National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Canada

The Role of Reductive Enzymes in *Trametes versicolor*-Mediated Kraft Pulp Biobleaching

by

Brian Paul Patrick Roy

A Thesis

Submitted to the Faculty of Graduate Studies and Research

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the

Department of Natural Resource Sciences

Microbiology Unit

McGill University

Montreal, Quebec, Canada

February, 1994

ISBN 0-612-00133-4

Canada

The Role of Reductive Enzymes in Pulp Biobleaching

PREFACE

This thesis consists of six manuscripts which have been or will be published elsewhere, of which four are co-authored by the candidate's supervisor, Dr. Frederick Archibald. The fifth manuscript was co-authored with Dr. Frederick Archibald and Michael Paice at the Pulp and Paper Research Institute of Canada and Drs. Sushil Misra and Lucjan Misiak at Concordia University. The sixth manuscript was co-authored with Dr. Frederick Archibald, Brian Walker and Michael Paice at the Pulp and Paper Research Institute of Canada and Drs. Sushil Misra and Lucjan Misiak at Concordia University. Included with the thesis are an introductory literature review, an overall conclusion, and appendices. The work involved was the sole responsibility of the candidate, under the guidance of the project superisor, except for all of the EPR measurements which were performed by Dr. Lucjan Misiak. Data presented in Figure 6.2 and Tables 6.3, 6.4, and 6.5 resulted from collaborative efforts with Brian Walker and Michael Paice. The material presented in Appendix A was initiated by Dr. F. Archibald and Tina Kouri and the author contributed the data related to kraft lignin (Indulin) and the material presented in Figures 3 and 4.

This dissertation is presented in accordance with the Guidelines Concerning Thesis Preparation as published by the Faculty of Graduate Studies and Research of McGill University. The format chosen is that of a compendium of six manuscripts, according to the following directions outlined in the guidelines:

"Candidates have the option, subject to the approval of their Department, of including as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis. If this option is chosen, connecting texts, providing logical bridges between the different papers, are mandatory. The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is more than a mere collection of manuscripts published or to be published. The thesis must include, as separate chapters or sections: (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion and/or summary. Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (e.g., in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis. In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent; supervisors must attest to the accuracy of such claims at the Ph.D. oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers."

ACKNOWLEDGMENTS

The author wishes to thank his supervisor, Dr. Fred Archibald, for his guidance, enthusiasm, and encouragement while this project evolved from ideas to hypotheses, experiments, and finally to the preparation of this manuscript. Your insight and criticism has made this work much stronger.

I thank my fellow students, Katherine Addleman and Louis Tremblay whose support and wit kept at least a thread of sanity when times were difficult. Dr. Ian Reid and Lubo Jurasek are sincerely thanked for their constructive comments and moral support throughout the course of this project. The contributions of Michael Paice and Robert Bourbonnais, both practical and intellectual are gratefully acknowledged. Sincere thanks to Francine Lafortune, Frank Young and Sylvie Renaud for their expert technical assistance with and operation of many of the analytical instruments used in this thesis. Sincere thanks is expressed to Dr. John Schmidt for translation of the papers from which the methods for synthesis of the orthoquinones were obtained. Finally, to all of the staff in Biotechnology, thank you for our shared camaraderie which made this whole project the great pleasure that it was.

To my parents Paul and Helena Roy who have supported me in all things, I thank you. To my daughters Chantelle and Miranda who have had to sacrifice time with their father while he finished "just one more experiment", yours was the most generous of gifts. This thesis is dedicated to my wife, Pauline, who has always encouraged me to pursue my dreams, though as a consequence she has borne the far greater burden of managing our home and family.

Financial support from the Pulp and Paper Research Institute of Canada in the form of two years from Clifford B. Purves Graduate Fellowship and one year from the

G.H. Tomlinson II Graduate Fellowship and F. St. Germain Merit Award have made these studies possible and are gratefully acknowledged.

ABSTRACT

The extracellular culture supernatants of the white rot fungus *Trametes versicolor* can bleach and delignify unbleached kraft pulps, however the process is too slow for commercial application. Though at least two oxidative enzymes, laccase and manganese peroxidase (MnP) produced by this organism can catalyze a partial delignification of kraft pulp, the effect observed is small relative to that obtained with the complete fungal system. To develop a synthetic (cell-free) delignification system, other protentially important components of the culture supernatant were identified and their contributions to biobleaching and delignification were evaluated. The presence of pulp did not significantly affect the overall carbon balance of the fungus, but a number of non-volatile metabolites (oxalic, fumaric, glyoxylic and phenyllactic acids) induced by the presence of pulp were identified. In *T. versicolor* 52J, the secretion of manganese peroxidase (MnP), and cellobiose:quinone oxidoreductase (CBQase) enzymes were inducible by pulp whereas cellulase and laccase were not. Several low molecular weight metabolites secreted by *T. versicolor* functioned as effective Mn(III) complexing agents at their physiological concentrations and promoted MnP activity.

Two distinct CBQase proteins are secreted by *T. versicolor* 52J, CBQase 4.2 a a 113kDa homodimer containing both heme and flavin cofactors and CBQase 6.4, a 48 kDa monomer with a flavin cofactor only. Superficially, these enzymes appear very similar to the cellobiose oxidase (CBO) and CBQase reported in *Phanerochaete chrysosporium*. CBQase 4.2 was shown to reduce insoluble manganese dioxide to its soluble Mn(II) and Mn(III) forms with the concommitant oxidation of cellobiose. The sugar acids formed by CBQase could function as effective complexing agents for Mn(III), and complement the Mn(II) to Mn(III) oxidation activity of MnP.

It is proposed that a redox cycling of lignin molecules by certain fungal oxidative and reductive enzymes occurs during delignification and that this cycling ultimately promotes net lignin degradation. A redox cycle was established between *T. versicolor* CBQase and laccase which allowed the O_2 consumption rate of laccase to remain at a constant level and the total O_2 consumption by the enzyme was much greater than if the substrate were incubated with laccase alone. A new assay for CBQase based on the ability of this enzyme to reduce the radical intermediates formed during laccase-mediated chlorpromazine oxidation was developed. A redox cycle for these two enzymes was established using both model substrates like CPZ, and with a kraft lignin preparation. CBQase inhibited the formation of polymeric material by laccase; however no evidence was found indicating that cycling with these two enzymes favors depolymerization of kraft lignin. However, the alkali extractability of residual lignin in kraft pulp was increased by a sequential treatment with MnP followed by CBQase.

