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COMMUNICATION

Investigating Oxalate Biosynthesis in Wood-decaying Fungus *Gloeophyllum trabeum* using ^{13}C Metabolic Flux Analysis

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Oxalate synthesis was rigorously investigated in a wood-decaying fungus, *Gloeophyllum trabeum*, using ^{13}C metabolic flux analysis, a method not previously explored in this type of system. Peroxisomal glyoxylate dehydrogenase and cytosolic oxaloacetate acetylhydrolase were found to contribute to the majority of oxalate synthesized under low and high nitrogen conditions, respectively.

Lignocellulose is the most abundant biomass resource in terrestrial environments[1], and wood fiber has seen growing use as an industrial feedstock for value-added chemical and biofuel production[2]. However, the recalcitrant structure of lignocellulosic biomass prevents it from being converted to fermentable sugar efficiently and research in this area is still needed. Brown rot fungi are the only organisms known that can depolymerize crystalline cellulose without removal of lignin[3]. *Gloeophyllum trabeum*, a brown rot fungus with high polysaccharide depolymerization activity has promising industrial application prospects. For example, it has been used in a corn stover pretreatment step for ethanol production[4], and also in a saccharification step in ethanol production via simultaneous saccharification and fermentation (SSF)[5]. A non-enzymatic oxidative "chelator-mediated Fenton" (CMF) system is reported to be involved in brown rot fungi cellulose depolymerization processes[6], but many aspects of how this system works are unknown. The process, involves the secretion of the low molecular weight metabolites, oxalate and hydroquinone/catecholate compounds to first sequester and then reduce iron in a sequence, which results in generation of Fenton chemistry at a distance from the fungal hyphae within the lignocellulose cell wall. A highly reactive hydroxyl radical is generated and has been demonstrated to be responsible for the deconstruction of the wood cell wall in brown rot non-enzymatic decay[7-9]. Previous research on the detection of oxalate and catechols in brown rot fungal liquid culture medium in the absence of wood has demonstrated that these compounds are generated by the fungus rather than being derivatives of wood [10-12]. Due to the important role that oxalate and catecholates play in brown rot fungal degradation mechanisms, study of their biosynthetic pathways will provide novel insight about how brown rot fungi regulate the production of these compounds to break down the recalcitrant structure of lignocellulose biomass.

In this study, we applied ^{13}C Metabolic Flux Analysis (^{13}C-MFA), a technique that has been widely applied to reveal previously unknown metabolic pathways in various non-model organisms[13-17], to provide valuable insight into the intracellular metabolisms of *G. trabeum* under different culture conditions[18-20]. We found that the major source for intracellular oxalate synthesis varied under different culture conditions. While peroxisomal glyoxylate dehydrogenase contributed to the majority of oxalate synthesized under low nitrogen conditions, cytosolic oxaloacetate acetylhydrolase was found to be the main pathway for oxalate synthesis when culturing *G. trabeum* in a high concentration of nitrogen. To our best knowledge, this is the first time that oxalate biosynthesis metabolism in *G. trabeum* has been rigorously and quantitatively determined by ^{13}C trace experiments using the metabolic flux analysis approach. Research in this area will facilitate future work in increasing the degradation efficiency for bioconversion of lignocellulosic biomass by *G. trabeum* and related organisms.

Table 1. Growth behaviour of *G. trabeum* under different nitrogen conditions.

Nitrogen Conditions	Growth rate (day^{-1})	Glucose uptake (g/g DCW/day)
LN	0.14±0.03	0.24±0.002
HN	0.15±0.03	0.15±0.001

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G. trabeum was cultured statically, in 150mL flasks at 36 ℃ in 20mL liquid medium containing the following basal salts per liter: 2g NH_4NO_3, 2g KH_2PO_4, 0.5g $MgSO_4•7H_2O$, 0.1g $CaCl_2•2H_2O$, 0.57mg H_3BO_4, 0.036 mg $CuSO_4•5H_2O$[21], and 1% glucose. All glassware was acid-washed and Fe and Mn was omitted to promote production of secondary metabolites. After 20 days, the medium in three flasks was homogenized and sterile water was added to bring the total volume to 150mL. The homogenate was diluted 1:40 and a 0.5 ml aliquot was used to inoculate a ^{13}C-glucose medium containing low nitrogen (LN), or high nitrogen (HN). These cultures contained 1% ^{13}C-glucose (a mixture of 80% [1-^{13}C] and 20% [U-^{13}C] glucose)[10] and the basal salts listed above with either 25mM NH_4NO_3 (HN) or 2.5mM NH_4NO_3 (LN). In separate work with HN and LN cultures using non-labeled glucose, growth was analysed by weighing the dried mycelial mass at 3-5d intervals, and HPLC of the culture filtrate was also conducted to assess glucose consumption (Table 1). The ^{13}C-labeled fungal cultures were harvested during the exponential growth phase, after 14 days. Our cultivation conditions were different to those previously used by Varela and Tien[22] in part to avoid uncontrolled levels of iron in the media and so that nitrogen levels could be modified.

