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# Induction of an Oxalate decarboxylase in the Filamentous Fungus *Trametes versicolor* by Addition of Inorganic Acids

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**Abstract** In order to improve yields and to reduce the cost of oxalate decarboxylase (OxDC, EC 4.1.1.2), the induction of OxDC in the white-rot fungus *Trametes versicolor* was studied in this work. OxDC was induced by addition of inorganic acids including hydrochloric acid, sulfuric acid, and phosphoric acid to culture media. The results showed that all the acids could enhance OxDC expression. The activity of the acid-induced OxDC rose continuously. All of the OxDC volumetric activities induced by the inorganic acids were higher than 20.0 U/L and were two times higher than that obtained with oxalic acid. OxDC productivity was around 4.0 $U \bullet L^{-1} \bullet day^{-1}$. The highest specific activity against total protein was 3.2 U/mg protein at day 8 after induction of sulfuric acid, and the specific activity against mycelial dry weight was 10.6 U/g at day 9 after induction of hydrochloric acid. The growth of mycelia was inhibited slightly when the pH values in culture media was around 2.5–3.0, while the growth was inhibited heavily when the pH was lower than 2.5.

**Keywords** Oxalate decarboxylase · Induction · *Trametes versicolor* · Low pH environment · Inorganic acid

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## Introduction

Oxalate decarboxylase (OxDC, EC 4.1.1.2) is a manganese-containing intracellular enzyme, which is able to convert oxalate to formate and carbon dioxide [1]. OxDC was firstly identified almost 50 years ago in the studies of basidiomycete fungi *Flammulina* (*Collybia*) *velutipes* and *Coriolus hirsutus* [2, 3]. Subsequent studies have shown that the enzyme is also present in the following basidiomycetes *Agaricus bisporus* [4], *Dichomitus squalens* [5], *Heterobasidion annosum* [6], *Phanerochaete chrysosporium* [6], *Phanerochaete sanguinea* [5], *Postia placenta* [7], *Trametes ochracea* [5], *Trametes* (*Coriolus*) *versicolor* [8], and in ascomycetes *Aspergillus niger* [9, 10], *Aspergillus phoenicis* [9], *Myrothecium verrucaria* [8], and *Sclerotinia sclerotiorum* [11], as well as in the bacterium *Bacillus subtilis* [12]. OxDC is localized in vesicles close to the plasma membrane [8] and belongs to the cupin superfamily, which is defined by their conserved motifs and a proposed common β-barrel fold [13, 14]. OxDC has been used in the clinical assay of oxalate in blood and urine and could be used to decrease toxic oxalate levels in foods and in the environment [15]. Recently, the enzyme has been shown to confer fungal disease resistance when expressed in plants [16] and can selectively eliminate oxalic acid to prevent scaling in the pulp and paper industry [17, 18].

For a long time, OxDC was regarded as an enzyme to be induced by oxalic acid and to control excess oxalic acid concentrations in microbiological systems. Magro et al. [11] showed that the production of *S. sclerotiorum* OxDC was regulated by the composition and pH value of culture medium and required the presence of oxalate or its precursor, succinic acid, as inducers. The OxDCs of both *T. versicolor* [8] and *P. placenta* [7] could be induced by oxalic acid. However, Tanner et al. [12] found that *B. subtilis* OxDC (bacterial enzyme) was not oxalate induced but acid induced. Subsequently, Azam et al. [19] showed that the induction of OxDC from *F. velutipes* was pH dependent and suggested that increased transcription of the gene-encoding OxDC in *F. velutipes* in response to low pH may be mediated by the interaction of a specific transcription factor with the OxDC promoter.

