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Analysis of D-glucose metabolism of wood decay fungi using ^{13}C-NMR and ^{13}C-labeled substrates

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Abstract

D-Glucose metabolism is thought to be important during wood decay by fungi, not only for anabolic and catabolic purposes of central metabolism, but also as a potential source of peroxide required by extracellular peroxidases. There has been some confusion in the literature as to whether this peroxide-generating activity is of the glucose 1-oxidase or pyranose 2-oxidase (glucose 2-oxidase) type with various fungi or even within the same fungal species. Definitive classification requires accurate identification of the enzymatic products D-glucono-1,5-lactone and D-*arabino*-2-hexosulose (glucosone) with glucose 1-oxidase and pyranose 2-oxidase, respectively. We used ^{13}C-NMR to distinguish these reactions starting with ^{13}C-labeled glucose. The use of labeled substrates simplifies analysis and greatly increases detection sensitivity without requiring the isolation or derivatization of metabolites. We synthesized ^{13}C-1-glucosone to study subsequent metabolism with crude enzyme preparations. Preliminary results with *Phanerochaete chrysosporium* are presented.

Key words: *Phanerochaete chrysosporium,* glucose 1-oxidase, pyranose 2-oxidase, D-glucono-1,5-lactone, glucosone, pyranosone dehydratase.

Introduction

Lignin peroxidase, manganese peroxidase, and laccase are thought to be major players in lignocellulose degradation that directly, or indirectly (via mediators), oxidize lignin. The peroxidases use peroxide as co-substrate and therefore peroxide-generating oxidases are also important. Candidates for this role include glucose 1-oxidase, pyranose 2-oxidase, glyoxal oxidase, and aryl alcohol oxidase. The microbiology, biochemistry and molecular biology of lignocellulose biodegradation has been reviewed (5, 9, 13, 15, 16, 20, 29).

After the first reports of lignin peroxidase with *P. chrysosporium,* two different glucose oxidases were reported from this species, both of which were suggested to play a role in supplying the extracellular peroxide for the fungal peroxidases. These oxidases were glucose 1-oxidase from *P. chrysosporium* ME-446 (18), and pyranose 2-oxidase from *P. chrysosporium* K-3 (8).

Glucose 1-oxidase oxidizes glucose at C-1 to give glucono-1,5-lactone and peroxide (figure 1). Characterization of glucose 1-oxidase purified from *P. chrysosporium* ME-446 mycelia indicated that the primary substrate was glucose and that there was very little activity with cellobiose, mannose and galactose (17-19). About the same time of these reports of glucose 1-oxidase from *P. chrysosporium* ME-446, a pyranose 2-oxidase was reported from *P. chrysosporium* K-3 (8, 30). In contrast to the glucose

1-oxidase, pyranose 2-oxidase oxidizes D-glucose at C-2 to give D-glucosone and peroxide (figure 2). The enzyme is non-specific (1, 8). At 50 mM substrate concentration in pH 6.5 buffer, the following relative activities were determined with various substrates (8): glucose 100%; glucono-1,5-lactone 60 %; L-sorbose 52%; D-xylose 37%; D-galactose 5%; D-mannose < 5%; D-, L-arabinose < 5%; cellobiose < 5%.