RÉSUMÉ

Le surnageant du milieu de culture du champignon *Trametes versicolor* peut blanchir et délignifier les pâtes kraft non-blanchies. Malheureusement, ce procédé est trop lent pour être utilisé de façon commerciale. Au moins deux enzymes oxidatives qui catalyzent la délignification partielle de la pâte kraft, soient la laccase et la manganèse péroxidase (MnP), sont produites par cet organisme, mais leur effet est faible comparé a celui du système fongique complet. Quelques autres composantes potentiellement importantes du surnageant ont été identifiées et leurs contributions au processus de bioblanchiment et de délignification ont été évaluées en vue du développement d'un procédé de délignification entièrement synthétique. La répartition du carbone métabolisé est demeurée à peu près la même en présence de pâte, mais un certain nombre de métabolites non-volatiles (acides oxalique, fumarique, glyoxylique et phényllactique) ont été identifiés et résultent de la présence de la pâte. Bien que la sécrétion de MnP et de la cellobiose:quinone oxidoréductase (CBQase) par *T. versicolor* 52J a été induite par la pâte, aucune sécrétion additionnelle de cellulase ou de laccase n'a été induite. Plusieurs des métabolites de faible masse moléculaire sécrétés par *T. versicolor* forment efficacement des complexes avec le Mn(III), même à des concentrations physiologiques. Ces métabolites ont aussi amélioré l'activité du MnP.

Deux CBQases sont sécrétées par *T. versicolor* 52J, soient CBQase 4.2 et CBQase 6.4. La première est un homodimère de 113 kDa contenant deux cofacteurs, la hème et la flavine, et la deuxième est un monomère de 48 kDa contenant un seul cofacteur, la

flavine. Ces protéines ressemblent beaucoup à la cellobiose oxidase et la CBQase sécrétées par *Phanerochaete chrysosporium*. La CBQase réduit le dioxyde de manganèse, insoluble, en ses formes de Mn(II) et de Mn(III) solubles lors de l'oxidation concomitante de la cellobiose. Les acides de glucose formés par la CBQase pourraient être des agents complexant efficaces pour le Mn(III) et pourraient également jouer un rôle dans l'oxidation du Mn(II) au Mn(III) par la MnP.

Nous proposons donc un cyclede réactions d'oxydoréduction par certains enzymes fongiques oxidatives et réductives. De plus, ce cycle serait à la base de la dégradation de la lignine. Un cycle d'oxydoréduction a été établi entre la CBQase de *T. versicolor* et la laccase. Ce cycle permet à la fois un taux de consommation d'oxygène de la laccase constant et une consommation totale d'oxygène par l'enzyme beaucoup plus élevée que celle qui aurait été observée si le substrat n'avait été incubé qu'avec de la laccase. Une nouvelle méthode de quantification basée sur la capacité de la CBQase à réduire les radicaux intermédiaires formés lors de l'oxidation de la chlorpromazine par la laccase a été développée. Nous avons utilisé le CPZ et l'ABTS comme modèles de substrats pour établir le cycle d'oxidoréduction de la laccase de même que celui de la CBQase. Nous avons aussi utilisé une solution de lignine kraft pour établir ce cycle. La CBQase inhibe la formation de substances polymériques catalysée par la laccase bien que rien n'indique que le cycle entre les substrats modèles ou la lignine et les enzymes favorise la dépolymérisation de la lignine kraft. Toutefois, un traitement séquentiel de MnP suivi de CBQase augmente la solubilité de la lignine dans des conditions alkalines.

CLAIMS OF CONTRIBUTION TO KNOWLEDGE

1. Identified the volatile and non-volatile metabolites which are produced during kraft pulp biobleaching by *Trametes versicolor* and demonstrated that certain of these metabolites can form relatively stable oxidizing complexes with Mn(III). These organic acid metabolites, at their physiological concentrations, effectively promoted the MnP-mediated oxidation of phenol red.

2. Demonstrated that laccase, manganese peroxidase (MnP) and cellobiose: quinone oxidoreductase (CBQase) are secreted into the culture medium during pulp biobleaching. Lignin peroxidase, glyoxal oxidase, and glucose oxidase, enzymes identified in the literature as being important in delignification were not detectable in biobleaching cultures of *T. versicolor*.

3. Showed that CBQase is present during *Trametes versicolor* biobleaching but, when assayed under aerobic conditions, its activity is partially or completely masked by the presence of laccase.

4. Developed a novel colorimetric assay for CBQase-like reductive enzymes based on their ability to reduce free radical intermediates formed by oxidative enzymes such as laccase which carry out one electron oxidations of their substrates. This assay was used to demonstrate the presence of CBQase under aerobic conditions in the presence of laccase.

5. Isolated, purified, and characterized CBQase proteins produced by *T. versicolor* and demonstrated that the total reductive activity in the culture is catalyzed by two proteins with distinctly different molecular weights and cofactors. Both enzymes reduce quinone and free radical substrates and have approximately the same kinetic parameters. These parameters were determined for a large number of substrates.

6. It was shown by electron paramagnetic resonance spectroscopy (EPR) that CBQase acts predominantly by reducing free radical reaction intermediates produced by laccase, manganese peroxidase, and lignin peroxidase. In contrast to its oxidation by lignin peroxidase, the oxidation of veratryl alcohol by veratryl alcohol oxidase was not affected by the presence of CBQase. CBQase did not reduce laccase, lignin peroxidase or manganese peroxidase proteins directly when oxidizable substrates for these enzymes were present. CBQase did reduce oxidized laccase, albeit at a rate 100-fold lower than it reduced reaction intermediates formed by this enzyme. Direct reduction of lignin peroxidase protein (compound II) by CBQase protein was not observed.

7. The presence of CBQase and cellobiose allowed for the continued consumption of oxygen by laccase for extended periods by reducing phenoxy radical intermediates formed from 2-methoxyphenol (guaiacol) by reforming a phenolic laccase substrate. In the presence of sufficient cellobiose extensive phenolic substrate cycling by CBQase and laccase is possible. Similar redox cycling of these two enzymes was demonstrated when a polymeric kraft lignin preparation as substrate. CBQase inhibited the polymerization of this lignin preparation, but depolymerization by CBQase was not observed under the conditions tested.

8. Using EPR spectroscopy, the dynamics of free radical cycling by laccase and CBQase were directly observed using two cation radical-forming compounds, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) and chlorpromazine. The free radical formed from these substrates by laccase formed a characteristic EPR signal. In the presence of CBQase and cellobiose, the strength of the EPR signal was reduced or eliminated in proportion to the amount of CBQase added. This is direct and conclusive evidence that CBQase reduces free radical species and not just the quinone oxidation products. These findings confirm that the free radicals are the key species mediating the interaction between CBQase and laccase.

9. It was determined that the high molecular weight (HMW) fraction of a biobleaching *T. versicolor* culture supernatant is an effective Mn(III) chelator, able to support the oxidation of phenol red, a commonly used phenolic substrate for assaying MnP activity. It was determined that the HMW material is primarily carbohydrate with a glucose:mannose ratio of 100:6.

10. It was shown that CBQase can form sugar acids on the reducing ends of cellulose, and that the resulting sugar acids are effective MnP activity promoting (Mn(III) complexing) agents. Similarly, simple sugar acids are also Mn(III) complexing agents able to promote MnP activity.