As described above, OxDC has extensive usages in foods, biomedicine, environment, agriculture, and industry; however, the low production of this enzyme limits its applications. Although Dutton et al. [8] reported that the white-rot fungus *T. versicolor* was able to produce OxDC, very little research was done to study the induction of OxDC in the fungus in detail. In order to improve yields, to reduce the cost of OxDC, and to understand the regulatory mechanism of OxDC expression in *T. versicolor*, the induction of OxDC in the fungus by addition of inorganic acids, including hydrochloric acid, sulfuric acid, and phosphoric acid, was firstly studied in this work. The results showed that all the acids could induce OxDC expression evidently.

## Materials and Methods

### Microorganism and Culture Media

Cultures of *T. versicolor* PRL572, which were given as a gift by Professor Leif Jönsson in Karlstad University in Sweden, were maintained on potato dextrose agar (PDA, 20% potato, 2.0% dextrose, and 1.5% agar) slants at 4 °C. *T. versicolor* was cultivated in a defined liquid culture medium (pH 5.0) containing 2.0% (*w*/*v*) glucose, 0.3% (*w*/*v*) peptone, 0.1% (*w*/*v*) $KH_2PO_4$, 0.02% (*w*/*v*) $Na_2HPO_4{\cdot}2H_2O$, and 0.05% (*w*/*v*) $MgSO_4{\cdot}7H_2O$, 0.1% (*v*/*v*) trace element stock solution [20]. The medium was autoclaved at 121 °C for 20 min.

## Cultivation of *T. versicolor* and Production of OxDC

A 250-mL flask containing 50 mL of the defined liquid medium was inoculated with two 12-mm pieces of 10-day-old PDA plate culture and was incubated stationarily at 30 °C for 6 days to prepare inocula. After incubation, the culture was transferred into a sterile container with some glass beads. The container was shaken until the mycelia were broken, and a homogeneous inoculum suspension was obtained.

For enzyme production, 250-mL flask cultures containing 50 mL of medium with 10% inocula (from the inoculum suspension) were incubated at 25 °C and 160 rpm. After a 3-day growth, the pH of the cultures was adjusted to 2.5–3.0 by adding 2 mol/L sterile hydrochloric acid, sulfuric acid, or phosphoric acid in the cultures. All the diluted acids were prepared from the concentrated acids, which were of chemical purity and were purchased from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China. Samples were taken everyday and were filtrated to obtain filtrates for determination of pH value (electronic pH meter PHSJ-4A, Shanghai REX Instrument Factory, Shanghai, China) and residual glucose concentration (3,5-dinitrosalicylic acid colorimetric method [21]). The presented results are the mean values of triplicates.

## Extraction of Oxalate Decarboxylase

After addition of inorganic acid, mycelial pellets of each culture were harvested everyday and were washed three times with acetate buffer (200 mmol/L, pH 3.7). The washed mycelia were frozen in liquid nitrogen and were ground in a mortar using a pestle to fine paste. In order to extract enzyme, 2 mL of acetate buffer (200 mmol/L, pH 3.7) was added in the paste, and then the mixture was enclosed in a 15-mL centrifuge tube and centrifuged at 9,000×*g* for 30 min at 4 °C. The supernatant was frozen in liquid nitrogen and stored at −20 °C for later analysis of oxalate decarboxylase activity and protein concentration. The mycelial pellet was collected and dried at 105±1 °C to constant to determine the dry weight of the mycelia. Protein concentration in the crude extracts was determined by the Bradford method [22] with bovine serum albumin as standard protein.

## Determination of OxDC Activity

Enzyme activity was measured according to Magro et al. [4]. Oxalate is decomposed to $CO_2$ and formate at pH 3.0 and 30 °C, and the formate is measured at pH 7.0.