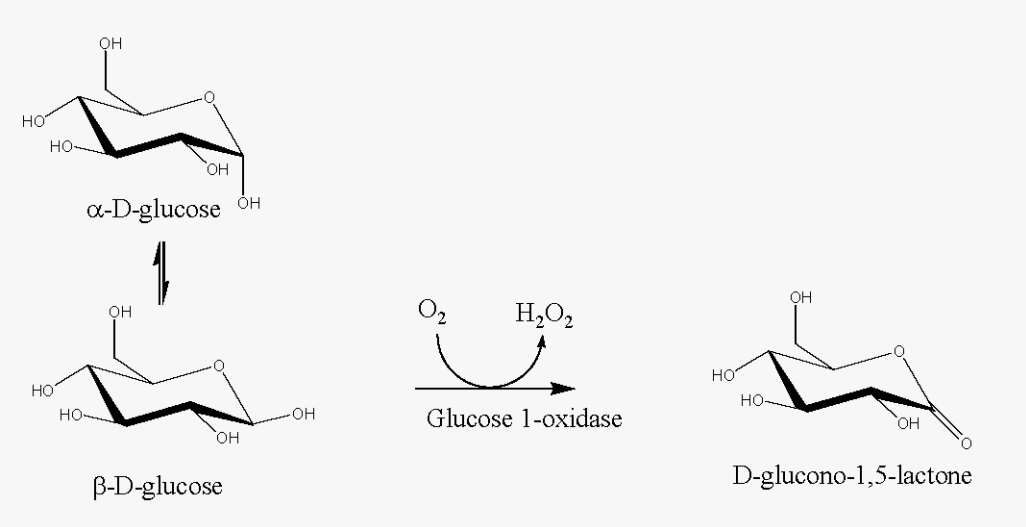

Fig. 1. Reaction catalyzed by glucose 1-oxidase. Glucose 1-oxidase of *Aspergillus* oxidizes β-D-glucose at C-1 to give D-glucono-1,5-lactone which hydrolyzes non-enzymatically to gluconate.

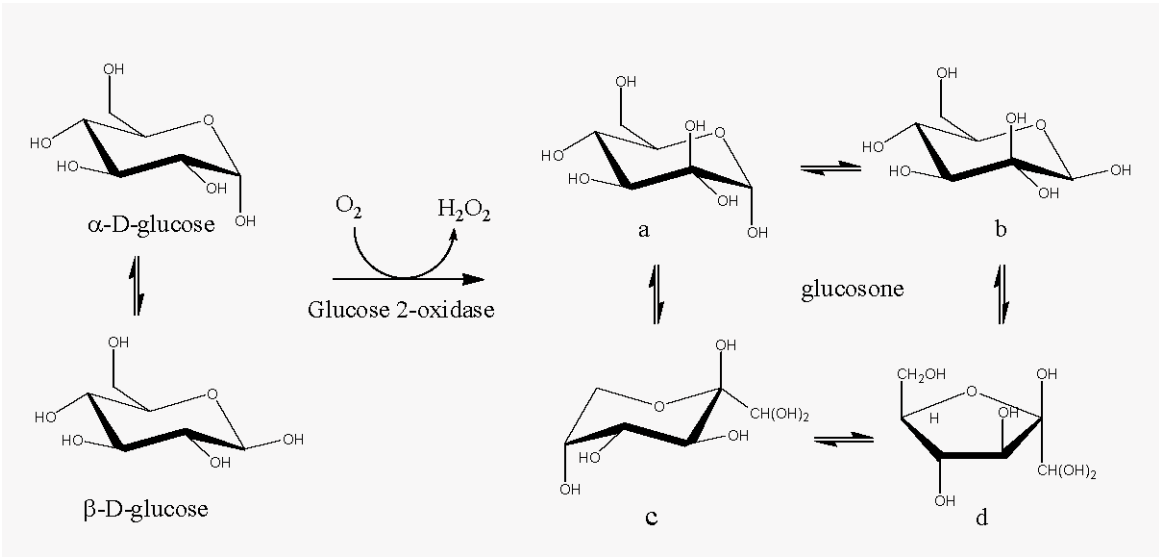

Fig. 2. Reaction catalysed by pyranose 2-oxidase. Pyranose 2-oxidase oxidizes both α- and β-D-glucose at C-2 to give D-glucosone, which exists in aqueous solution in four isomeric forms that are differentiated by NMR. The approximate proportions of the four isomers a-d under our experimental conditions are 46%, 26%, 18% and 10%, respectively, based on ^{13}C-NMR peak intensities.

Daniel *et al.* (6) proposed that pyranose 2-oxidase is a major source of H_2O_2 during wood degradation by *P. chrysosporium* K-3, *Oudemansiella mucida,* and *Trametes versicolor.* These results contradicted the findings of Kelly and Reddy with ME-446 (18) and presented obvious questions. Is glucose 1-oxidase found only in *P. chrysosporium* ME-446 and pyranose 2-oxidase in *P. chrysosporium* K-3? Or are both oxidases produced with both fungi but under different conditions? Volc *et al.* (31) addressed these questions and grew the ME-446 and K-3 strains under three different culture conditions and found only pyranose 2-oxidase. Cultures conditions used by Kelly and Reddy were also duplicated by Volc *et al.* (31) but they only found pyranose 2-oxidase and no evidence of glucose 1-oxidase.