11. In addition to the free radical and quinone reducing activity of CBQase, the enzyme was shown to reduce insoluble $Mn(IV)O_2$ to a soluble form, either Mn(III) or Mn(II), rendering the Mn available for MnP-mediated Mn(III)-complex formation. The reduction of Mn(IV)

to Mn(II) was demonstrated by the appearance of the characteristic six line EPR signal of the latter. Reduction of Mn(IV) to Mn(III) was demonstrated by the appearance of the characteristic absorbance profiles of various Mn(III)-chelator complexes. The room-temperaure chelator-dependent line broadening of the Mn(II) EPR signal was also observed and quantified. The dependance of the Mn(IV) reduction rate on the nature of the complexing agents present was also shown.

12. It was shown that important interactions occur between MnP and CBQase when unbleached kraft pulp treatments are attempted. Co-incubation of these enzymes generally decreased the effect of MnP on pulp demethylation and delignification whereas a sequential MnP-CBQase treatment was found to augment the MnP-mediated demethylation and delignification with some of the pulps tested.

TABLE OF CONTENTS

Table of Contents (continued) Page

Table of Contents (continued) Page

LIST OF TABLES

LIST OF TABLES (continued) Page

LIST OF TABLES (continued) Page

LIST OF TABLES (continued) Page

LIST OF FIGURES

LIST OF FIGURES (continued) Page

LIST OF ABBREVIATIONS

λ	wavelength
4-AAP	4-aminoantipyrine
A	amperes
ABTS	2,2' -azino-di-(3-ethylbenzthiazoline)-6' -sulfonate
ATCC	American Type Culture Collection
Bq	Bequerel
cb	cellobiose
CBQase	cellobiose:quinone oxidoreductase
CBO	cellobiose oxidase
Ci	Curie
cm	centimeter
CPPA	Canadian Pulp and Paper Association
CPZ	chlorpromazine
d	day
D	dextrorotatory
d.w.	dry weight
Da	dalton
DCIP	dichlorophenolindophenol
DEAE	diethylaminoethyl
df	dilution factor
DHP	dehydrogenation polymerizate of (lignin precurssors)
dpm	disintegrations per minute
DPPH	diphenyl-picryl-hydrazyl
EPR	electron paramagnetic resonance
eV	electron volts
FAD	flavin adenine dinucleotide
FID	flame ionization detector
FPLC	fast protein liquid chromatography
g	gram
GC	gas chromatograph
GHz	gigahertz
GO	glucose oxidase
h	hour
HMW	high molecular weight

h	hour
HMW	high molecular weight
HPLC	high performance liquid chromatography
HR	high resolution
HWKP	hardwood kraft pulp
i.d.	internal diameter
IEF	isoelectric focusing
ISO	International Standards Organization
IU	International unit
k	kilo
K	Kelvin
K_{cat}	catalytic constant of an enzyme equivalent to turnover number (s^{-1})
K_m	substrate concentration at one half of maximum reaction velocity
l	litre
LP	lignin peroxidase
m	milli
M	molar
MB	mycological broth
min	minute
ml	millilitre
mm	millimetre
MnP	manganese peroxidase
μ	micro
mPa.s	centipoise viscosity in millipascal seconds
MS	mass spectrometer
mT	milliTesla
MW	molecular weight
nm	nanometers
No.	number
ODU	optical density unit (Absorbance unit)
PAGE	polyacrylamide gel electrophoresis
pH	log H^+ ion concentration
pI	isoelectric point
pO_2	partial pressure of oxygen
r	radius
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulphate
SWKP	softwood kraft pulp

TCD	thermal conductivity detector
TDM	*Trametes* defined medium
TLC	thin layer chromatography
U	enzyme unit
UL	uniformly labeled
UV	ultraviolet light
VAO	veratryl alcohol oxidase
Vis	visible light
V_{max}	maximum rate of an enzyme catalyzed reaction
w/v	weight to volume
x	times
X	X-ray
Z-span	zero-span breaking length (of paper)
°C	degrees Celsius

CHAPTER 1. GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

The pulp and paper industry is concerned with the production of non-woven fibers appropriate for the needs of a number of industries, including the printing industry. Traditionally, research in pulp and paper has been directed at decreasing the costs of raw materials and production through improvements in plant efficiency as well as increasing the value of the end products that are produced. However, there is an increasing research effort being directed at decreasing the environmental impact of the pulp and paper industry.

Though of relatively recent application, wood has proven to be an ideal raw material for the manufacture of paper. Simple and inexpensive processes have been developed for the conversion of wood to a fibrous material suitable for the manufacture of paper and other products. One of these processes, kraft pulping, is particularly well suited for the production of strong, high value pulps. Consequently kraft mills comprise nearly half of all pulp mills in Canada.

Unfortunately, the lignin which is not removed during the kraft pulping process substantially darkens the pulp, making it unacceptable for the production of many end products where its other properties make it the ideal starting material. Traditionally, the removal of the residual lignin to increase the brightness of kraft pulp has been achieved relatively easily using chlorine compounds. However, recent concern over the fate of

many of the compounds formed during the chlorine-based bleaching processes has led to an intensive search for alternative methods for brightening and delignifying kraft pulp.

Previous work has shown that a white rot fungus, *Trametes versicolor*, when incubated with unbleached kraft pulps, substantially increased the brightness and decreased the residual lignin content of these pulps (Paice *et al.*, 1989; Reid *et al.*, 1990). The process was however inherently slow, requiring days to weeks to achieve the desired product improvements. Therefore, a method of increasing the rate of the observed bleaching and delignifying effect is being sought.

The *T. versicolor* kraft pulp biobleaching system, as it has been developed at Paprican, is being used as a model system to determine how the fungus effects the delignification and bleaching of the hardwood kraft pulp. As much as was practical, I have used this "real-world" system to investigate the delignification system of *T. versicolor*. The approach is not without its inherent difficulties, however the results obtained are with the actual substrate of interest and can be applied immediately.

1.2 Historical Overview of Papermaking

The conversion of cellulosic fibers from various plant sources to produce non-woven fiber mats is an industry which has existed for nearly two thousand years. Historically, the development of paper was first achieved by the Chinese whose knowledge of felting undoubtably led to the idea of forming an intertwined fiber mat into paper (Hunter, 1947). The invention has been attributed to Ts'ai Lun who reported the development of a process for converting hemp waste, old rags and the bark of trees to

paper to the emperor of China in 105 AD (Hunter, 1947). The process was likely the culmination of many years of development which led to a method for forming a paper of adequate quality (Hunter, 1947). The technology of papermaking slowly spread around the world, though for the first 1500 years the industry evolved using non-woody plant fibers such as flax, hemp, jute and cotton.

1.2.1. Pulp from Trees

The manufacture of paper from trees is a much more recent event which began with the development of efficient machines capable of reducing woody tissues to fibrous materials which could be formed into paper sheets. The earliest recorded mention was in 1719 when Réaumur alluded to the similarity between the composition of the nest of the American wasp which is made from wood, and that of paper (Hunter, 1947). However, it was not until 1840 in Saxony that the weaver Friedrich Keller obtained a patent for a wood grinding machine (Hunter, 1947). By 1852 a commercial groundwood pulp machine built by I.M. Voith was in regular production (Hunter, 1947). The technology was soon widely adapted and with the forest now serving as a source of papermaking fiber, the cost of newsprint fell from $0.25 per pound in 1860 to less than one cent by 1897 (Hunter, 1947).