Reaction mixtures contained 100 μL of 50 mM oxalic acid (Sigma-Aldrich, St. Louis, MO, USA), 300 μL of acetate buffer (200 mmol/L, pH 5.6), and 100 μL of enzyme (the crude extract). Reaction mixtures were incubated at 30 °C for 30 min, then 2 mL of 14 mM nicotinamide-adeninedinucleotide-Li salt ($NAD^+$, Yuanju Bio-Tech Co., Ltd., Shanghai, China), and 0.3 mL deionized water were added. The reaction mixture was mixed and then incubated at 30 °C for 2 min. After incubation, the absorbance at 340 nm was measured with a UV-visible spectrophotometer. Thereafter, 200 μL of formate dehydrogenase (5.27 U/mL, Sigma-Aldrich, St. Louis, MO, USA) was added and incubated for 30 min. The absorbance at 340 nm was also measured after the incubation. The changes in absorbance were calculated, and the amount of generated formate was obtained. One unit of OxDC activity equals the formation of 1 μmol formate from 1 μmol of oxalate per minute at pH 3.0 and 30 °C. The experiments were repeated in triplicates, and the mean values were given.

Total OxDC activity was defined as gross activity in crude enzyme extract obtained from one bottle of culture (50 mL). Specific activity I was that obtained from 1 g dry mycelial

weight, and specific activity II was that obtained from 1 mg intracellular protein. Volumetric activity and OxDC productivity were calculated based on Eqs. 1 and 2.

$$\text{Volumetric activity} = \frac{\text{Total OxDC activity(U)}}{\text{Total volume of culture media(L)}} \quad (1)$$

$$\text{OxDC productivity} = \frac{\text{Volumetric activity(U/L)}}{\text{Induction time(day)}} \quad (2)$$

Analysis of Oxalic Acid by HPLC

The oxalic acid concentration in the cultures was analyzed using a LC2000 HPLC system (Techcomp, Shanghai, China) equipped with a chromatographic column Aminex HPX-87H (300×7.8 mm, 9 μm, Bio-Rad, Hercules, CA, USA) and a UV detector (210 nm) at 25 °C with 5 mmol/L $H_2SO_4$ as mobile phase at a flow rate of 0.5 mL/min. The samples were filtered through membrane filters (0.45 μm). Twenty microliters of filtrate sample was injected into the HPLC system. All analyses were carried out in duplicates, and mean values are presented.

## Results and Discussion

Effects of Inorganic Acids on the Growth of *T. versicolor*

The effects of inorganic acids on the growth of *T. versicolor* were investigated by adding inorganic acids to pH 2.5–3.0 in the culture media. The pH values and residual glucose concentration of culture media, the dry weight of mycelia, and total protein concentration of the crude enzyme extracts were measured. The results are shown in Fig. 1. Table 1 shows the difference in mycelial pellet size and color in the end, changes in pH values, average sugar consumption rate after acid addition, mycelial growth rate, and protein in the crude enzyme extracts.

The pH values of the control decreased from 5.0 to 3.3 during the first 4 days, then changed very slowly (around 3.0–3.2). After addition of inorganic acids, the pH values decreased from 3.9 to 2.6, and little change (2.5–2.8) was found during the latter cultivation (Fig. 1a).

The residual glucose concentrations of the control decreased continuously from 20.0 g/L (initial) to 2.6 g/L (day 9) during the cultivation stage, with an average sugar-consuming rate of 1.94 $g \bullet L^{-1} \bullet day^{-1}$ (Table 1). The residual glucose concentrations in the acid-added cultures had the same change trend as the control, but the concentrations were higher than that of control. The results showed that the sugar-consuming rate of the sulfuric-acid-added culture was lowest, only 0.98 $g \bullet L^{-1} \bullet day^{-1}$, and the sugar consumption of the cultures in the order: control>phosphoric-acid-added>hydrochloric-acid-added>sulfuric-acid-added (Fig. 1b and Table 1). This is because the pH value of the sulfuric-acid-added culture was lowest, which affected the sugar consumption negatively.