Following harvest, isotopomer analysis of proteinogenic amino acids and intracellular oxalate was conducted using a previously developed protocol [23-25]. In brief, the biomass was hydrolyzed using 6 M HCl (20h at 100 °C) and the amino acids and intracellular oxalate were silyated using 50 µl tetrahydrofuran and 50 µL N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide (Sigma-Aldrich) to form tert-butyl dimethylsilyl (TBDMS) derivatives. Gas Chromatography-Mass Spectrometry (GC-MS) was performed to analyse fungal metabolites using a Shimadzu GC2010 GC with a SH-Rxi-5Sil column and a Shimadzu QP2010 MS. Three types of charged fragments were detected by GC-MS for various amino acids (Table S1): the $[M-57]^+$ group (containing unfragmented amino acids); and the $[M-159]^+$ or $[M-85]^+$ group (containing amino acids that had lost an α-carboxyl group). For each type of fragments, the labeling patterns, mass distribution vectors (MDVs), were represented by M0, M1, M2, etc., which were fractions of non-labeled, singly-labeled, and doubly-labeled amino acids. The effects of natural isotopes on isotopomer labeling patterns were corrected by previously reported algorithms[26]. MDVs were used to calculate the summed fractional labelling (SFL) values which were directly used in the MFA (Biomet Toolbox 2.0[27], based on MATLAB, MathWorks, Inc. MA). The central carbon metabolic model for the MFA was developed based on the KEGG database (http://www.genome.jp/kegg/) and previous reports on metabolic pathways of brown rot fungi[28, 29], which included glycolysis pathways, pentose phosphate pathways, futile pathways, the tricarboxylic acid (TCA) cycle, the glyoxylate cycle in peroxisomes, cytosolic oxaloacetate acetylhydrolase and transport pathways among different cell compartments (Fig. 1A). Of particular interest, two oxalate biosynthesis pathways, i.e., cytosolic oxaloacetate acetylhydrolase (OAH) and peroxisomal glyoxylate dehydrogenase (GLX)[30] were examined in our metabolic models (Fig. 1A) to assess oxalate biosynthesis. These two pathways have been reported to contribute to fungal extracellular oxalate accumulation in other species[31]. Although little research has been done to elucidate the genes associated with oxalate biosynthesis pathways in *G. trabeum*,