1.2.2. Chemical Pulps

Though wood provided an inexpensive new source of fiber, the groundwood process produced pulps of inferior strength due to the very short length of fibers produced

and large amounts of debris which hindered fibre bonding. There was therefore still a requirement for a stronger pulp with a lower cost than rag pulps. A method of selectively removing lignin from wood tissue which did not destroy the cellulose fibers was needed.

One of the first methods for selective lignin removal resulted from the work of Tilghman who in 1857 discovered that at high temperature and pressure, sulphurous acid dissolved the intracellular material of wood, producing red-stained fibers (Hunter, 1947). Inclusion of unslaked lime (CaO) in the cooking liquor favored the dismutation of sulfuric acid to neutral sulphite, and the red staining problem disappeared. This led to the development of a commercially viable pulping process and by 1887, the first sulphite mills were being operated at Cornwall and Merriton Ontario (Hunter, 1947).

In 1854, H. Burgess and C. Watt patented a process in which the lignin in wood was removed by heating wood chips in a closed vessel at high pressure and temperature in the presence of alkali (Soda process)(Hunter, 1947). When resinous wood was used, the "pulping" process was followed by a treatment with chlorine or chlorine compounds to whiten the pulp.

1.2.3. Kraft (Sulphate) Pulping

It was observed that pulp delignification was accelerated when sulphur compounds were included in the caustic pulping of straw (Clayton *et al.*, 1989). This was the basis of the sulphate (kraft) process patented by Carl F. Dahl(1884). The use of the term "sulphate" reflects the use of sodium sulphate to replace sodium and sulphur ions lost from the pulping liquor during the pulping and chemical recovery steps, whilst kraft is

the German word for strong. Not surprisingly, the most important characteristic of pulps produced by this process, are their superior strength properties when compared to pulps produced using mechanical, thermomechanical, chemi-thermomechanical, sulphite or soda processes (Clayton *et al.*, 1989).

Sulphate pulp was first produced in North America at the Brompton mill in East Angus, Quebec in 1907 (Hunter, 1947). In Canada in 1992, the production of chemical pulps accounted for 48% of total wood pulp production, 93% of which was kraft pulp (CPPA, 1993). The production of kraft pulp is expected to increase in the short term (CPPA, 1993a). Though new pulping technologies, such as solvent pulping (Young, 1993; Aziz and Sarkanen, 1989) are being developed, it is unlikely that the production of chemical pulps by the kraft process will be replaced by other technologies in the near future. The Alcell process is one example of solvent pulping technology developed here in Canada (Jamieson, 1991). The application of the Alcell process is currently limited to pulping of hardwood species (Jamieson, 1991).

The process of alkaline delignification in the presence of sulphide has remained virtually unchanged since the process was first patented (Dahl, 1884). The method favors the depolymerization and solubilization of lignin with a much lower loss of the cellulose component. This is achieved by heating wood chips or shavings under pressure to 160-180°C in the presence of one molar sodium hydroxide and 0.2 molar sodium sulphide (Dahl, 1884). The sulphide in the cooking liquor accelerates the delignification when compared to alkali alone, minimizing the attack on cellulose and consequent negative impact on the strength properties of the pulp (Fig. 1.1; Dahl, 1884). Most of the

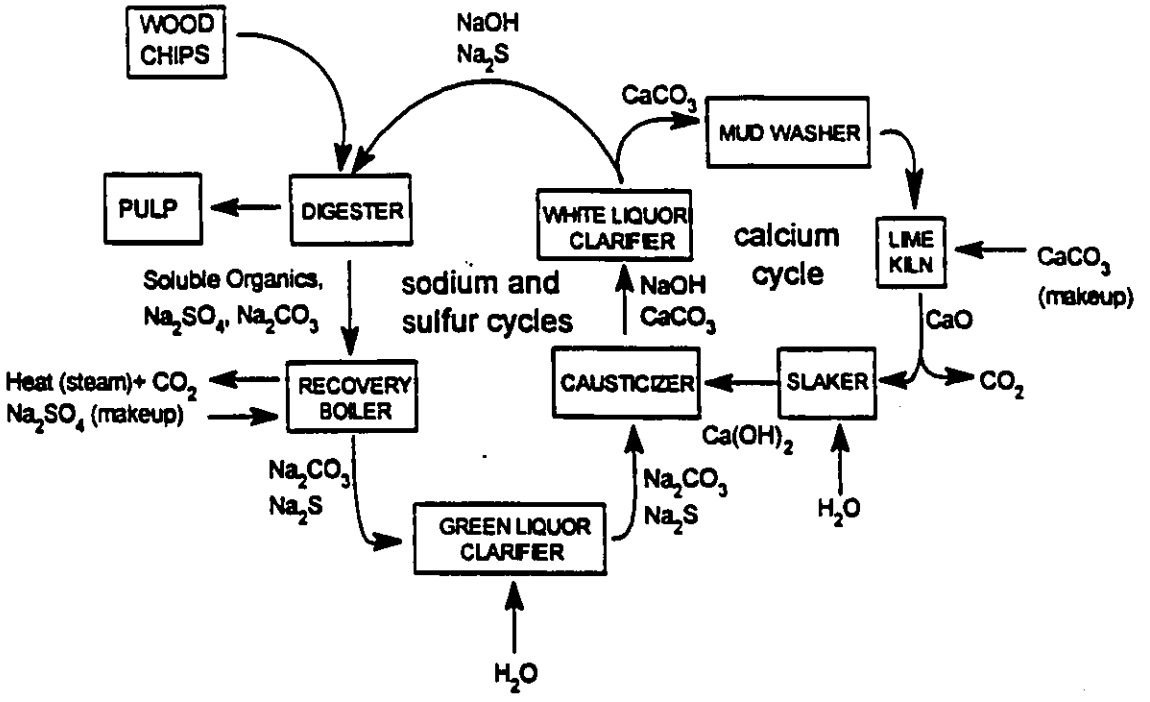


Figure 1.1. Diagrammatic representation of the kraft pulping and liquor recovery cycles.

chemicals consumed in solubilizing the lignin can be recovered by burning the dissolved organic material in the recovery furnace which also produces energy in the form of steam which can be used in the process cycle. A simplified diagrammatic representation of the liquor side of the kraft process is shown in Fig. 1.1.

1.2.4. Wood Composition and Its Modification by Kraft Pulping

1.2.4.1. Structure

The three dimensional structure of woody tissues is an important consideration if wood is used to form non-woven fiber networks (paper). There are a number of properties of wood which make it a suitable raw material. Structurally, the porous nature of the wood structure allows for the diffusion of chemical reagents into the cellular matrix where delignification must occur (Fig. 1.2; Wardrop, 1962). The differences in the chemical composition (and reactivity) of the components of woody tissues allows for selective removal of some while leaving others relatively unaffected (see section 1.2.4.2). Finally, the ability of delignified wood fibers to form a hydrogen bonded randomly oriented network (paper formation) following the pulping process is an essential property for making paper from wood (Clayton *et al.*, 1989). In designing a process for making pulp, these properties must be considered since the loss of any of them would make wood an unsuitable raw material for making paper.