The dry mycelial weights of the acid-added cultures were lower than that of the control, which means the growth of mycelia was inhibited slightly by all inorganic acids. The dry mycelial weight of the phosphoric-acid-added culture was maximal among the three acid-

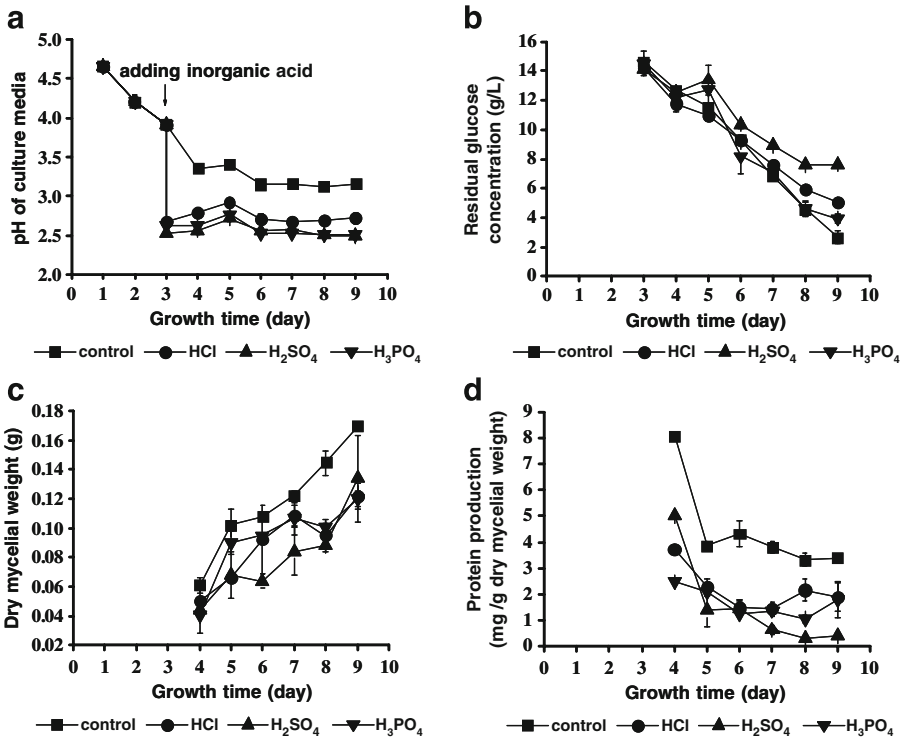


**Fig. 1** Effects of inorganic acids on the growth of *T. versicolor*. **a** pH of culture media; **b** residual glucose concentration; **c** dry mycelial weight; **d** total protein production. Three parallels were carried out, and mean values were given. The *bars* show the absolute deviations

added cultures, but the mycelial weight of the sulfuric-acid-added culture was minimal (Fig. 1c). Combined with Fig. 1a–c, it is concluded that the pH value affected the growth of *T. versicolor* biomass negatively if pH value of culture media was lower than 3.0. The lower the pH value was, the less biomass amount obtained. Biomass of the control and the phosphoric-acid-added culture reached peaks at day 12, but biomass of the hydrochloric-acid-added and sulfuric-acid-added cultures grew continuously until day 16 (data not shown).

**Table 1** Effects of inorganic acids on the growth of *T. versicolor*.

| Items | Control | Hydrochloric acid | Sulfuric acid | Phosphoric acid |
|---|---|---|---|---|
| Mycelial pellet size and color in the end | 5.0 mm diameter, milk white | 1.6 mm diameter, dark gray | 1.8 mm diameter, gray | 3.4 mm diameter, white |
| Changes in pH values | pH 3.2–3.4 | pH 2.6–2.9 | pH 2.5–2.7 | pH 2.6–2.7 |
| Average sugar-consuming rate ($g \cdot L^{-1} \cdot day^{-1}$) | 1.94 | 1.35 | 0.98 | 1.65 |
| Mycelial growth rate (g/day) | 0.022 | 0.014 | 0.011 | 0.016 |
| Protein in enzyme extract (μg) | 350–500 | 150–250 | 30–80 | 100–250 |

The total protein concentration of crude enzyme extract from the control was highest (Fig. 1d). This may be because all inorganic acids would inhibit protein production in mycelia heavily.

