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Evaluation of Oxalate Decarboxylase and Oxalate Oxidase for Industrial Applications

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Abstract Increased recirculation of process water has given rise to problems with formation of calcium oxalate incrusts (scaling) in the pulp and paper industry and in forest biorefineries. The potential in using oxalate decarboxylase from *Aspergillus niger* for oxalic acid removal in industrial bleaching plant filtrates containing oxalic acid was examined and compared with barley oxalate oxidase. Ten different filtrates from chemical pulping were selected for the evaluation. Oxalate decarboxylase degraded oxalic acid faster than oxalate oxidase in eight of the filtrates, while oxalate oxidase performed better in one filtrate. One of the filtrates inhibited both enzymes. The potential inhibitory effect of selected compounds on the enzymatic activity was tested. Oxalate decarboxylase was more sensitive than oxalate oxidase to hydrogen peroxide. Oxalate decarboxylase was not as sensitive to chlorate and chlorite as oxalate oxidase. Up to 4 mM chlorate ions, the highest concentration tested, had no inhibitory effect on oxalate decarboxylase. Analysis of the filtrates suggests that high concentrations of chlorate present in some of the filtrates were responsible for the higher sensitivity of oxalate oxidase in these filtrates. Oxalate decarboxylase was thus a better choice than oxalate oxidase for treatment of filtrates from chlorine dioxide bleaching.

Keywords Oxalic acid · Calcium oxalate scaling · Oxalate decarboxylase · Oxalate oxidase

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Introduction

Due to environmental considerations, the pulp industry in the Nordic countries has replaced chlorine bleaching with elementary chlorine-free and totally chlorine-free processes. To reduce industrial wastes into nature and to optimize the use of bleaching agents and energy, the bleach plants have increased the recirculation of process waters. Unfortunately, these closed-loop bleaching systems give rise to enrichment of inorganic and organic substances that either are produced during the bleaching or dissolved from the pulp. Oxalic acid is one of the most troublesome compounds, since it together with calcium forms hard deposits on the process equipment, e.g., filter tanks, pipelines, and heat exchangers. This can cause reduction in heat transfer and flow rate leading to negative consequences for profitability and product quality [1–3]. Wood contains 0.1 to 0.4 kg oxalic acid per ton plus calcium ions in various amounts depending on the wood species [4]. The oxidation of lignocellulosic material during bleaching with oxygen (O stage), chlorine dioxide (D stage), ozone (Z stage), and hydrogen peroxide (P stage) produces additional amounts of oxalic acid, which also accumulates in the process [5–7].

Oxalate oxidase (oxalate:oxygen oxidoreductase, E.C. 1.2.3.4) [8, 9] catalyzes the oxidation of oxalate to hydrogen peroxide and carbon dioxide in the presence of oxygen. Oxalate oxidase from barley is well studied, and it belongs to a group of proteins called germins [10]. Previously, we have shown that barley oxalate oxidase can be used to decrease the levels of oxalic acid in industrial bleach plant filtrates [11]. However, bleaching filtrates can be more or less inhibitory to oxalate oxidase [11, 12].

Oxalate decarboxylase (E.C. 4.1.1.2) [10, 13, 14] degrades oxalic acid to carbon dioxide and formate. Since the first discovery of oxalate decarboxylase in the mycelia of the white-rot fungi *Coriolus hirsutus* (*Trametes hirsuta*) and *Collyvia veltipes* (*Flammulina velutipes*) [15], the enzyme has been identified in many species of filamentous fungi such as *Aspergillus niger* [16], *Myrothecium verrucaria* [17], and *Trametes ochracea* [18]. Oxalate decarboxylase activity has also been reported in *Bacillus subtilis* [19] and in guinea pig liver [20]. Oxalate decarboxylases from *A. niger*, *M. verrucaria*, and *F. velutipes* are highly specific for oxalic acid and display no activity towards other organic acids, such as formic, succinic, and citric acid [15–17, 21, 22]. *B. subtilis* oxalate decarboxylase and barley oxalate oxidase are manganese-containing enzymes [23, 24]. The manganese ions are thought to be vital for the catalytic action of both enzymes.

