels of copper tolerance common among brown-rot basidiomycetes (Clausen et al., 2000). *Antrodia vaillantii* 90877 is known to produce and accumulate significant quantities of oxalic acid from copper citrate-treated Southern pine (Clausen and Green, 2003). In the presence of copper, oxalic acid production by *A. vaillantii* in copper citrate-treated wood exceeds that in untreated wood after just two weeks in a soil block test.

While extensive work has been done on soluble and total oxalate determinations from foods (Hönow and Hesse, 2002), there has been considerable variability in oxalate reporting methods, as well as discrepancies in reporting results from brown-rotted wood. Oxalate (mmol) based on wood weight or extraction solution is the typical method of reporting, particularly if relative comparisons between treatments or fungi are being reported. Extractions at or near neutral pH have generally been assumed to solely represent soluble oxalate which has commonly been determined with HPLC (Dutton et al., 1993; Jordan et al., 1996; Urzua et al., 1998), GC–MS (Munir et al., 2001), and a commercially available colorimetric assay for oxalate (Espejo and Agosin, 1991; Jordan et al., 1996; Clausen et al., 2000; Clausen and Green, 2003; Green and Clausen, 2003).

The colorimetric assay is based on the quantitation of oxidation products generated by the catalytic activity of the enzyme oxalate oxidase. The assay is specific for oxalate and will not react with other structurally related compounds (Sugiura et al., 1979).

$$\text{Oxalate} + O_2 \xrightarrow{\text{Oxalate oxidase}} 2CO_2 + H_2O_2$$

$$H_2O_2 + \text{MBTH} + \text{DMAB} \xrightarrow{\text{Peroxidase}} \text{Indamine dye} + H_2O$$

MBTH is 3-methyl-2-benzothiazoline hydrazone and DMAB is 3-dimethylaminobenzoic acid.

Jordan et al. (1996) reported quantitative differences between HPLC and the colorimetric assay method for oxalic acid detection in *Postia placenta*-decayed wood blocks extracted in 1 N HCl. They found that detection levels for HPLC were 2 μg whereas levels for the oxalate colorimetric assay were 16–126 mg.

Accurate quantification of both insoluble and soluble oxalic acid is equally valuable (Schilling and Jellison, 2004). Ion-exchange HPLC is the most widely reported technique for soluble oxalic acid analysis, but the method suffers from interfering peaks and low sensitivity. Total oxalate (acid-extractable) has not been reported from decayed wood due to conditions required for analysis or difficult methodology. Schilling and Jellison (2004) developed an HPLC method for use on fungal culture filtrates to resolve oxalate from medium components. A related study, Schilling and Jellison (2006) reported that soluble and acid-extractable oxalate levels in metal-enriched wood were not different from each other. Acid extraction of total oxalate from wood remains difficult to accurately assess.

Accurate oxalate determinations are important for a variety of fungal physiology studies and the studies of fungal effects on their environment. The objective of this study was to compare HPLC with the colorimetric assay for oxalate from brown-rotted southern yellow pine. Extraction methods near neutral, acidic and alkaline pH were utilized to assess soluble oxalate versus total oxalate from whole or ground wood blocks. Verification of oxalate and validation of extraction method was confirmed by enzymatic destruction of oxalate with oxalate decarboxylase.

2. Materials and methods

2.1. Decayed wood specimens

Soil block culture bottles were prepared according to AWPA E-10 (2006) with a modification of wood block size to 1 × 1 × 1 cm. Southern yellow pine feeders were inoculated with the brown-rot fungus, *A. vaillantii* strain 90877 and incubated at 27 °C and 70% relative humidity (RH) for three weeks until the fungus completely colonized each feeder. Sixteen pre-weighed southern yellow pine blocks, conditioned at 27 °C and 70% RH, were vacuum-treated for 40 min at −165.5 kPa gage pressure with 1.2% copper citrate. Treated blocks were conditioned at 25 °C for seven days, steam-sterilized, placed on actively growing feeders, and incubated at 27 °C, 70% RH for four weeks. Following incubation, blocks were brushed free of mycelium, oven-dried at 60 °C, reconditioned to 6% equilibrium moisture content, and reweighed. Percent weight loss was calculated.