## Effects of Inorganic Acids on OxDC Production

The results with regard to the effects of inorganic acids on OxDC production are shown in Fig. 2 and Table 2. The total activities of OxDC induced by the inorganic acids were very similar and rose continuously in the late growth stage, but OxDC activity of the control was almost not detected, which means that inorganic acid could induce OxDC obviously (Fig. 2a). Further experimental results indicated that total inorganic-acid-induced OxDC activity peaked at day 10 or 12 (data not shown). Among the three acid-added cultures, the specific activity I against dry mycelial weight was similar. It was 10.6 (hydrochloric acid), 10.0 (sulfuric acid), and 9.6 (phosphoric acid) U/g dry mycelial weight at day 9, respectively (Fig. 2b). However, the specific activity II against protein was different. It was 1.2 (hydrochloric acid), 3.2 (sulfuric acid), and 1.6 (phosphoric acid) U/mg protein at day 8 (Fig. 2c), respectively.

So far, oxalic acid has been regarded as the best inducer for OxDC production. In order to compare the effects of inorganic acids on the production of OxDC, the activity induced by oxalic acid was studied in this paper as well. The result showed that the total OxDC activity induced by oxalic acid reached the highest during the first 2 days after acid addition

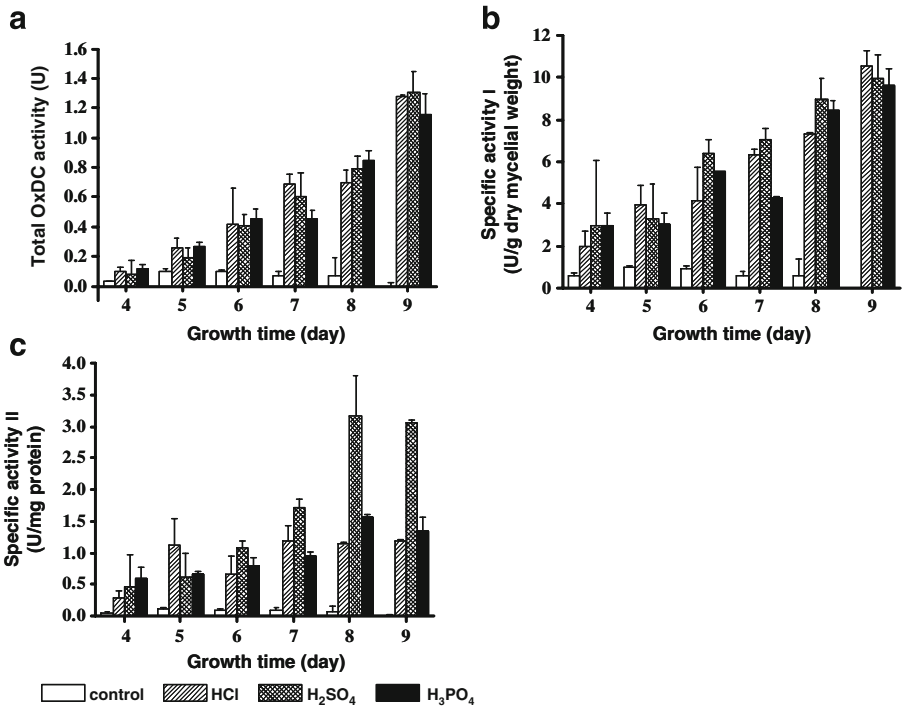


**Fig. 2** Effects of inorganic acids on OxDC activity. **a** Total OxDC activity; **b** specific activity I (U/g dry mycelial weight); **c** specific activity II (U/mg protein). Three parallels were carried out, and mean values were given. The *bars* show the absolute deviations

**Table 2** Effects of inorganic acids on the production of OxDC.

| Items | Control | Oxalic acid (2 days)[a] | HCl (6 days)[a] | $H_2SO_4$ (6 days)[a] | $H_3PO_4$ (6 days)[a] |
|---|---|---|---|---|---|
| Inducer concentration (mM) | 0 | 2.7 | 5.5 | 3.6 | 9.1 |
| Volumetric activity (U/L) | ND | 8.6 | 25.6 | 26.0 | 23.2 |
| OxDC productivity ($U•L^{-1}•day^{-1}$) | ND | 4.3 | 4.3 | 34.3 | 3.9 |