Ten different filtrates from Kraft pulp production were used to compare the performance of oxalate decarboxylase from *A. niger* to that of the better known oxalate oxidase from barley. In addition, analysis of the chemical compositions and model experiments with potential inhibitors were made to elucidate differences between oxalate decarboxylase and oxalate oxidase. This investigation differs from a previous study [25] by the inclusion of experiments with separate compounds that inhibit oxalate decarboxylase and oxalate oxidase and by the focus on filtrates from Kraft pulping. The results are important for the selection of the most suitable enzyme type to lower critical concentrations of oxalic acid from process water from the pulp and paper industry.

Materials and Methods

Samples, Chemicals, and Enzymes

Ten authentic filtrates from Swedish pulp mills producing bleached softwood Kraft pulps were selected for enzymatic treatment: EP (alkali extraction stage reinforced with hydrogen

peroxide), EOP (alkali extraction stage reinforced with both oxygen and hydrogen peroxide; this sample was from a bleaching sequence using chlorine dioxide), O (oxygen stage), PO (peroxide stage reinforced with oxygen), Q (complexing agent stage), QP^a (peroxide stage with no washing after the addition of complexing agent), QP^b (QP filtrates from different mill), D^a (chlorine dioxide stage), D^b, and D^c (D0 filtrates from different mills). Prior to treatments with oxalate oxidase and oxalate decarboxylase, the pH of the filtrates was adjusted to 3.8 and 5.6, respectively. The pH was adjusted with HCl (37%) or NaOH (1 M).

Analytical grade chemicals were used: formic and glycolic acid; hydrogen peroxide (H_2O_2); sodium salts of chlorate (ClO_3^-), chlorite (ClO_2^-), and sulfite (SO_3^{2-}); vanillin [121-33-5]; vanillic acid [121-34-6]; acetovanillone [498-02-2]; and syringol [91-10-1]. *A. niger* oxalate decarboxylase was obtained from Roche (Mannheim, Germany), and barley oxalate oxidase (0.71 $U mg^{-1}$ solid) was obtained from Sigma-Aldrich (St. Louis, MO, USA). The oxalate decarboxylase suspension was dialyzed against 20 mM acetate buffer (pH5.6) (for 20 h at 4°C) prior to treatment of filtrates. The dialysis was performed with Spectra/Por membrane tubing (cut-off of 12,000–14,000) (Spectrum Laboratories, Rancho Dominguez, CA).

Enzymatic Reactions

In order to compare the performance of oxalate oxidase and oxalate decarboxylase in different filtrates, the activity (calculated from the rate of consumption of oxalic acid in a buffered aqueous solution of oxalic acid) of the oxalate-degrading enzymes was the same in all samples. The optimal pH of each enzyme was used in the reactions: pH3.8 for oxalate oxidase and pH5.6 for oxalate decarboxylase. The oxalate oxidase assay mixture consisted of 0.6 mM oxalic acid, 50 mM succinate buffer (pH3.8), and 0.017 $U ml^{-1}$ oxalate oxidase (1 U of oxalate oxidase will consume 1.0 μmol of oxalic acid per minute at pH3.8 and 37°C). The oxalate decarboxylase assay mixture contained 0.6 mM oxalic acid, 50 mM acetate buffer (pH5.6), and 0.017 $U ml^{-1}$ oxalate decarboxylase (1 U of oxalate decarboxylase will consume 1.0 μmol of oxalic acid per minute at pH5.6 and 37°C). The reaction mixtures were incubated with stirring at 37°C. Samples for analysis were taken directly after the addition of the oxalate-degrading enzyme and at the end of the incubation (16 min).