Many other wood decay fungi in addition to *P. chrysosporium* are reported to produce pyranose 2-oxidase. These include *Polyporus obtusus* (26), *Phlebiopsis gigantea* (27), and *Trametes multicolor* (33). In general, pyranose oxidase is a FAD homotetramer with subunit MW of 68 - 76 kDA (7, 23, 30, 33). Substrates include D-glucose, D-xylose, L-sorbose, and D-glucono- 1,5-lactone. Pyranose 2-oxidase from *T. versicolor* oxidizes both α and β anomers of glucose essentially equally well and therefore can be used in the micro-determination of glucose without the addition of mutarotase (28).

One apparent function for intracellular pyranose 2-oxidase is the synthesis of cortalcerone (2, 11, 12, 21, 32). However, conversion of glucosone to cortalcerone occurred in only 43 macrofungi out of the 315 surveyed, suggesting it is not the only role for pyranose 2-oxidase (2). *P. chrysosporium* K-3 can synthesize cortalcerone from glucosone, a pathway requiring pyranosone dehydratase (12). Not all wood decay fungi that produce pyranose 2-oxidase also produce pyranosone dehydratase. For example, *T. versicolor, Lenzites betulinus, O. mucida* are known to produce pyranose 2-oxidase but are incapable of converting glucosone to cortalcerone, apparently because they lack pyranosone dehydratase (22, 32). Volc *et al.* (34) suggest that pyranose 2-oxidase from *O. mucida* has a second role in glucose metabolism based on the oxidation of glucosone itself by pyranose 2-oxidase to give the tricarbonyl product D-erythro-hexos-2,3-diulose or 2,3-diketo-D-glucose.

Materials and Methods

Organism and culture conditions. P. chrysosporium BKM-F-1767 was grown on wheat bran solid substrate supplemented with cornsteep liquor (24). Cultures were harvested after 2 weeks of growth.

Chemicals and enzymes. ^{13}C-1-glucose, acetone-d_6 and ^{13}C-labeled acetone were purchased from Cambridge Isotope Laboratories, Inc. Andover, MA. D-Glucosone was synthesized from glucose (3, 25) and, likewise, ^{13}C-1-glucosone was synthesized from ^{13}C- 1-glucose. D-Glucono-1,5-lactone and gluconic acid were from Aldrich. Glucose 1-oxidase (type II-S) from *Aspergillus niger* was purchased from Sigma. Pyranose 2-oxidase was prepared from *P. chrysosporium* (24) using purification procedures similar to that previously reported. Crude enzyme preparations from *P. chrysosporium* were prepared by water extraction of whole cultures (500 ml H_2O/100g wheat bran culture) for 30 minutes at 4°C followed by filtration through Mira-cloth and centrifugation. These are mild extraction conditions and are not expected to appreciably lyse the fungal mycelia.

Reaction conditions. Enzymatic reactions were run at pH 6 with 20 mM 2,2-dimethylsuccinic acid as buffer. The reaction solution with glucose 1-oxidase contained 5 mM ^{13}C-l-glucose, 10 µg glucose 1-oxidase, and 3 µg catalase in 1 ml. Similar reactions were run with pyranose 2-oxidase, except that horseradish peroxidase and phenol red were used, instead of catalase, to consume the peroxide generated. The reactions with glucosone contained 5 mM ^{13}C-1-glucosone and 50 µl of crude enzyme from *P. chrysosporium* grown on wheat bran. To these reactions was added 50 µl of 0.1% ^{13}C-acetone (1,3-$^{13}C_2$, 99%) in D_2O prior to NMR analysis.

NMR analyses. A Bruker DPX-250 spectrometer at 62.9 Mhz was used for NMR analyses. Acetone-d_6 was used for reference signal (29.83 ppm) with authentics. In enzymatic reactions, ^{13}C-acetone (1,3-$^{13}C_2$, 99%) was used for reference signal (30.56 ppm) and the instrument shimmed against D_2O. NMR reference data include that reported for aldono-1,4-lactones (35), aldono-1,5-lactones (36), pentonolactones (14), monosaccharides (4) and glucosone (10). The chemical shift assignments for glucosone, glucono-1,5-lactone, gluconic acid and gluconate were determined with authentics and agree well with reference literature.