[a] Induction duration. ND: not detectable. The pH in the oxalic acid-added culture changed from 2.9 to 3.4

and then decreased quickly in the following days. The pH value in the oxalic-acid-added culture recovered to the same level as the control quickly in 1 day and then changed just like the control did (data not shown). This is because the added oxalic acid was degraded by the induced OxDC quickly because oxalic acid is the substrate of OxDC. The pH in the oxalic-acid-added culture subsequently increased after the added acid was consumed.

The result significantly indicated that the production of enzyme in the mycelium was affected by the pH value of culture media. In the range of pH 2.5–3.0, the specific activities of the acid-induced OxDC were higher if the pH values in the culture were lower. Figure 2 and Table 2 showed clearly that the effects of the inorganic acids on the production of OxDC were similar, especially on OxDC productivity (around 4.0 $U•L^{-1}•day^{-1}$, Table 2), though low pH slightly inhibited the biomass growth (Fig. 1 and Table 1). Among all the cultures, the volumetric OxDC activities induced by inorganic acids were higher than 20 U/L, which were two times higher than that induced by oxalic acid (Table 2).

After adding inorganic acids, the pH values of media were around 2.5 and changed weakly during the following days. The production of OxDC might be affected by hydrogen ion concentration in the media. The influence might be environmental stress and biological defense reactions. When the hydrogen ion concentration increased to have the growth environment changed to acidic, in order to survive, cells must produce many OxDC and probably some other enzymes to try to adapt or change the environment [23]. In order to study the effects of hydrogen ion concentration in the culture media further, more experiments were made as follows.

## Effects of pH Values on Mycelial Growth and OxDC Production

Since it affects the biomass growth among the studied acids less, phosphoric acid was selected to adjust pH values in the media to pH 2.5, 2.0, and 1.5, respectively. The results showed that the growth of biomass at pH 2.0 and 1.5 was inhibited severely (Fig. 3b), and only the activity of OxDC produced at pH 2.5 could be detected (Fig. 3c).

Dutton and Evans [1] reported that in white-rot fungi, the pH of the media continues to rise slowly throughout the growth phase despite oxalate accumulation, indicating that a buffering system is in action in the media. After adding inorganic acids in our study, the pH of the media decreased to lower values and changed weakly, while the pH value in the oxalic-acid-added culture recovered to the same level as the control quickly in 1 day and then changed just like the control. This difference implies that the buffering system may be efficient for the generated organic acids, including oxalic acid from microorganism, but may have no function on the added inorganic acids. Azam et al. [19] found that the induction of OxDC from *Flammulina velutipes* was pH dependent and the *F. velutipes* OxDC gene was induced by acidic pH but not by exogenous oxalate. The increased