2.2. Sample preparation

Decayed blocks were either selected for analysis as individual whole blocks or ground to 20 mesh (850 μm screen) for a homogenous sawdust mixture. Of the sawdust mixture, nine 0.1 g samples were taken for extraction and analysis.

2.3. Extraction methods

Tenth-gram samples of sawdust ($n = 3$) were extracted in one of three ways:

1. Phosphate buffer (0.1 M, pH 5.0) for 1 h at 25 °C with rotary mixing, acidified with 0.25% H_2SO_4 and filtered through a 0.45-μm Whatman filter (Clifton, NJ, USA) (Clausen and Green, 2003).
2. NaOH (0.1 N) for 1 h at 65 °C, centrifuged (21,000 × g, 10 min), supernatant was acidified with 0.25% H_2SO_4, centrifuged (21,000 × g, 10 min) and filtered through a 0.45-μm filter (Kenealy et al., 2007).
3. H_2SO_4 (0.1 N) for 1 h at 25 °C with rotary mixing, centrifuged (21,000 × g, 10 min), and filtered through a 0.45-μm filter (Hunt et al., 2004).

Whole blocks ($n = 3$) were individually extracted in 3 ml of the same extraction solutions in the same manner as described for the sawdust samples.

2.4. HPLC analysis

Oxalic acid was determined by HPLC using detection at 210 nm. Extracts (80 μl) were injected into an Ion-300 organic acid analysis column (Phenomenex, Torrance, CA, USA) with 0.005 N H_2SO_4 eluant at 0.5 ml min^{-1} and 67 °C. An oxalic acid standard was prepared and run before and after the extracts (Hunt et al., 2004).

2.5. Colorimetric assay analysis

Ten microliters of test solution and oxalic acid standards were placed in 96-well uncoated polystyrene microplates (Dynex Technologies, Chantilly, VA, USA) (Clausen and Green, 2003; Green and Clausen, 2003). To each well, 200 μl DMAB/oxalate oxidase reagent was added followed by 20 μl of MBTH reagent (Trinity Biotech Co., Wicklow, Ireland). The integrants were mixed gently and incubated for 5 min at 37 °C. Optical density was measured at 590 nm in a Dynatech MRXII microplate reader (Dynex Technologies, Chantilly, VA, USA) and oxalate (mmol) was determined from a standard curve.

2.6. Identification of oxalate

Extracted samples were neutralized to pH 3.0 by addition of 1 M sodium tartarate. Neutralized extracts were treated with 0.4 units of oxalate decarboxylase (ODC) kindly provided by Alexander Rostovtsev, OxThera, Inc (activity at pH 4.0 = 58 μmol formate/min^{-1}) for 1 h at room temperature. The samples were acidified, centrifuged (21,000 × g, 10 min) at room temperature, and filtered through a 2-ml Whatman filter (Clifton, NJ, USA) before analysis by HPLC and colorimetric assay. Oxalate presence was confirmed by disappearance of the HPLC peak or lack of colorimetric reaction (1 unit = 1 μmol oxalate converted to formate and CO_2 min^{-1}).

2.7. Statistical analysis

A means model was fit in SAS® Version 9.1.2 (SAS, 2004) and mean comparisons were based on the Tukey–Kramer multiple comparison procedure to control the experiment-wise error rate at 0.05. The analysis did not include the controls.

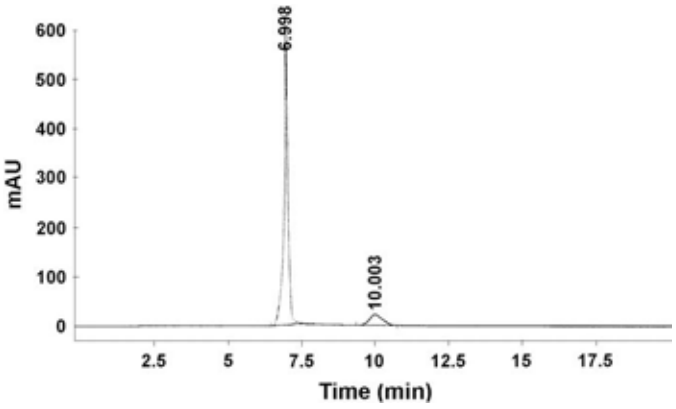

Fig. 1. HPLC chromatograph showing the separation of oxalate ($t_R = 6.99$) from wood extracts and fungal metabolites.