After adjustment of pH to pH3.8 (oxalate oxidase) or 5.6 (oxalate decarboxylase), degradation of oxalic acid in the filtrates was performed by addition of 0.017 $U ml^{-1}$ of oxalate oxidase or oxalate decarboxylase. The samples were incubated at 37°C with stirring, and samples were taken immediately after the addition of enzyme and at the end of the incubation (1 h). Before analysis, the samples were diluted 60 times. Negative controls were made in which heat-denatured enzyme was added. The oxalic acid content in the filtrates was not affected in the negative controls. The inhibitory effect of chlorate on oxalate decarboxylase was studied at 0.2, 2, 3, and 4 mM. The inhibitory effect of chlorite on oxalate decarboxylase was studied at 0.2, 1.0, 2.0, 4.0, 8.0, and 16 mM. The effect of formic acid (pH5.6), glycolic acid (pH5.6), hydrogen peroxide, and copper (II) chloride was studied at 0.1, 1, 10, and 20 mM, while the effect of sulfite was studied at 0.001, 0.01, 0.1, and 1 mM. The lignin model vanillic acid was studied at 0.1, 1.0, and 5.0 mM and vanillin, acetovanillone, and syringol at 5 mM. The concentrations chosen emanate from previous results [12, 26]. The remaining activity was calculated as the rate of degradation of oxalic acid in the reaction mixture containing the inhibitor divided by the degradation rate in a buffered aqueous oxalic acid solution without any inhibitor. All model experiments were performed in duplicates. Samples (100 μl) for analysis were taken directly after

initiation of the reaction and after 16 min of incubation. Before analysis, the samples were diluted 20 times and filtered through a 0.45-μm cellulose acetate filter (Whatman, Maidstone, UK).

Analysis of Oxalic Acid

The oxalate oxidase and oxalate decarboxylase activity was quantified by determining the rate of oxalic acid decomposition using a Dionex 2020i-series ion chromatography system (Dionex, Sunnyvale, CA). Separation was performed using an IonPac AS4A-SC anion-exchange column (250×4 mm or 250×2 mm). The separation columns were protected by AG4A-SC guard columns. The mobile phase consisted of a mixture of 1.7 mM $NaHCO_3$ and 1.8 mM Na_2CO_3. The flow rate was 0.6 ml min^{-1} for the 250×2 mm column and 2.0 ml min^{-1} for the 250×4 mm column. The precision of the method, which is well documented [27], is estimated to 3% (given as the coefficient of variation) with regard to analysis of oxalic acid in process liquors from the pulp and paper industry.

Filtrate Analyses

The analysis of aliphatic monoacids was performed using a P/ACE MDQ capillary electrophoresis instrument equipped with a 60 cm×50 μm I.D. fused silica capillary (Beckman Coulter, Fullerton, CA). The samples were filtered prior to hydrodynamic injection at 15 psi for 4 s. The voltage was set to 20 kV at reversed polarity. The electrolyte, composed of 5.0 mM trimellitic acid, 50 mM tris(hydroxymethyl)-aminomethane, 1.0 mM tetradecyltrimethylammoniumbromide, and 0.5 mM calcium chloride, had a pH of 9.8. Before use, it was filtered through a 0.45-μm cellulose acetate filter and degassed with helium. Detection was performed by indirect UV at 220 nm. Succinic acid was used as internal standard.

Chlorite and chlorate were determined using a DX 500-series ion chromatography system (Dionex) equipped with a conductivity detector (ED-40). An IonPac AS9-HC column (250×4 mm I.D.), equipped with an AG9-HC guard column, was used for the separation. The suppressor fluid consisted of 13 mM sulfuric acid. The eluent consisted of a mixture of 5.0 mM $NaHCO_3$ and 15.7 mM Na_2CO_3. For all analyses, a flow rate of 2.0 mL min^{-1} was used. Quantifications of all different compounds measured were performed by peak area calculations and comparison with calibration curves using the EZChrom Elite software system version 2.31 (Scientific Software Inc., Pleasanton, CA).

Results and Discussion

The performance of *A. niger* oxalate decarboxylase in ten different process waters from the pulp and paper industry was investigated. Treatment with oxalate decarboxylase resulted in a decrease in the concentration of oxalic acid in the following filtrates: D^a, D^b, D^c, EP, PO, Q, QP^a, and QP^b (Table 1). The highest rate of oxalic acid degradation by oxalate decarboxylase was observed in the D^c stage filtrate, whereas the lowest rate was found in the EOP and O stage filtrates, where no degradation was detected (Table 1).

Oxalate oxidase decreased the oxalic acid content of the EP, O, Q, QP^a, and QP^b stage filtrates (Table 1). The rate of oxalic acid consumption by oxalate oxidase was highest in the O stage filtrate, while no activity was observed in the D^a, D^b, D^c, EOP, and PO stage filtrates (Table 1).