In the pulping process, the objective is to remove the middle lamella (ML), while the attack on the cell wall (see Fig. 1.2) should be minimized so as to maintain the strength properties of the fiber (Clayton *et al.*, 1989; Waldrop, 1962). The major

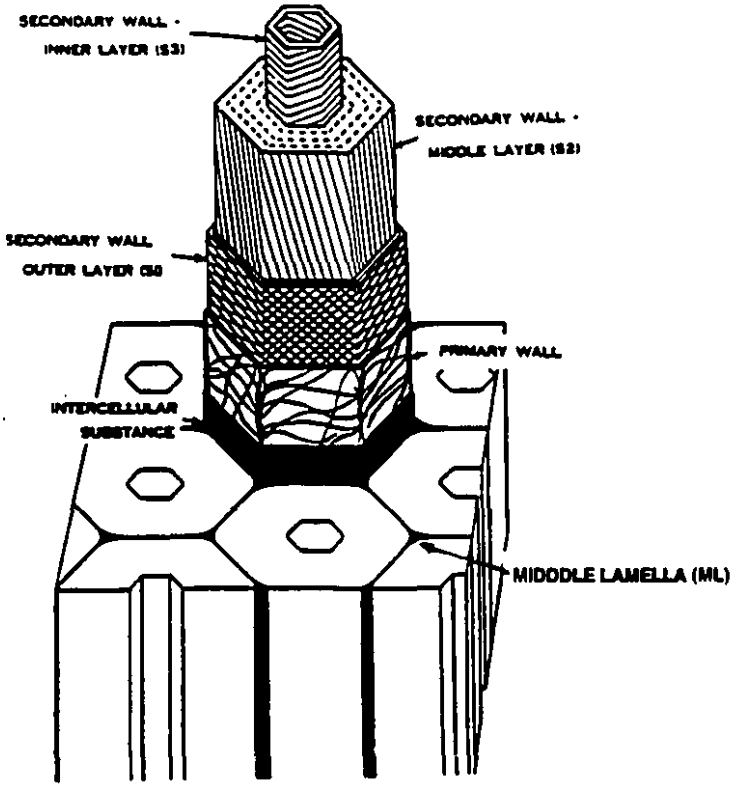


Figure 1.2. A schematic representation of the cell wall organization in a typical wood fibre. Figure taken from Wardrop (1962).

component of the ML is lignin, and lignin reactions are most important in the kraft process. Unfortunately, not all of the lignin is located in the ML. The S_2 layer (Fig. 1.2) contains more lignin by weight and reacts with delignifying reagents at the same or greater rate than observed with ML lignin (Goring, 1983). This substantially increases the amount of lignin that must be removed from woody tissues before enough is removed from the ML to allow fiber separation (Goring, 1983). The removal of lignin from the S_2 layer also affects the properties of the delignified fibers.

1.2.4.2. Composition

In plants, wood has two distinct elements, the structural and the extraneous or non-structural material (Clayton *et al.*, 1989). The extraneous materials comprise all of the water and solvent soluble substances present in the untreated woody tissues. These "extractives" vary considerably among plant species. In hardwood, they typically make up ≈2% by weight of the plant dry weight, whereas in certain softwood species they can account for up to 25% of the material present (Clayton *et al.*, 1989). These are efficiently removed from the pulp by the kraft process and will not be discussed further.

The structural element of wood is composed of three principal parts which together make up the cell wall and the intracellular matrix, namely, cellulose (40-50%), hemicelluloses (20-35%), and lignin (20-30%). The objective of the kraft process is to remove the lignin while minimizing the degradation of the other two components.

The polysaccharide components (cellulose and hemicellulose) of wood each have unique properties. Cellulose $(C_6H_{10}O_5)_n$ is a linear polymer typically having 7000-15000

anhydroglucose units. These polymers are organized into microfibrils and fibrils having crystalline and amorphous regions in the cell wall (Fengel and Wegener, 1984). The kraft process normally reduces the average chain length to between 1000-1500 units. Cellulose is generally regarded as a stable macromolecule which can, as a consequence of the large number of hydroxyl groups, form numerous hydrogen bonds with adjacent cellulose chains (Reeve, 1989). Hemicelluloses are also carbohydrate polymers, but of more variable structure than cellulose. They are non-crystalline polymers composed of a number of five carbon (xylose and arabinose) and six carbon (glucose, mannose, and galactose) sugars which form branched networks with a number of acetyl and uronic substituents (Fengel and Wegener, 1984). The hemicellulose content is quite variable among plant species, with hardwoods generally having a higher glucuronoxylan content (25-35%), while glucomannan is predominant in most softwood species (25-30%)(Reeve, 1989). The hemicelluloses and many of the acidic side chains are quite susceptible to degradation during the kraft process and hemicelluloses are more soluble than cellulose in the alkaline cooking liquor (Reeve, 1989).

The third structural component of wood is lignin. Lignin is a large, branched and crosslinked noncrystalline three dimensional polymer composed of randomly linked phenylpropane subunits with the general structure shown in Fig. 1.3. Lignin comprises approximately 20-30% of the dry weight of wood (Reeve, 1989). Lignin is synthesized in plants via free-radical coupling reactions initiated by the action of plant peroxidases (Higuchi and Ito, 1958; Harkin and Obst, 1973) and/or laccases (Sterjiades *et al.*, 1993). Generally, the inter-unit bonds are comprised of the alkali -hydrolyzable ether bonds, and

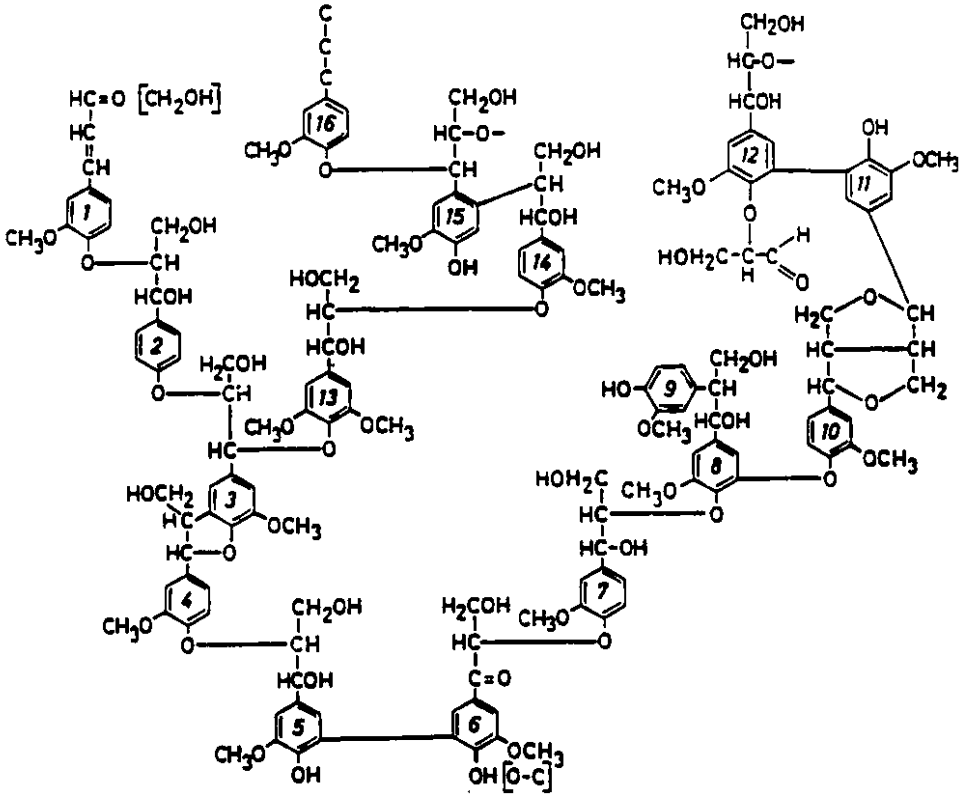


Figure 1.3. Structural features of softwood lignin. Figure taken from Adler (1977).