Results

Differentiation of glucose 1 -oxidase and pyranose 2-oxidase reactions. Reactions of the oxidases with ^{13}C-l-glucose as substrate could be easily followed by observing the chemical shifts at 92.47 and 96.29 ppm corresponding to α-D-glucose and β-D-glucose, respectively. Reactions with glucose 1-oxidase gave products with chemical shifts of 173.98 and 178.83 ppm, in good agreement with the formation of glucono-1,5-lactone and gluconate, respectively. With time the peak at 173.98 disappeared as the peak at 178.83 increased, consistent with the slow hydrolysis of the lactone.

Reactions of pyranose 2-oxidase gave four new peaks at 94.99, 95.41, 89.80 and 90.44 ppm corresponding to the C-1 of glucosone isomers a-d (see figure 2). Under our experimental conditions, the relative peak heights corresponding to the four glucosone isomers a-d were approximately 46%, 26%, 18% and 10 %, respectively.

Metabolism of glucosone by P. chrysosporium crude enzyme. Figure 3 shows the NMR spectrum of synthetic ^{13}C-1-glucosone with dominant peaks corresponding to the isomers a-d depicted in figure 2. Upon addition of crude enzyme, extensive changes in the spectrum were observed with the generation of five new easily detectable species. The kinetics of glucosone disappearance gave no indication that a specific isomer was preferentially metabolised (figure 4). Perhaps the kinetics of isomer equilibration was fast enough that if an isomer were preferentially metabolised, it would not be detected. The half-life of glucosone in this reaction was approximately 8 h.

The products of the enzymatic reaction gave chemical shifts at 60.08, 59.96, 94.38, 90.20, and 89.55 ppm due to the labelling at C-1 of the substrate glucosone. The kinetics of formation of these products suggest that the compound corresponding to the 94.38 ppm peak was an immediate product, followed by 90.20, and 89.55 ppm, then finally by 60.08 and 59.96 ppm. There is insufficient adjustment in kinetic parameters (e.g. adjusting substrate or enzyme concentration) to know if the sequence

is obligatory. The continued increases in peak intensities at 60.08 and 59.96 ppm, after the depletion of glucosone, indicates that the corresponding species cannot be direct products. DEPT analysis indicates that these end products with chemical shifts at 60.08 and 59.96 ppm are methylenes at the labeled carbons. This would exclude the identification of these products as cortalcerone or 2,3-diketo-D-glucose.