Table 1 Enzymatic degradation of oxalic acid in bleaching stage filtrates using oxalate decarboxylase and oxalate oxidase.

Filtrate	Rate of oxalic acid removal (mg $l^{-1}h^{-1}$)	
	Oxalate decarboxylase	Oxalate oxidase
D^a	4.4	ND
D^a*	–	3.1
D^b	7.8	ND
D^b*	–	0.7
D^c	102	ND
EP	4.9	3.7
EOP	ND	ND
O	ND	12.3
PO	0.8	ND
Q	7.4	1.9
Q*	–	3.0
QP^a	5.6	3.6
QP^b	15.3	5.0

D^a*, D^b*, and Q* were treated with three times higher dosage of oxalate oxidase

ND no activity detected

Oxalate decarboxylase performed better than oxalate oxidase in all filtrates except in the O stage filtrate. As shown in Table 1, the rate of consumption of oxalic acid differed in particular in the D^a, D^b, D^c, O, PO, and Q stage filtrates. The D^a, D^b, and Q stage filtrates were treated separately with a three times higher dosage of oxalate oxidase. Despite the addition of more oxalate oxidase, the oxalic acid degradation rates were still slower than for oxalate decarboxylase (Table 1).

In an attempt to find an explanation why the efficiency of oxalic acid degradation by oxalate oxidase and oxalate decarboxylase differed, the chemical composition of selected filtrates was analyzed (Table 2). Furthermore, in order to identify potential oxalate decarboxylase inhibitors, the effect of compounds encountered in bleaching filtrates on the oxalate decarboxylase activity was studied (Table 3). Although there are some reports regarding inhibitors of oxalate decarboxylase [15–17, 21, 22, 28], most of the results are of little relevance to process waters from the pulp and paper industry. The effect of selected compounds on oxalate decarboxylase (this study) was compared with the effect on oxalate oxidase [12].

Table 2 Analysis of oxalic acid and some potential inhibitors in bleaching filtrates (mM).

Filtrate	Oxalic acid		Formic acid	Glycolic acid	Chlorite	Chlorate	Hydrogen peroxide
	pH3.8	pH5.5					
D^a	0.17	0.08	2.9	0.1	0.5	1.7	ND
D^b	0.18	0.13	5.9	0.2	ND	3.7	ND
EOP	0.91	0.72	5.3	0.1	1.4	2.8	ND
O	0.31	0.27	0.2	0.3	ND	ND	ND
PO	0.09	0.11	2.2	0.1	–	–	16
Q	0.23	0.16	1.3	ND	–	–	6.8

ND not detected

Data in a previous report indicated that *A. niger* oxalate decarboxylase does not lose its activity for several months when it is incubated in acetate buffer (0.2 M, pH5.6) [28]. Twenty millimolars acetic acid had no inhibitory effect on oxalate oxidase [12]. Acetic acid is generally present in filtrates, but it is most likely not relevant as an inhibitor of either oxalate decarboxylase or oxalate oxidase and was therefore not included in the comparison.

Among the filtrates analyzed, the highest concentration of formic acid (5.9 mM) was found in the D^b stage filtrate followed by the EOP, D^a, PO, Q, and O stage filtrates, which contained from 0.2 to 5.3 mM (Table 2). Formic acid had no effect on the reaction rate of oxalate decarboxylase at concentrations of 0.1 and 1.0 mM and only weak effects of the high concentrations, 10 and 20 mM (Table 3). In compliance with this observation, it has been demonstrated that 1 mM of formate did not affect *A. niger* oxalate decarboxylase [16]. Earlier, we have reported that formic acid (pH3.8) had no effect on oxalate oxidase in concentrations up to 1 mM, whereas at 10 mM, it had a negative effect (approximately 60% inhibition) [12]. Although oxalate oxidase is somewhat more sensitive than oxalate decarboxylase towards the formic acid concentration in the D^a and D^b stage filtrates, this

Table 3 Effect of various compounds on the activity of oxalate decarboxylase from *A. niger* at pH5.6 and a temperature of 37°C.