3. Results and discussion

3.1. Oxalate analysis

Following exposure to *A. vaillantii*, (average weight loss $8.4 \pm 3.9\%$) in a soil block test for four weeks, partially decayed southern yellow pine blocks were analyzed in two configurations (whole block or sawdust) by two methods of analysis (HPLC and oxalate assay) following three methods of extraction (acidic, neutral, or basic). Fig. 1 typifies the HPLC chromatographs for the wood extracts analyzed in this study and demonstrates separation of oxalate ($t_R = 6.99$) from wood components and fungal metabolites. Table 1 summarizes oxalate analyses for each set of conditions. In the initial analysis by both HPLC and colorimetric assay, results for whole block extractions were statistically the same as results for sawdust extractions for the volume and geometry of wood block used in this study ($1 \times 1 \times 1$ cm). The greatest difference was noted in the NaOH-extracted samples analyzed by HPLC with an average of 11 mmol in whole blocks and an average of 15.1 mmol in sawdust. Results from NaOH extracts were significantly different by both HPLC and colorimetric assay than results from either phosphate or acid extractions.

Phosphate buffer extracts, presumed to only represent soluble oxalic acid, were two to three times higher than sulphuric acid extracts, for both the colorimetric assay and the HPLC method of analysis. One possible explanation is that the multiple anions in phosphate buffer may be replacing ionically bound oxalic acid and releasing precipitated oxalic acid. In a study on oxalate regulation by brown-rot fungi in non-amended and oxalate-amended wood, Schilling and Jellison (2005) reported higher oxalate values for acid-extractable oxalate in non-amended wood than for soluble oxalate resulting from extraction in deionized water. That finding from an unbuffered extraction conflicts with our results from the buffered system of the colorimetric assay.

Table 1
Oxalate determinations of wood extracted in acid, base, or neutral conditions by colorimetric assay and HPLC

Test method	Extraction method	Average OA (mmol)			
		Original extract		Freeze/thaw	
		Colorimetric assay	HPLC	Colorimetric assay	HPLC
Whole block	Phosphate	4.5 (0.2)^{ef}	6.6 (2.7)^{bcdef}	6.1 (3.1)^{cdef}	7.2 (2.8)^{bcde}
	H_2SO_4	1.4 (0.3)^{ef}	3.1 (0.4)^{ef}	2.1 (1.0)^{ef}	2.7 (0.3)^{ef}
	NaOH	3.6 (0.8)^{ef}	11.0 (2.8)^{abcd*}	12.2 (3.4)^{ab}	11.9 (3.4)^{abc}
Sawdust	Phosphate	4.4 (0.1)^{ef}	5.3 (0.3)^{def}	4.8 (1.5)^{def*}	–
	H_2SO_4	1.2 (0.1)^{f}	3.8 (0.4)^{ef}	2.4 (0.5)^{ef}	–
	NaOH	3.0 (0.3)^{ef}	15.1 (2.0)^{a}	11.4 (4.2)^{abcd}	–
OA controls (mmol)					
0.25		0.25			
0.50		0.48			
1.00		1.00			
2.50		2.26			

Mean (±SD) followed by the same letters are not significantly different;
*In addition to the letter display, these two groups are significantly different as a result of one of the groups having fewer observations than the other groups, and some mean difference comparisons having a different standard error associated with them.

There was no significant difference between colorimetric assay or HPLC as method of analysis for phosphate buffer or sulphuric acid extractants. Most notable was the difference between colorimetric assay and HPLC results for the NaOH extractions; the original result for sawdust was five times higher by HPLC analysis and the original result for the whole block extract was three times higher by HPLC analysis. These results suggest that NaOH extracts were outside the pH range for the pH-dependent colorimetric reaction, but both methods of analysis were conducted on identical extracts that had been neutralized prior to analysis. pH was confirmed on original samples.