alkali-resistent carbon to carbon linkages (Reeve, 1989). In addition, the methoxyl units constitute approximately 20% of hardwood and 15% of softwood lignins (Reeve, 1989).

1.2.4.3 Chemistry of Kraft Pulping

The conventional kraft process is used to reduce the lignin content of hardwood and softwood from 25% to 2% (kappa number 12-15) and 30% to 5% (kappa number 26-33), respectively (Clayton *et al.*, 1989). The alkaline pulping process is not completely selective in its removal of lignin, and carbohydrate losses often equal lignin losses (Matthews, 1974). In a pulp cooked to give a 50% yield, approximately 5% of the material lost was cellulose, 20% was hemicelluloses, and the balance was lignin (Matthews, 1974). The pulp resulting from a kraft cook is therefore substantially different in composition than the wood tissue from which it is derived. Thus, what occurs is not just a simple solubilization and removal of the lignin. Appreciation of this is important for understanding the subsequent processes, including any biologically based treatment that can be envisaged for the kraft pulp.

The carbohydrate components of kraft pulp are modified by at least four types of reactions: (1) The saponification of acetyl groups and the removal of methoxyl groups from hemicelluloses; (2,3) the "peeling" sugar oxidation and cleavage reactions which are initiated early in the kraft cook before significant delignification has occurred and take place at the reducing ends of glucose polymers and proceed releasing isosaccharinic acid monomers until an alkali-stable stopping end group (metasaccharinic acid) is formed; (4) random cleavage of the polysaccharide chain, leading to a decrease in the

degree of polymerization of the pulp as well as forming sites for further "peeling" reactions (Matthews, 1974; Clayton *et al.*, 1989). Following the initial losses due to the peeling reaction, Matthews (1974) found that only a small fraction (1%) of the additional carbohydrate losses in a kraft cook were attributable to cellulose degradation under standard pulping conditions. Much greater losses occur upon solubilization and subsequent decomposition of hemicelluloses (Clayton *et al.*, 1989). It is the relative insolubility and low reactivity of wood xylans compared to lignin under alkaline pulping conditions which contribute to their retention in pulps produced by the kraft process (Clayton *et al.*, 1989). The solubilized xylans are also believed to be reprecipitated as the alkali is depleted during the course of a kraft cook (Clayton and Stone, 1963). A kraft cook yields a pulp with a composition that is primarily cellulose, with hemicellulose yields in the order arabinoxylan >> galactoglucomannan > glucomannan (Thompson *et al.*, 1962).

Improvements to the basic kraft process have come from optimization of the reaction conditions, however two important methods for removing additional lignin with only a moderate effect on pulp quality have been developed. These are the so called extended modified continuous cook (EMCC) and the oxygen delignification processes which are now being widely adopted by kraft pulp producers (MacLeod, 1993). The EMCC process is an extension of the cooking time in the digester combined with a continuous, rather than all at the start, addition of the pulping chemicals (NaOH and Na_2S). This results in a reduction of the residual lignin content of the pulp from approximately 5% to 3-4% (kappa number of 20-25) (MacLeod, 1993).

Oxygen delignification is the treatment of kraft pulp with an excess of gaseous oxygen and 1-5% NaOH at temperatures of 90-120°C (Reeve, 1989). It is possible to remove up to an additional 40% (kappa number of 12-15) of the residual lignin before the pulp moves to the bleach plant (MacLeod, 1993).

Unfortunately, the kraft process also darkens the pulp significantly, and bleaching agents are required to increase the pulp brightness for many applications (see section 1.2.5). During the kraft cook, delignifying reactions occur concurrently with condensation reactions. In one study, a conventional kraft cook was used to produce a kappa 30 softwood pulp. Analysis of this pulp showed that only 9 mol % of the residual lignin phenyl nuclei present had a non-condensed structure, whereas 54 mol % of the residual lignin occurred as various diphenylmethane type structures and the balance (37 mol %) as other condensed structures (Chiang and Funaoka, 1988).

In hardwoods, the presence of syringyl aromatic nuclei favored an increased lignin solubilization rate in the kraft cook (Chaing and Funaoka, 1990). Though condensation reaction rates also increased, the syringyl-guaiacyl condensation products were much more soluble in the kraft cooking liquor which increased their removal from the pulp (Chaing and Funaoka, 1990). The residual lignin in hardwood is predominantly composed of condensed phenyl nuclei of the guaiacyl-guaiacyl type (92 mol %) and is very similar to that found in softwood pulps (Chaing and Funaoka, 1988). Thus the residual lignin produced from softwood and hardwood is likely to be quite similar in how it responds to subsequent delignification.

The chromophoric structures of residual kraft lignin are of great interest, since their removal substantially increases the value of kraft pulp. Some of the structures which have been suggested as the source of color in kraft pulps include transition metal-lignin complexes, conjugated unsaturated carbon-carbon bond systems and quinones (Falkehag *et al.*, 1966).

In a comprehensive study of one lignin preparation, quinones were the major visible-light absorbing chromophores found (Furman, 1986). Iiyama and Nakano (1973) have estimated the quinone content of isolated kraft lignins at 0.03-0.04 ortho-quinone structures per C_9 unit accounting for 40-50% of lignin's absorbance in the visible range. Quinones also participate in the formation of charge transfer complexes with free phenolic groups, futher enhancing their contribution to chromophore formation (Furman, 1986). Neither transition metal-lignin complexes nor extended conjugated systems were a significant source of color in the residual kraft lignin studied (Furman, 1986). Still others have suggested that the color in kraft pulps is not lignin-derived but rather is due to keto-enols formed from carbohydrates during the kraft cook (Ziobro, 1989).

1.2.5. Kraft Pulp Bleaching

Bleaching is the treatment of cellulosic fibers to increase brightness and can be achieved by decolorizing or removing the colored components, primarily lignin. Bleaching processes are chosen on the basis of their operating and capital costs, selectivity for lignin, and more recently, their potential impact on the environment (Reeve, 1989a).

