



Oxalate fermentation by wood-rotting fungi vs. normal respiration

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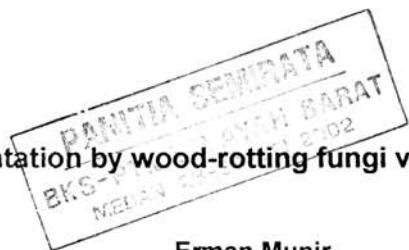
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Abstract

Oxalic acid, the simplest dicarboxylic acid, has been receiving much attention from different aspects of wood-decaying processes. A new biosynthetic pathway of the acid in the wood-rotting fungus *Fomitopsis palustris* has been discovered; glucose was mainly fermented to oxalate (COOH)₂ rather than oxidized to CO₂ and H₂O. Enzymatic analyses showed that the fungus lacks of the decarboxylation steps as required for the normal respiration. Therefore this paper describes the differences between these two systems in acquiring biochemical energy needed for the fungal growth.

Key words: Oxalic acid biosynthesis; wood-rotting fungi; decarboxylation; biochemical energy; *Fomitopsis palustris*

Introduction

Metabolic pathway of oxalic acid, which is widely found in various plants, culture of molds, and human urine as the end product, has long been investigated from a medicinal viewpoint (Hodgkinson 1977). In addition to the strong acidity with pK_{a1} of 1.23 and pK_{a2} of 3.83, oxalic acid is also a strong chelating agent to precipitate many metal ions, including calcium ions (Voet and Voet 1990). Therefore, it has been receiving much attention from various ecological aspects.

Wood-rotting basidiomycetes, the principal organisms able to degrade all wood components, are group into white- and brown-rot fungi on the basis of physiological and biochemical features with respect to their ability to degrade wood and to accumulate oxalate. The white-rot fungi can completely mineralize all cell wall components. However, the brown-rot ones are unique, they mineralize cellulose and hemicellulose without decomposing lignin (Crawford 1981; Green and Highley 1997). The brown-rotters produce large amounts of oxalate, which is accumulated in the culture media, whereas the white-

rotters do not accumulate the acid but rather decompose it to carbon dioxide (Akamatsu *et al.* 1993; Green and Clausen 1999).

Nevertheless although the production of oxalic acid by wood-rotting fungi has been widely reported, the biosynthetic pathway of the acid has hardly been known in these organisms, except in following report. By using the brown-rot fungus *Fomitopsis palustris* as a model fungus, it has been discovered the unprecedented metabolic pathway in which the fungus mainly fermented glucose to oxalate rather than oxidized it to carbon dioxide. Uniquely, enzymatic analyses showed that the fungus did not operate the normal respiration pathway as commonly known for other organisms (Munir *et al.* 2001a). Here, it is discussed the energy acquiring steps of oxalate biosynthesis in comparison to normal respiration.

Materials and Methods

Chemicals. All chemical reagents were of reagent grade.

Organism and growth conditions. The oxalate-producing basidiomycete *Fomitopsis palustris* known as a copper-tolerant fungus was used in this investigation. The fungus was cultivated on a modified Kirk medium with 2% (w/v) glucose and 24 mM ammonium tartarate as the carbon and nitrogen sources, respectively (Kirk *et al.* 1978). Cultivation condition was described in the previous report (Munir *et al.* 2001b).

Analysis of glucose, oxalate, and other organic acids. Amount of glucose consumed, oxalic and acetic acids produced during cultivation was determined enzymatically by using the kits provided by Boehringer (Mannheim, Germany). GC-MS analysis (Shimadzu GC-MS QP-5050A) was also performed to determine other organic acids including oxalic acid.

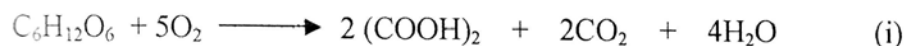
Preparation of cell-free extracts and enzyme assay. Cell-free extracts were prepared from the fungal mycelia in the same way as previously described (Munir *et al.* 2001b). Enzyme activity in the fungal extracts was determined spectrophotometrically (Hitachi model U-3000, Hitachi, Tokyo) equipped with a temperature controller and external recorder. Each of the enzyme assays was carried out on the basis of the reported or modified methods, as listed in Table 1. Protein content was determined by the Bio-Rad method with bovine serum albumin as the standard (Bradford 1975).

The enzyme activities were expressed in terms of total activity ($\mu\text{mol min}^{-1} \text{ culture}^{-1}$) and specific activity ($\mu\text{mol min}^{-1} \text{ mg}^{-1} \text{ protein}$), unless otherwise stated. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol product per minute or the consumption of 1 μmol substrate per minute under the conditions described.