3.2. Oxalate confirmation

All samples were stored at 0 °C until reactions with ODC. Samples were first thawed and assayed to assess the freeze/thaw stability of oxalate (Table 2). Oxalate results by colorimetric assay following freeze/thaw cycle were higher (26–33%) for phosphate buffer and sulphuric acid whole block extracts than original assay results. Oxalate results by HPLC following freeze/thaw cycle were 8% higher for phosphate buffer whole block extract, but 13% lower for the sulphuric acid whole block extract than original results. Results for the NaOH extracts following freeze/thaw were 11–12 mmol for both methods of analysis, which was three times higher than the original colorimetric result, suggesting that this

Table 2
Verification of presence of oxalate following treatment of wood extracts with oxalate decarboxylase (ODC)

Test method	Extraction method	Colorimetric assay			HPLC		
		Original	+ODC	Quenched (%)	Original	+ODC	Quenched (%)
Whole block	Phosphate	6.1 (3.1)	1.2 (1.4)	80.3	6.6 (2.7)	1.6 (0.6)	75.8
	H_2SO_4	2.1 (1.0)	0.4 (0.8)	81	3.1 (0.4)	1.8 (0.8)	41.9
	NaOH	12.2 (3.4)	11.9 (3.3)	2.5	11.0 (2.8)	13.3 (2.8)	0
Sawdust	Phosphate	4.8 (1.5)	5.0 (0.3)	0	5.3 (0.3)	5.9 (0.4)	0
	H_2SO_4	2.4 (0.5)	1.4 (0.9)	41.7	3.8 (0.4)	2.8 (0.8)	26.3
	NaOH	11.4 (4.2)	11.2 (3.8)	1.8	15.1 (2.0)	11.4 (3.8)	24.5
OA control[a]		2.26	0	100			

Mean (±SD).
[a] OA control, 2.5 mmol.

method of extraction might represent total versus soluble oxalic acid if confirmed by decarboxylation with ODC.

Decarboxylation of oxalate with ODC is expressed as percent of original oxalate for each method of analysis (Table 2). For the colorimetric assay, percent oxalate quenched represents the change in activity from oxalate results following the freeze/thaw cycle. Eighty and 81% oxalate activity in the phosphate and sulphuric acid whole block extracts, respectively, was neutralized by the ODC treatment, while virtually no change occurred in the NaOH extract. Forty percent of the oxalate colorimetric reaction was quenched in the sulphuric acid sawdust extract, but no change was seen in the other sawdust extracts for this method.

For HPLC, percent oxalate quenched by ODC represents the change in activity from oxalate result in original unfrozen samples, due to freeze/thaw whole block samples showing virtually identical results to initial test results, plus insufficient sample remained to assay sawdust samples following freeze/thaw cycle. For whole block extracts, 75.8 and 41.9% oxalate was quenched in the phosphate and sulphuric acid extracts. The NaOH extract was not neutralized by ODC. Sulphuric acid and NaOH sawdust extracts showed 26 and 25% reduction in oxalate activity following treatment with ODC, but the oxalate in the phosphate extract was unchanged.

4. Conclusions

Partially decayed southern pine blocks ($1 \times 1 \times 1$ cm) can be extracted as efficiently as sawdust. Soluble oxalic acid extracted in phosphate buffer was two to three times higher than sulphuric acid extracts, which was previously presumed to represent total oxalic acid for both colorimetric assay and HPLC method of analysis. While oxalic acid results for NaOH extracts were initially higher than results for either phosphate or sulphuric acid extracts, the colorimetric reaction gave inconsistent results following extraction in NaOH. The sole presence of oxalate could not be validated by either HPLC or colorimetric analysis of NaOH extracts. Neither HPLC nor the colorimetric assay provided convincing evidence that total oxalic acid was being determined in acid-extracted wood. Oxalate confirmation with ODC indicates that the colorimetric assay is specific for oxalate and that there is no interference from wood components or fungal metabolites. Oxalate extracted in NaOH could not be verified by quenching with ODC. Both the HPLC and the colorimetric assay remain valid methods of reporting soluble oxalic acid from decayed wood, particularly if relative determinations are being made between treatments or fungal isolates.

Acknowledgements

The authors would like to thank Diane Dietrich for technical assistance.

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