A series of empirical tests for evaluating the efficacy of bleaching have been developed to establish manufacturing standards and aid in the development of new processes.

Brightness is a specific test which compares the reflectance of a paper sheet compared to that observed using a standard material at a wavelength of 457 nm using a standard light source (Reeve, 1989a). Both the absorption and scattering coefficients affect the brightness of a given pulp sample (Schmidt and Heitner, 1993). The absorption coefficient is proportional to the concentration of chromophores for a given sample; however brightness is not, and for optically thick samples, the relationship between brightness and reflectance are related by the Kubelka-Munk function $f(R_\infty)$ shown in Eq. 1 (Schmidt and Heitner, 1993).

$$f(R_\infty) = K/S = (1-R_\infty)^2/2R_\infty \quad (1)$$

where R_∞ = measured reflectance
K = absorption coefficient
S = scattering coefficient

The brightness is determined by comparing the reflectance of a pulp sample to that obtained for a $BaSO_4$ brightness standard. Changes in the scattering coefficient of a given pulp (as can happen when pulps are extensively delignified) will affect the brightness value observed (Schmidt and Heitner, 1993). Brightness is a key property of paper and is the most widely used test for evaluating the efficacy of bleaching processes. Brightness values for typical commercial papers vary between 55-60% for newsprint, 20-40% for kraft bags, and 85-93% for fine papers photographic paper grades.

Other properties which must be considered when developing bleaching technologies are the ability to decolorize "dirt" particles, originating from the wood, such

as uncooked fiber bundles (shives), knots, pitch, and bark. The strength of kraft pulps and the degree of polymerization of the cellulose component are important properties, and the bleaching process should not decrease them substantially. The kappa number is used to measure the quantity of residual lignin in kraft pulps determined by measuring consumption of acidic potassium permanganate by a pulp sample under carefully controlled conditions (CPPA method G18). The kappa number of a pulp corresponds to approximately 6 times its lignin content in percent of dry weight. Kappa number is also used in this work to monitor the progress of delignification during bleaching.

1.2.6. Chemical Bleaching Methods

Chemical oxidants which are most often used for bleaching kraft pulps include chlorine, hypochlorite, chlorine dioxide, and to a lesser extent hydrogen peroxide and oxygen, the latter most commonly as a part of an oxygen delignification stage or in an alkali-oxygen bleaching stage (Reeve 1989a). The removal of lignin from kraft pulps has historically been dependent upon the use of chlorine (Cl_2) for lignin oxidation, and chlorine compounds (ClO_2) for pulp brightening (Reeve, 1989a). For example, the tendency of hypochlorite to form low levels of chloroform has led to a tremendous decrease in its use in the bleach plant (Reeve, 1989a). Increasing concern over the potential toxicity and fate of chlorinated organic material produced during chlorine (and to a lesser degree ClO_2) bleaching has created an opportunity for chlorine-free or low chlorine technologies.

One of the first of the new technologies, xylanase pulp pretreatment, can decrease chlorine and chlorine dioxide use by up to 20-25% (Viikari *et al.*, 1986). This technology is used on a regular basis in many mills and is the first successful application of biotechnology in the pulp and paper industry (Paice *et al.*, 1992).

Numerous other chemical-based technologies are being developed to bleach pulp. Paprican recognized and applied ozone as a pulp bleaching agent in the 1970s (Liebergott *et al.*, 1975; 1977; Liebergott and van Lierop, 1981) and environmental concerns have generated renewed interest in this area and many pilot scale studies of ozone stages have been run (Lachenal, 1991). A Canadian pulp mill in Espanola, Ontario, has done pilot studies with ozone and Union Camp Corporation in the United States has a full scale ozone bleaching stage in their 1000 tonne per day kraft mill (Lachenal, 1992) and recently a commercial bleaching process using ozone has been developed (Sixta *et al.*, 1991). Other non-chlorine technologies being developed include the use of peroxy acids and metal catalysts (Dence and Francis, 1991) and the application of reductive agents (Liebergott, 1991). Presently, these new approaches have various problems with high costs, the toxicity of the effuents produced and efficiency of the processes. Therefore there is still no really good alternative to bleaching sequences containing chlorine dioxide.

1.3.1. Biological Delignification

Biological bleaching using *T. versicolor* or its enzymes is one alternative technology being pursued and in this thesis, the possible role(s) of enzymes and metabolites produced by this fungus in kraft pulp biobleaching and delignification are

explored. In many ways, kraft pulp can be considered a xenobiotic material, since wood heated at high temperature in the presence of alkali does not exist in most natural environments. Nonetheless, the fungus was first isolated from decaying wood where its marked ability to bleach and delignify woody tissue is readily observed.

All of the improvements in the kraft process to date still yield a pulp that contains sufficient chromophores to keep its brightness below what is required in many applications, and the residual lignin must be bleached or removed. Technology based on *T. versicolor* (or components thereof) for pulp delignification or bleaching must be compatible with the kraft process. A brief review of what is known about other microorganisms which can degrade the lignin component of wood tissues is given in sections 1.3.1.1-1.3.3.4

1.3.1.1. Bacteria

That bacteria are present and form an integral part of natural wood decay processes is well established (Shigo, 1967; Blanchette and Shaw, 1978). What is less well understood is the precise role of bacteria in wood degradation. Some species of bacteria can hydrolyze the cell wall carbohydrates of woody plants. In a survey of 60 bacterial species isolated from decaying trees, none of the organisms surveyed were able to attack the lignified cell walls of pine or beech sapwood (Schmidt and Liese, 1982). A mild chlorite treatment, to partially delignify the plant tissues led to substantial weight loss due to attack by these bacteria(Schmidt *et al.*, 1987).

The ability of bacteria to degrade lignin and has been studied most extensively in the actinomycetes (Kirk and Farrell, 1987). Incubation of ^{14}C-lignin with a number of streptomycetes resulted in the release of less than 20% of the radioactivity as $^{14}CO_2$ (McCarthy and Broda, 1984; Mason *et al.*, 1988). The formation of water soluble acid precipitable polymeric lignin (APPL) is associated with bacterial attack on lignin (Pometto and Crawford, 1986), however the compositon of this material is not very different from that of native lignin (Mason *et al.*, 1988). Certainly the low numbers of bacteria which exhibit ligninolytic activity as well as the low rate of lignin mineralization obtained using the known delignifying bacteria has limited the amount of research in this area (Vicuña, 1988). The enzyme lignin peroxidase (LP), implicated in fungal-mediated lignin degradation (section 1.3.2), is produced by some actinomycetes (Ramachandra *et al.*, 1988; Spiker *et al.*, 1992). However, bacterial lignin degradation is limited even in the presence of this enzyme. No bacterial species, or even consortia of bacteria has been shown to completely degrade lignin.

1.3.2 Wood-rotting Fungi

Though an extensive literature exists in the broad field of fungal wood colonization and degradation, only the major lignin-degrading fungi are reviewed here. The total number of wood-rotting basidiomycete species in North America has been estimated to be between 1600-1700 (Gilbertson, 1980). This group of fungi comprise by far the majority of organisms capable of lignin degradation. Fungi having wood-degrading activities have been classified into three categories, namely the white-rot (*Basidiomycetes*),

brown-rot (*Basidiomycetes*), and soft-rot (*Ascomycetes*) fungi (Leisola and Feichter, 1985).