Results and Discussion

Glucose consumption and oxalic acid formation during cultivation

Taking advantage of the fast growing wood-rotting basidiomycete *Fomitopsis palustris* known to produce oxalate, the amount of glucose consumed and oxalate produced was determined during the cultivation period. Results (Table 2) show that there is a positive correlation between the glucose consumption and oxalate formation; decrease of glucose followed with the accumulation of oxalic acid in the culture fluid, which was also consistent with increase of acidity indicating with the reduction of pH (from 5.0 at the beginning of growth to 2.08 at the end of cultivation). Furthermore, the fungus exhibited active growth at the early cultivation reaching the maximum biomass of 1.5 g at day 13 (data not shown). At this cultivation time, the amounts of glucose consumed and oxalic acid produced were 10.505 and 7.048 g, respectively. On the basis of glucose consumed, the ratio of conversion of glucose to oxalate and biomass was 67, and 14 %, respectively, and the rest of 19% might be oxidized to carbon dioxide or other metabolites. These results strongly suggest that glucose was not completely oxidized to CO_2 through the normal respiration pathway. Therefore, on the basis of higher conversion of glucose to oxalic acid, the following equation has been proposed (Munir *et al.* 2001a):



The above equation is quite different from the normal respiration equation as shown below:



In normal respiration, as commonly known (eq. ii), 6 mol oxygen are required to completely oxidized 1 mol glucose. However, 5 mol oxygen are needed to oxidized glucose yielding 2 mol oxalate, 2 mol CO₂ and 4 mol H₂O. Thus it is speculated that 4 mol carbon dioxide, which should be respired through the normal respiration, are converted to 2 mol oxalic acid. Therefore, it reveals that the fungus conducts oxalate fermentation (eq. i), probably for the energy acquisition. GC-MS analysis has also confirmed; no any other organic acid was detected in the culture fluid other than oxalic acid. Interestingly, the oxalate fermentation equation (eq. i) has never been proposed in other study, although the oxalate fermentation process has been introduced since the nineteen century. Thus it is important to ask why the fungus converted glucose to mainly oxalic acid rather than to carbon dioxide and what is biochemical role of its biosynthesis.

Physiological role of oxalic acid biosynthesis

To answer the above curiosities, at first, the activity of decarboxylating enzymes, isocitrate dehydrogenase (IDH) and 2-oxoglutarate dehydrogenase (ODH) both the common enzymes in Krebs cycle, were investigated in comparison with the activity isocitrate lyase (ICL) known as cleavage enzyme usually located in glyoxisomes, because IDH and ICL, the two branch point enzymes, compete for the same substrate, isocitrate. Figure 1 shows the profiles of total (A) and specific (B) activity during cultivation time indicating that ICL activity was much greater than IDH activity, which sharply contrasts with the reported findings that ICL was not or little detected, whereas IDH was predominant when microorganisms were grown on glucose (Rua *et al.* 1989; Schmidt *et al.* 1996). Much greater ICL activity (up to 12 times) than that of IDH was observed when *F. palustris* was cultivated on the different growth conditions. Importantly, ODH as the other decarboxylating enzyme was not detected at all cultivation time. All these results demonstrate that *F. palustris* does not operate the classical Krebs TCA cycle, which oxidizes 1 mol acetyl-CoA to 2 mol carbon dioxide catalyzed by IDH and ODH (dotted line in Figure 2, cycle A), but used the modified TCA cycle (solid line cycle A); the biochemical role of ICL in the cycle is to cleave isocitrate yielding succinate and glyoxylate. Therefore, it clearly shows that there is no decarboxylation process in this cycle, which is also consistent with the Equation (i). A small amount of oxoglutarate formed after decarboxylation by IDH may be bypassed to succinate via γ -aminobutyrate

(GABA) route as a minor pathway, as found in other fungi (Moore 1984; Kumar and Puneekar 1997).