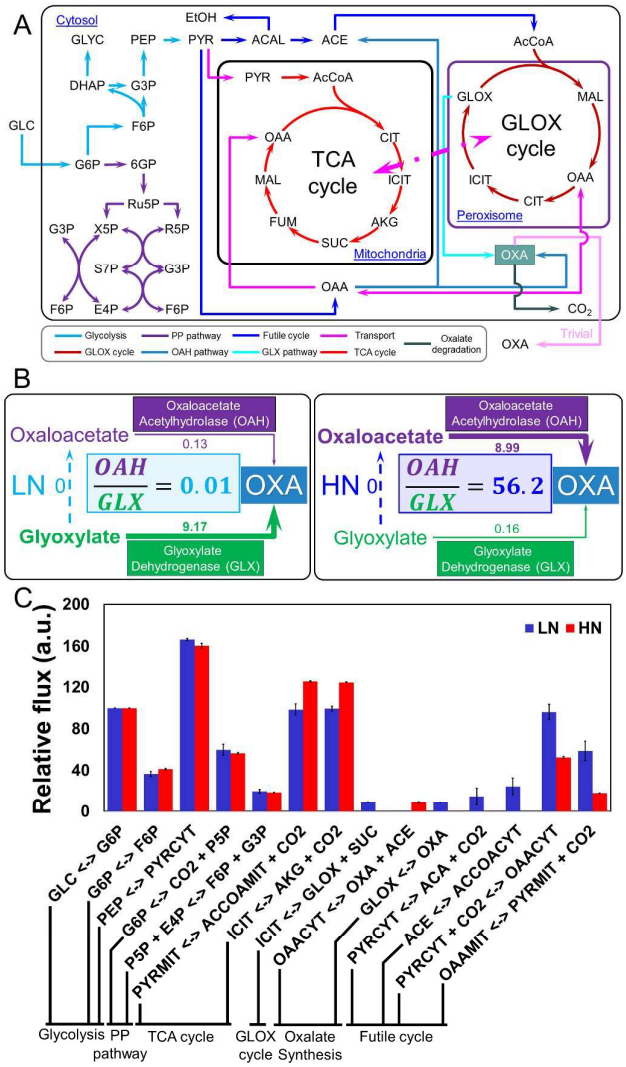

Fig 1. Metabolic pathway map and key flux distributions for *G. trabeum* under different C/N ratios. (A) Central carbon metabolic pathways and oxalate synthesis pathways of *G. trabeum*. Transport fluxes between mitochondria and peroxisome were simplified to a single dash-dot line. (B) The flux ratio between OAH and GLX pathways for LN and HN conditions with the OAH/GLX ratio highlighted. (C) Key metabolic flux distributions for *G. trabeum* under LN and HN conditions.

Abbreviations: GLC: Glucose, 6PG: 6-Phosphogluconate, ACAL: Acetaldehyde, AcCoA: Acetyl-CoA, ACE: Acetate, AKG: α-Ketoglutarate, CIT: Citrate, DHAP: Dihydroxyacetone phosphate, E4P: Erythrose 4-Phosphate, EtOH: Ethanol, F6P: Fructose 6-phosphate, FUM: Fumarate, G3P: Glyceraldehyde 3-phosphate, G6P: Glucose 6-Phosphate, GLOX cycle: Glyoxylate cycle, GLYC: Glycerol, GLOX: Glyoxylate, ICIT: Isocitrate, MAL: Malate, OAAMIT/OAACYT: Oxaloacetate (Mitochondria/Cytosol), OXA: Oxalate, PEP: Phosphoenolpyruvate, PYRMIT/PYRCYT: Pyruvate (Mitochondria/Cytosol), R5P: Ribose 5-Phosphate, Ru5P: Ribulose 5-Phosphate, S7P: Sedoheptulose 7-phosphate, SER: Serine, SUC: Succinate, TCA cycle: Tricarboxylic acid cycle, X5P (P5P): Xylulose 5-phosphate.

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the homologs of oxaloacetate acetylhydrolase (fpOAH) and glyoxylate dehydrogenase (fpGLOXDH) in *Fomitopsis palustris* have been indicated in the *G. trabeum* genome using Blastp[32] with 77% and 67% similarity respectively, which suggested these two pathways could potentially be exploited by *G. trabeum* for oxalate synthesis. For our metabolic flux analysis, flux estimation was repeated at least 50 times starting with different initial values generated by a genetic algorithm for all fluxes to find a likely global solution. A fit of the simulated and measured summed fractional labelings was determined to be a global solution only after the solution fit was obtained at least twice using this method (Fig. S1).

In addition to the MDVs detected by GC-MS, we also used a similar protocol to detect oxalate concentrations for both soluble and insoluble oxalate from the cultivation medium and cell biomass, respectively. To measure the concentrations of soluble oxalate, we dried 5mL of the medium and followed the same derivatization process for GC-MS analysis. We detected no soluble oxalate and therefore repeated this work with a more sensitive HPLC analysis. Again, no soluble oxalate was detected (at levels down to 0.01 mM), which is consistent with previous results that *G. trabeum* accumulates less oxalate compared to other brown rot fungi [33], mainly due to the presence of oxalate decarboxylase, which has previously been shown to decompose oxalate in *G. trabeum*. To measure the concentrations of insoluble oxalate, we followed similar protocols for biomass hydrolysis followed by derivatization. We found that oxalate was produced at levels of 0.19±0.09 mM and 0.56 ±0.09 mM under LN and HN conditions, respectively. The observation that greater amounts of oxalate were produced under high nitrogen levels was also consistent with previous research[34].

Based on the production of insoluble oxalate, we next investigated the central metabolic flux of *G. trabeum* when cultured using different nitrogen levels. In general, for most of the central metabolic pathways such as glycolysis and pentose phosphate pathways, the fluxes were similar despite changes in nitrogen culture levels (Fig. 1A). However, when we next evaluated the effects of peroxisomal glyoxylate dehydrogenase (GLX) and cytosolic oxaloacetate acetylhydrolase (OAH) on oxalate synthesis, we found that usage of these two pathways was significantly different when high and low nitrogen conditions were used (Fig. 1B). While the flux ratio of OAH/GLX for the low nitrogen condition was only 0.01; it was as high as 56.2 for the high nitrogen condition (Fig 1B), indicating that the preferred pathway for oxalate production (OAH or GLX) shifts dramatically when the fungus was grown at different nitrogen levels. Correspondingly, the fluxes of metabolic pathways related to OAH and GLX were also found to be different. For example, the metabolic flux of the futile pathway that supplies cytosolic oxaloacetate from cytosolic pyruvate in the LN condition was dramatically higher than that in the HN condition (Fig 1C). The varied flux distribution in the peroxisomal glyoxylate dehydrogenase, cytosolic oxaloacetate acetylhydrolase and futile pathways clearly indicated that *G. trabeum* metabolism was regulated in response to the C/N ratio, which confirmed that the C/N ratio was a key factor in oxalate biosynthesis for this fungus[10].