Compound	Concentration (mM)	Determined activity (%)
Hydrogen peroxide	0.1	105
	1.0	66
	10	23
	20	5
Chlorite	0.2	101
	1.0	81
	2.0	70
	4.0	32
	8.0	26
	16	0
Sulfite	0.001	107
	0.01	52
	0.1	11
	1.0	0
Formic acid	0.1	112
	1.0	112
	10	80
	20	75
Glycolic acid	0.1	106
	1.0	105
	10	90
	20	88
Copper (II)	0.1	78
	1.0	8
	10	0
	20	0

Neither chlorate nor the lignin-derived phenolic compounds inhibited oxalate decarboxylase. These compounds are therefore not included in the table

difference in sensitivity is not sufficient to explain why oxalate decarboxylase performed better than oxalate oxidase in these filtrates.

Among the filtrates analyzed, the concentration of glycolic acid did not reach higher than 0.3 mM (Table 2). Glycolic acid displayed only weak effects on the oxalate decarboxylase activity and no inhibition at concentrations ≤1 mM (Table 3). Low concentrations of glycolic acid (≤1 mM) are not inhibitory to oxalate oxidase, either [12]. Consequently, glycolic acid was not responsible for the observed differences in oxalic acid degradation rate by oxalate oxidase and oxalate decarboxylase.

Hydrogen peroxide was found in the PO and Q stage filtrates (Table 2). Hydrogen peroxide had no effect on oxalate decarboxylase at a concentration of 0.1 mM but caused strong inhibition at 10 and 20 mM (Table 3). Hydrogen peroxide (1 mM) has been found to cause a severe inhibition of oxalate decarboxylase from *C. veltipes* [15]. Previously, we have shown that barley oxalate oxidase is inhibited by 10–20 mM hydrogen peroxide but not by lower concentrations (≤1 mM) [12]. The concentration of hydrogen peroxide in the PO stage filtrate, 16 mM, was thus sufficient to partially inhibit both oxalate decarboxylase and oxalate oxidase [Table 1, [12]]. These results can be compared with an investigation based on multivariate data analysis of the performance of oxalate-degrading enzymes in bleaching filtrates from mechanical pulping, which suggests that hydrogen peroxide is not correlated with low enzymatic activity [25].

Chlorite, formed during bleaching with chlorine dioxide, was observed in the EOP and the D^a stage filtrates (Table 2). Chlorite did not inhibit oxalate decarboxylase at a concentration of 0.2 mM, whereas strong inhibition was obtained when the concentration was 4–16 mM (Table 3). Since oxalate decarboxylase displayed 81% activity in the presence of 1 mM chlorite, it is most likely that the chlorite concentration in the EOP filtrate partially inhibited the activity of oxalate decarboxylase (Table 2). The activity of oxalate oxidase decreased to 54% and 45% of the original when the concentration of chlorite was 0.2 and 1 mM, respectively. When the chlorite concentration was 2 mM or higher, no oxalate oxidase activity was observed. It can therefore be concluded that the concentration of chlorite in the EOP and D^a stage filtrates had a more inhibitory effect on oxalate oxidase than on oxalate decarboxylase (Table 2).

The D^a, EOP, and D^b stage filtrates contained chlorate, also formed from chlorine dioxide, in concentrations ranging from 1.7 to 3.7 mM (Table 2). Even at the highest chlorate concentration tested (4 mM), no inhibitory effect on oxalate decarboxylase was detected. In contrast, 1.5 mM chlorate almost completely inhibits oxalate oxidase (97% inhibition) [12]. Considering these results, it is likely that the difference between the performance of oxalate decarboxylase and oxalate oxidase in the D^a and D^b stage filtrates (Table 2) could be attributed to chlorate. In a previous study based on multivariate data analysis and filtrates from mechanical pulping [25], it was not possible to draw conclusions about the effect of chlorate. This study clearly shows the difference between the oxalate decarboxylase and the oxalate oxidase with regard to their sensitivity to chlorate.