Furthermore, because it was previously known that the fungus has two oxalate producing enzymes, glyoxylate dehydrogenase (GDH) and oxaloacetase (OXA), their activities were also measured in addition to other TCA and glyoxylate cycle enzymes. The results, as in Table 3, show that the activity of OXA was much greater than the activity of GDH suggesting that most oxalate was produced from oxaloacetate formed by oxidation of malate catalyzed by malate dehydrogenase (MDH). In fact, the profile of all enzyme activity in Table 3 was determined through the culture period but results are not shown in this report. Interestingly, the activity of the key enzymes of glyoxylate cycle, ICL and malate synthase (MS), were detected at high levels, which sharply contrast with most reported cases as described above. Therefore, both enzymes have been purified and characterized for the first time for any basidiomycetes in the series of this investigation (Munir *et al.* 2002a,b). The reason for high activity of MDH ($6548 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein) may be that the enzyme is required at four reaction sites as shown in Figure 2. Furthermore, acetyl-CoA synthase catalyzing the conversion acetate to acetyl-CoA was also detected to a significant level. Although its activity was relatively lower than the other detected enzymes, it suggests that the enzyme plays an important role to bring the acetate liberated from oxaloacetate back to the glyoxylate cycle. Evidently, this metabolic pathway explains why acetate was not detected in the culture fluid.

Based on enzymatic analyses for the glucose metabolism, a new metabolic pathway of oxalic acid biosynthesis has been discovered, as shown in Figure 2. The metabolic pathway consists of modified TCA (A) and glyoxylate (B) cycles, which are interdependent each other and also coordinate with acetate-recycling routes (C and D). The physiological role of this metabolic pathway is to oxidize acetyl-CoA to yield oxalate, which accumulates in the culture media. Theoretically, 2 mol acetyl-CoA derived from 1 mol glucose through glycolysis shunt are finally converted to 2 mol oxalate in recycling route C and D. Furthermore, MDH which has the highest activity plays an important role in generating biochemical energy (NADH) by oxidation of oxaloacetate, which is the major precursor of oxalic acid. Therefore, it is suggested that oxalic acid biosynthesis is coupled with energy formation. With the assumption that no other metabolic pathway oxidized acetyl-CoA, the over all oxidation of 2 mol acetyl-CoA to 2 mol oxalic acid generates 14 ATPs, whereas 24 ATPs are formed in normal respiration by classical TCA

cycle. If it is considered that acetyl-CoA derived from glucose is obtained through the common glycolysis pathway, the net yield ATP from the conversion of 1 mol glucose to 2 mol oxalic acid will be 28 ATPs or 10 ATPs less than the ATP generated by normal respiration pathway. Thus, it has been speculated that the more oxalic acid produced, the greater biochemical energy will be acquired.

Finally, this study provides new evidence for metabolic pathway of oxalic acid biosynthesis, termed as oxalate fermentation, consisting of the modified TCA and glyoxylate cycles, which is quite different from the normal respiration process. The wood-rotting fungus *F. palustris* acquires biochemical energy needed for the growth by oxidizing glucose to oxalate.

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Table 1. Method for enzymatic analyses

Enzymes	Methods
1. Isocitrate lyase	Modified of Dixon and Kornberg (1959)
2. Isocitrate dehydrogenase	Moore and Ewaze (1976)
3. 2-Oxoglutarate dehydrogenasae	Moore and Ewaze (1976)
4. Succinate dehydrogenase	Modified of Moore and Ewaze (1976)
5. Fumarase	Cooper and Beavers (1969)
6. Malate dehydrogenase	Laboru and Clonis (1997)
7. Citrate synthase	Modified of malate synthase assay
8. Aconitase	Kenedy <i>et al.</i> (1983)
9. Malate synthase	Modified of Dixon and Kornberg (1959)
10. Oxaloacetase	Akamatsu <i>et al.</i> (1991)
11. Acetyl-CoA synthase	Londesborough <i>et al.</i> (1973)
12. Glyoxylate dehydrogenase	Tominatsu <i>et al.</i> (1998)

Table 2. Consumption of glucose and production of oxalic acid by *Fomitopsis palustris*

Culture days	Glucose consumed (g/l)	Oxalic acid produced (g/l)	pH	Ratio of glucose to oxalate conversion (%)
2	0.966	0.126	4.65	13
4	2.667	0.843	4.05	32
7	4.144	2.304	3.40	56
10	5.870	4.326	2.63	74
13	10.505	7.048	2.08	67

Table 1. Activities of the enzymes involved in ABC cycles

Number	Enzyme	Activity (nmole min ⁻¹ mg ⁻¹ protein) ^a
1 ^b	Isocitrate lyase (ICL)	501
2.	Isocitrate dehydrogenase (IDH)	82
3.	2-Oxoglutarate dehydrogenase (ODH)	0
4.	Succinate dehydrogenase	11
5.	Fumarase	365
6.	Malate dehydrogenase (MDH)	6548
7.	Citrate synthase	1115
8.	Aconitase	494
9.	Malate synthase (MS)	440
10.	Oxaloacetase (OXA)	166
11.	Acetyl-CoA synthase	17
12.	Glyoxylate dehydrogenase (GDH)	22

^a All values are obtained from 7 days old cultures, except acetyl-CoA synthase which was obtained from 2 days old cultures.