While it is clear that the conversion of oxaloacetate to oxalate by peroxisomal glyoxylate dehydrogenase occurred in the peroxisome, oxalate synthesis via cytosolic oxaloacetate acetylhydrolase could potentially be from several sources since oxaloacetate, the precursor of oxalate in synthesis via cytosolic oxaloacetate acetylhydrolase, can be generated by either of three pathways in different regions of the cell: 1) the futile pathway, where pyruvate is converted to oxaloacetate in the cytosol to supply the TCA cycle in mitochondria; 2) the TCA cycle, where oxaloacetate is generated from malate in mitochondria; and 3) the glyoxylate cycle, where oxaloacetate is generated in the peroxisome. However, based on ^{13}C-MFA, we found the flux for oxaloacetate synthesis in the glyoxylate cycle was trivial under both LN and HN conditions (Fig. 1B), indicating that glyoxylate was mainly used to produce oxalate directly rather than being converted to oxaloacetate in *G. trabeum*. Therefore, it is likely that the oxalate produced by action of cytosolic oxaloacetate acetylhydrolase is generated directly from oxaloacetate synthesized in the futile cycle or the TCA cycle, rather than from oxaloacetate that is synthesized in the glyoxylate cycle. In addition, in prior research with a related fungus, *F. palustris* [11, 35] under high nitrogen conditions (although with a different nitrogen source than in our research), cytosolic oxaloacetate acetylhydrolase was the dominant precursor enzyme active in oxalate synthesis. Both *F. palustris* and *G. trabeum* have previously been reported to utilize the non-enzymatic chelator-mediated Fenton (CMF) system in lignocellulose deconstruction, and therefore it is possible that these two fungi would adopt a regulatory system for oxalate synthesis as it is required by CMF chemistry. It has been reported that high levels of ammonium ions may inhibit the activity of glyoxylate dehydrogenase, the key enzyme for oxalate production in the glyoxylate cycle[36] [37], which could explain the limited role of the peroxisomal glyoxylate dehydrogenase on oxalate synthesis when nitrogen (ammonium) concentrations were high.

To further investigate the regulation of cytosolic oxaloacetate acetylhydrolase and peroxisomal glyoxylate dehydrogenase, we performed qPCR to determine the expression level of the genes that encode these two enzymes. We followed an established protocol[38] to extract the total RNAs of *G. trabeum* grown under both LN and HN conditions using a FastRNA spin kit (MP Biomaterials, USA), and measured the mRNA levels. It was found that there was no significant difference in gene expression levels between the LN and HN conditions for both cytosolic oxaloacetate acetylhydrolase gene and peroxisomal glyoxylate dehydrogenase gene (Figure S2). Comparing with the distinct flux results between LN and HN conditions, this indicates that the regulation of the pathways for oxalate synthesis was not executed at the transcriptional level. Considering the complex regulatory machinery in eukaryotic metabolism (e.g., *G. trabeum*), the peroxisomal glyoxylate dehydrogenase and cytosolic oxaloacetate acetylhydrolase could be regulated in translational, post-translational or even at the metabolic level (e.g., ammonium inhibition). The systemic investigation of the regulatory mechanism is beyond the scope of this manuscript but will be investigated by our group in the future.

In sum, this study examined the effects of peroxisomal glyoxylate dehydrogenase and cytosolic oxaloacetate acetylhydrolase on oxalate biosynthesis in *G. trabeum* using ^{13}C tracer experiments and metabolic flux analysis; approaches not previously explored in fungi for oxalate production. As revealed by the metabolic flux results, the pathway selected for oxalate biosynthesis was highly dependent on the C/N ratio, with cytosolic oxaloacetate acetylhydrolase being the dominant pathway for the

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oxalate synthesis under low C/N (i.e., high nitrogen) conditions, and peroxisomal glyoxylate dehydrogenase contributing to oxalate synthesis under high C/N (i.e., low nitrogen) conditions. Combined with transcriptional analysis, it was determined that such metabolic reprogramming was not transcriptionally controlled. Overall, ^{13}C-MFA proved to be a useful approach in evaluating the pathway usage in *G. trabeum* for oxalate production and could help improve our understanding of oxalate metabolism in wood-decaying fungi and to facilitate further improvement of the lignocellulose bioconversion technology with other fungal species.

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