Low molar mass lignin-oxidation compounds are formed from lignins during pulping and oxygen bleaching. The concentrations are strongly reduced as bleaching proceeds, and the amount of monomeric phenols are low in the following Kraft pulp bleaching filtrates [26, 29, 30]. Methyl tertiary butyl ether extractions of acidified filtrates, followed by silylation and analysis by GC, revealed only low concentrations. Four frequently occurring lignin model compounds were selected and tested as inhibitors for oxalate oxidase and oxalate decarboxylase: vanillic acid, vanillin, acetovanillone, and syringol. No inhibition was observed at 5 mM for any of the lignin models with either oxalate decarboxylase or oxalate oxidase.

The filtrates from stage O and EOP totally inhibited the activity of oxalate decarboxylase (Table 1). Glycolic acid, chlorate, and hydrogen peroxide were not present in inhibitory concentrations in either the O or the EOP stage filtrates (Tables 2 and 3). Since oxalic acid was present in substantial amounts (Table 2) and since the concentrations of formic acid and chlorite in the O or the EOP stage filtrates (Table 2) have weak or no effect (Table 3), compounds that were not included in the chemical analyses should be responsible for the inhibition of oxalate decarboxylase in these filtrates. The high activity of oxalate oxidase in the O stage filtrate (Table 1) was probably due to its low content of inhibitors such as chlorite, chlorate, and hydrogen peroxide (Table 2).

Only 8% of the oxalate decarboxylase activity remained when the concentration of copper(II) was 1 mM, while 78% remained at 0.1 mM (Table 3). This is consistent with a previous study showing that 1 mM copper(II) had a strong inhibitory effect on *C. veltipes* oxalate decarboxylase [15]. Low concentrations of sulfite inhibited oxalate decarboxylase (Table 3). Emiliani and Bekes [16] reported complete inhibition of *A. niger* oxalate decarboxylase with 0.1 mM sulfite, which closely resembles our results. Earlier, we have shown that 1 mM sulfite extinguishes the activity of oxalate oxidase [12]. Since low concentrations of sulfite are difficult to handle experimentally, these results should be regarded cautiously. However, it is clear that sulfite strongly inhibits both oxalate oxidase and oxalate decarboxylase. Fortunately, sulfite is in general not a significant component in filtrates, except when mechanical pulp is reductively bleached with dithionite.

The Michaelis constant (K_m) of *A. niger* oxalate decarboxylase has been determined to be 4 mM at pH5.2 and 30°C [16]. Different K_m values have been reported for barley oxalate oxidase, 1.3 mM (at pH4.0 and 22°C) [23], 0.27 mM (at pH3.8 and 37°C) [31], and 0.42 mM (at pH3.5 and 22°C) [32]. Considering these K_m values and that the concentration of oxalic acid varied from 0.08 mM (at pH5.5) in the D^a stage filtrate to 0.91 mM (at pH3.8) in the EOP stage filtrate (Table 2), neither *A. niger* oxalate decarboxylase nor barley oxalate oxidase reached their maximal reaction velocity in any of the treated filtrates. Furthermore, the superior performance of oxalate decarboxylase in several filtrates could be expected to be a consequence of lower sensitivity to inhibitors rather than to kinetic characteristics of the enzyme.

To compare the enzymic reactions, they were all performed at 37°C. However, it is known that oxalate oxidase from barley retains 25% of its activity after incubation for 30 min at 70°C [31] and that oxalate decarboxylase from *A. niger* retains 50% of its activity after 10 min at 65°C [16]; so therefore, it is not unreasonable to perform enzymic treatments at much higher temperatures than at 37°C.

In conclusion, this study demonstrates that oxalate decarboxylase and oxalate oxidase show a marked difference in their ability to degrade oxalic acid in filtrates. Oxalate decarboxylase from *Aspergillus* appears to be more promising than oxalate oxidase from barley for treatment of most filtrates, particularly from D stages. The active sites of barley oxalate oxidase and *B. subtilis* oxalate decarboxylase have been found to display structural similarities [33, 34]. So far, no investigation has been made regarding the detailed mechanism behind the inhibition of oxalate oxidase or oxalate decarboxylase. It remains to be elucidated why some inhibitors affect oxalate oxidase and oxalate decarboxylase similarly and others very differently. Detailed studies of the mechanisms behind the inhibition of oxalate-degrading enzymes are underway.

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