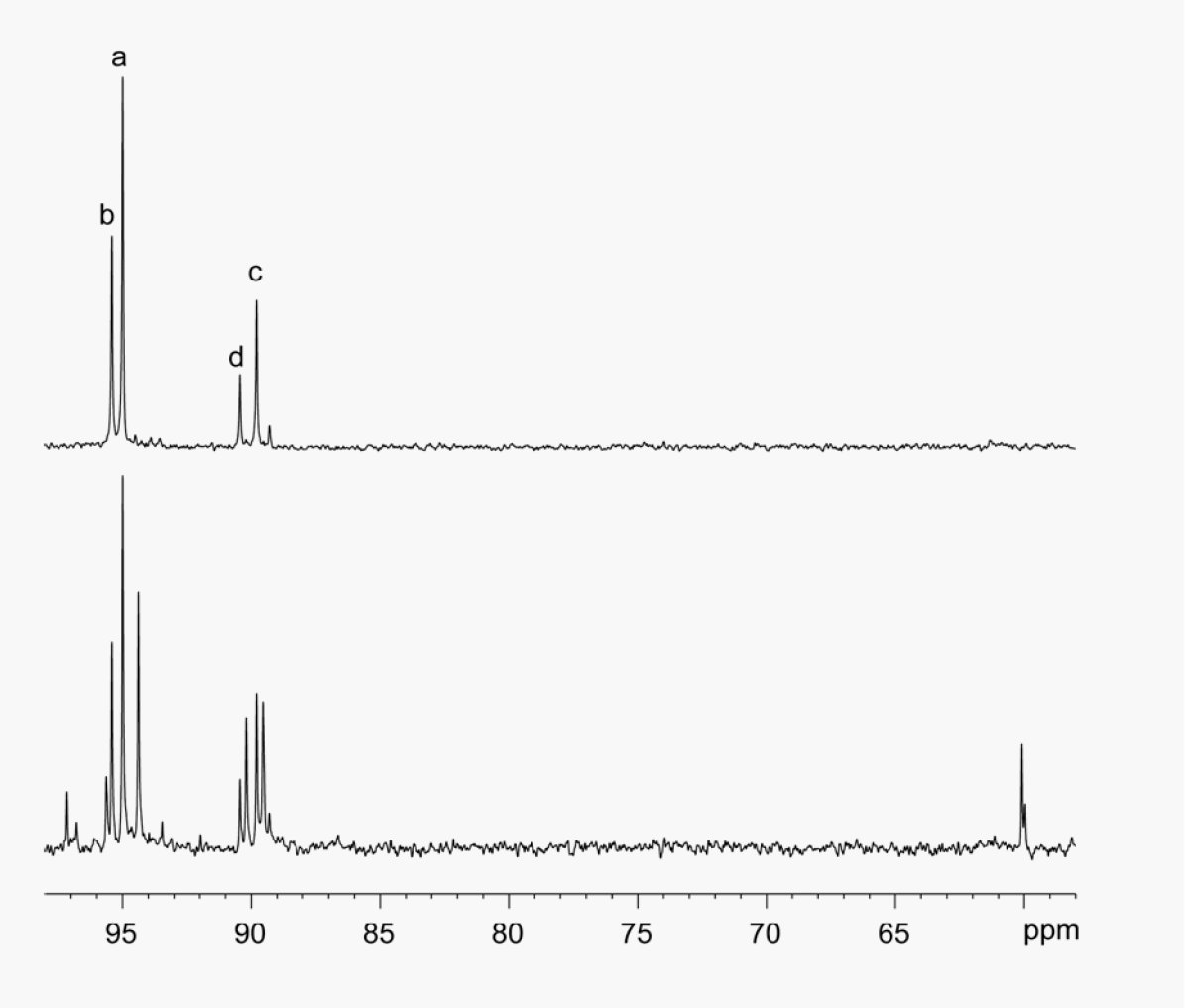

Fig. 3. ^{13}C-NMR analysis of reactions catalysed by crude enzyme from *P. chysosporium* with ^{13}C-1 -glucosone. *Top panel:* the NMR spectrum of synthetic ^{13}C-1 -glucosone is shown under the experimental conditions for enzymatic reactions. Chemical shifts at 94.99, 95.41, 89.80 and 90.44 ppm corresponding to the C-1 of glucosone isomers a-d are observed. In the absence of enzyme, the spectrum showed no appreciable change over a 16 h incubation. *Bottom panel:* Significant and extensive changes in the spectrum occurred in a 1 ml reaction with 50 µl of enzyme. Spectrum shown is from a reaction after an 8-h incubation when approximately 50% of the glucosone was consumed. At least five new peaks were detected, specifically at 60.08, 59.96, 94.38, 90.20, and 89.55 ppm.