^b The number of the enzyme corresponds with the enzyme catalyzes each reaction site numbered in Figure 2.

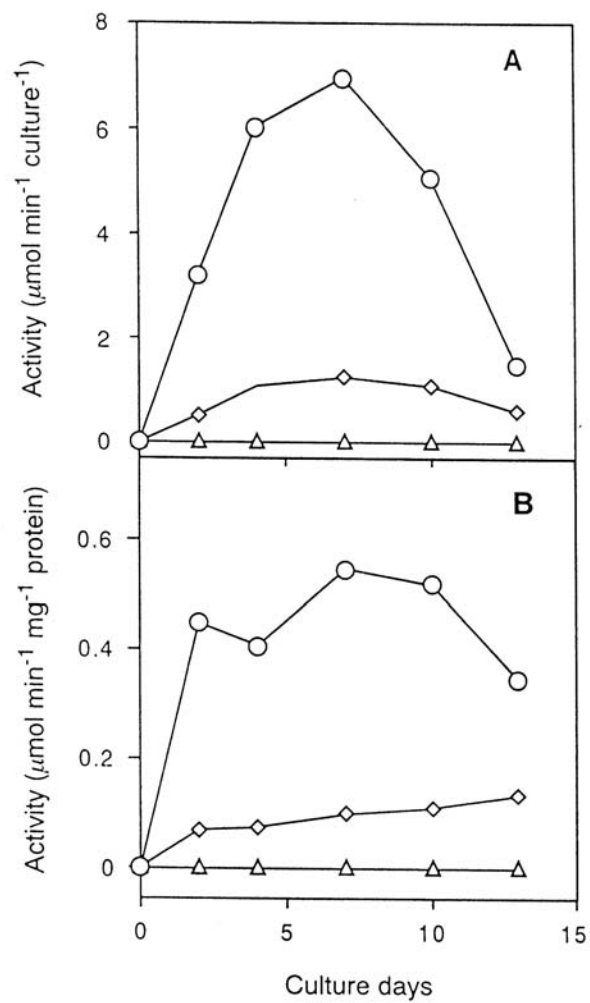


Figure 1. Profile of total (A) and specific (B) activities of ICL, IDH, and ODH during cultivation of *F. palustris*. Circles, ICL; diamonds, IDH; triangles, ODH.

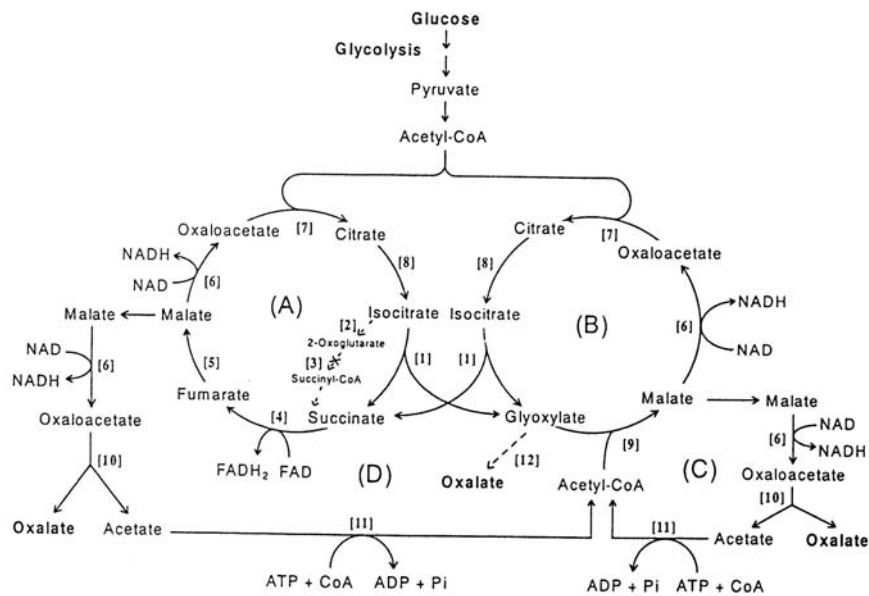


Figure 2. A proposed metabolic pathway for oxalic acid biosynthesis in the wood-rotting fungus *F. palustris*. (A), the modified TCA cycle; (B), glyoxylate cycle; (C) and (D), acetate-recycling-routes. (Munir *et al.* 2001a)