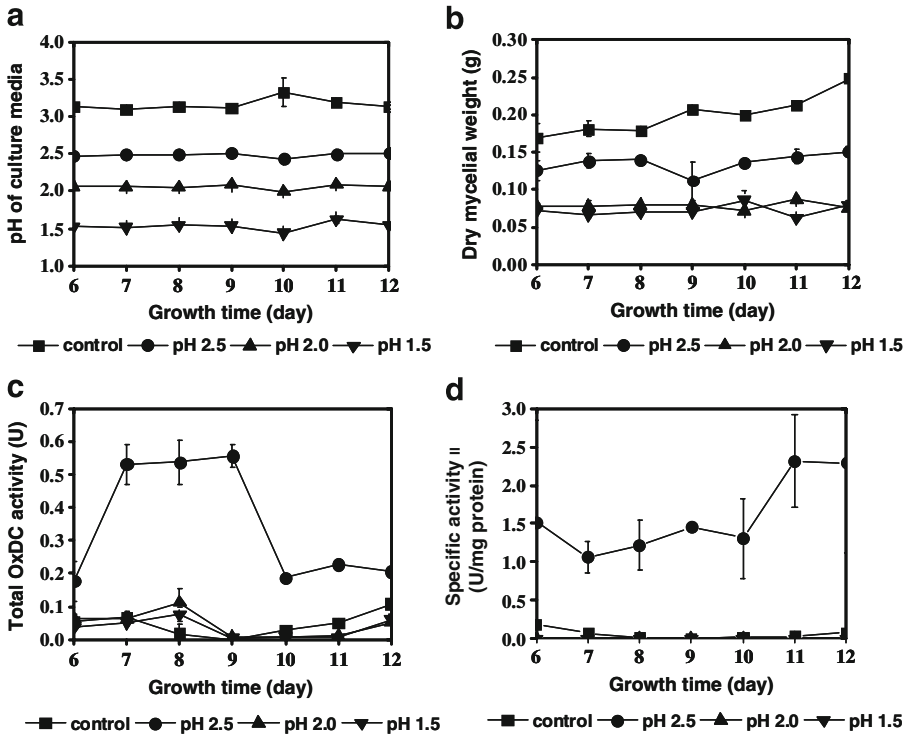


**Fig. 3** Effects of pH on mycelial growth and OxDC production. **a** pH of culture media; **b** dry mycelial weight; **c** total OxDC activity; **d** specific activity II (U/mg protein). Three parallels were carried out and mean values were given. The *bars* show the absolute deviations

transcription of the gene encoding OxDC in *F. velutipes* in response to low pH may be mediated by the interaction of a specific transcription factor with the OxDC promoter [19]. Our new finding indicates that both inorganic acids and oxalate are capable of inducing OxDC efficiently.

The oxalic acid concentration was found to be lower than 1 mM or even lower (data not shown) in the inorganic acid-added cultures. Oxalic acid is an organic acid, whose proton-releasing capacity is much weaker than the strong inorganic acids. The fact that low concentration (<1 mM) and weak proton-releasing capability of oxalic acid ruled out the possibility that oxalate decarboxylase would be induced by oxalic acid accumulation in late growth stage in the acid-added cultures. The result further demonstrates that the regulatory metabolism in the inorganic-acid-induced OxDC production is attributed to the low pH environment, which is different from the mechanism of substrate-induced OxDC production using oxalate/oxalic acid.

## Conclusion

The results showed that hydrochloric, sulfuric, and phosphoric acid could induce OxDC expression in *T. versicolor* obviously, though the biomass growth was inhibited in different degree. All of the OxDC volumetric activities induced by inorganic acids were higher than 20.0 U/L, which were two times higher than that induced by oxalic acid. All OxDC

productivities were around 4.0 $U \cdot L^{-1} \cdot day^{-1}$, similar with that of oxalic acid induced. Although the growth of mycelia was inhibited slightly when the pH value in culture media was around 2.5–3.0, the growth was heavily inhibited when the pH was lower than 2.5, and subsequently no activity was found. Among these acids, sulfuric acid had the best effect on specific activity production. The highest specific activity against total protein was 3.2 U/mg protein induced by sulfuric acid at day 8, and the highest specific activity against dry mycelial weight was 10.6 U/g induced by hydrochloric acid at day 9.

This work is the first detailed study of the induction of OxDC in *T. versicolor* by the addition of inorganic acids. The new finding indicated that both inorganic acids and oxalate/oxalic acid could induce OxDC efficiently. It is suggested that the production of OxDC may be induced by a low pH environment by the addition of inorganic acids, which is different from the oxalate substrate induction mechanism. In the study, the induction of OxDC was only explored in shaking flask cultivations, where the pH values were not able to be regulated accurately; therefore, the effects of pH on the production of *T. versicolor* OxDC in fermenters would be investigated in detail in future studies.

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