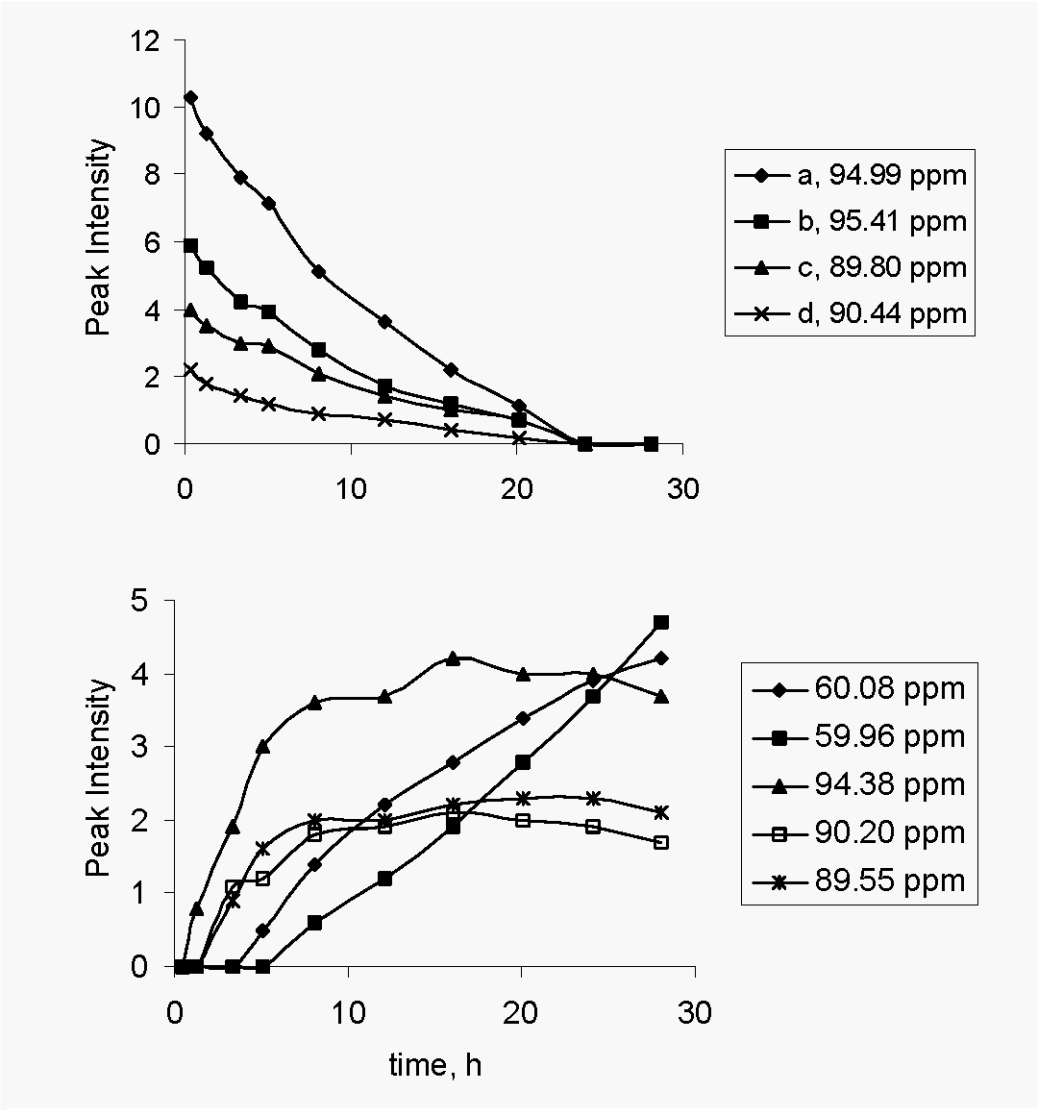

Fig. 4. Kinetic analysis of ^{13}C-1 glucosone metabolism and product formation. *Top panel:* The disappearance of the glucosone isomers in the enzymatic reaction of figure 3 was determined. The designations a-d correspond to isomers depicted in figure 2. There is no indication that any of the four isomers is preferentially degraded under these experimental conditions. *Bottom panel:* The kinetics of product formation suggests that a sequence of reactions is occurring with the products corresponding to 60.08 and 59.96 ppm being the most stable end products. Peak intensities were normalized against the methyl group peak corresponding to 2,2-dimethylsuccinic acid buffer.

Discussion

Advantages of the ^{13}C-NMR method using ^{13}C-labeled substrates include:

1) Analysis is greatly simplified. For example, glucosone with four isomers, and its corresponding 24 chemical shifts with natural-abundance ^{13}C-glucosone, is likely to obfuscate analysis. Although the detection of final products may eventually be known if the reaction goes to completion, the kinetics of intermediate formation and disappearance is likely to be obscured. The use of

^{13}C-label at a single position in glucosone allows the kinetics of substrate disappearance, and product appearance to be observed.

2) Detection sensitivity is greatly increased. The approximate 100-fold increase in sensitivity with the use of ^{13}C-labeled substrate allows detection of reactions with low levels of enzyme. This can be particularly important if a component of the reaction is present at limiting amounts e.g. oxygen in the oxidase reactions.

3) No isolation of intermediates or products is necessary for detection. This is critical especially when the identity of the intermediates is not known and therefore its reactivity for purposes of derivatization also is not known. Consequently, isolation or derivatization methods may not be appropriate and cannot be correctly assessed because of insufficient knowledge. These complications are circumvented by ^{13}C-NMR analyses of underivatized reaction mixtures.

Results presented here demonstrate the rapid differentiation between glucose 1-oxidase and pyranose 2-oxidase. Evidence supports the identification of the oxidase from *P. chrysosporium* as pyranose 2-oxidase, which produces glucosone from glucose. Additionally, by using ^{13}C-1-glucosone as substrate, we were able to demonstrate that it is further metabolised by extract of *P. chrysosporium* cultures. At least five chemical intermediates or products are detected. Research is in progress using glucosone labeled in the remaining five positions of the molecule to deduce the chemical structures of the intermediates, one carbon at a time, based on NMR characterizations and kinetic analyses.

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