The soft-rot fungi degrade lignin to a lesser extent than the white-rot fungi. This form of degradation is characterized by a loss of rigidity in the wood structure, without substantial loss of lignin. In one study, 1-8% of the added (^{14}C)-lignin was degraded to $^{14}CO_2$ after 25 days, compared to a release of 15-40% of the ^{14}C as $^{14}CO_2$ by white-rot fungi (Kirk *et al.*, 1975). Soft-rot fungi will attack lignin to a limited degree, especially in hardwoods where they cause some demethylation and perhaps a very limited attack on aromatic rings and side-chains (Ander and Eriksson, 1978).

The brown-rot fungi, a subset of the wood-rotting Basidiomycetes, arose relatively recently in evolutionary terms from the more widely distributed phenoloxidase-producing white-rot fungi (Gilbertson, 1980). In general terms, the brown-rot fungi degrade wood polysaccharides leaving most of the aromatic rings of lignin intact with only limited changes in lignin structure. Consequently, wood decayed by brown-rot fungi becomes increasingly lignin-enriched as the cellulose is degraded, resulting in increasingly brown-colored residual tissues. As a group, the brown-rot fungi comprise only a small fraction of wood-rotting Basidiomycetes (6%), and are mostly found growing on coniferous tree species (Gilbertson, 1980). Gilbertson (1980) has suggested that most species of brown-rot fungi arose as a consequence of the concurrent loss of mating type alleles and the ability to produce extracellular phenoloxidases, in a process of genome simplification that has occured more than once. Evidence cited includes the observation that species of brown-rot fungi are distributed throughout the whole range of wood-rotting Basidiomycete fungi (Gilbertson, 1980).

Generally, the brown-rot fungi do not degrade lignin; however as with most classification schemes, there are exceptions. One example is *Cyathus stercoreus* which degrades lignin as extensively as most white-rot fungi (Agosin *et al.*, 1985). Brown-rot fungi do mediate limited ring cleavage and hydroxylation of aromatic rings as well as extensive lignin demethylation when growing on wood substrates (Kirk, 1984). Generally, much more aggressive lignin modification and mineralization is seen with the white-rot fungi.

1.3.3. Biobleaching and White-rot Fungi

1.3.3.1 White-rot Fungi

Several thousand species of the Basidiomycotina produce a white-rot type of decay in softwoods and hardwoods (Gilbertson, 1980). The removal of lignin from the decaying tissues results in a progressive decolorization of the wood; hence the term "white-rot". It is possible to distinguish between the white- and brown-rot fungi on the basis of a secreted oxidase activity which produces a colored zone around colonies of white-rot fungi when they are grown on an agar medium containing certain phenolic compounds, including lignin (Nobles, 1958; Ander and Eriksson, 1977). There is a considerable literature which exists describing in detail the ability of certain white-rot fungi to either simultaneously degrade both lignin and cellulose/hemicellulose, or to preferentially degrade the lignin in wood. However within a single sample of wood being attacked by a single fungus it can be demonstrated that both simultaneous and preferential types of lignin degradation occur (Blanchette, 1984).

1.3.3.2 Biobleaching

Kirk and Yang (1979) reported that static ligninolytic cultures of *Phanerochaete chrysosporium* could significantly increase the alkali extractability of softwood kraft pulp lignin; however little change in pulp brightness was observed. Paice *et al.* (1989) were the first to describe a process whereby the fungus *T. versicolor* both decreased the lignin content and increased the brightness of hardwood kraft pulp. Hardwood kraft pulp inoculated with an actively growing *T. versicolor* culture gave about a 20 point increase in the ISO brightness of pulp pads after four or five days incubation (Paice *et al.*, 1989). The increase in the pulp brightness was accompanied by decreases in lignin content (kappa number), and the degree of polymerization of the cellulose fibers, however the overall strength properties of the pulp were improved (Paice *et al.*, 1989).

Reid *et al.* (1990) showed that a similar delignification was obtained, albeit over a longer 14 day incubation using softwood kraft pulp; however the brightening effect was much less pronounced than with hardwood pulps.

Subsequently, brightness values approaching those obtained with hardwood were attained using a softwood pulp. The authors attributed the differences in brightness observed in the earlier work to the higher initial lignin content of the softwood pulp (Reid and Paice, 1993). The rate and extent of delignification mediated by *T. versicolor* 52J were not dependent on the lignin content (measured as kappa number) or the lignin type (hardwood versus softwood) present in the pulp (Reid and Paice, 1993). A standard method (kappa test) which measures the amount of potassium permanganate oxidizable material (usually residual lignin) is routinely used to measure the lignin content of kraft

pulps. A kappa number of one corresponds to approximately one sixth of one percent of the dry weight of the wood as lignin (eg. a pulp containing 1% lignin by weight would have a kappa number of 6). It was found that after an initial lag period, the delignification of kraft pulps proceeded until a kappa number as low as four was attained (Reid and Paice, 1993). Fungal biomass accounted for $KMnO_4$ consumption equivalent to a kappa number of 2.5, suggesting that residual lignin levels as low as kappa number 1.5 can be attained by *T. versicolor* 52J in 14 days (Reid and Paice, 1993).

A biobleaching effect, similar to that observed with *T. versicolor*, has been reported for solid-state fermentations with a fungus identified only as IZU-154 which reduced subsequent bleaching chemical requirements by 72% (Fujita *et al.*, 1991; Fujita *et al.*, 1993). Furthermore, it was found that brightnesses of greater than 80% ISO for biobleached kraft pulps could be obtained in subsequent steps without the use of chlorine (Murata *et al.*, 1992). Addleman and Archibald (1993) showed that the use of a monokaryotic strain of *T. versicolor* was useful in eliminating dark particles often formed during biobleaching with the dikaryotic strain of this fungus (the same strain which was used in the work of Paice *et al.*, 1989; Reid *et al.*, 1990).

Intimate contact between the fungal hyphae and the pulp fibers does not seem to be a requisite for bleaching since microporous membrane-isolated *T. versicolor* biobleaching cultures could successfully bleach hardwood kraft pulp (Archibald,1992) . Similar results have been shown for the fungus IZU-154 (Kondo *et al.*, 1993). Kirkpatrick *et al.*(1990), using the flask bleaching system (Paice *et al.*, 1989) showed that foam-imobilized *T. versicolor* cultures could also biobleach pulp.

1.4.1. Chemistry of Lignin Biodegradation

Though at present it is unclear how the reactions of native wood lignin compare to what occurs with the more highly condensed and modified lignin-derived material in hardwood kraft pulp, a brief review of some of the ligninolytic reactions mediated by white-rot fungi follows.

Degradation of lignin by white-rot fungi occurs at the surface of the lignin, and as it is degraded, the lignin's alkali (Reid and Paice, 1993) and solvent (Chen and Chang, 1985) extractabilities increase. Analyses of the results of many studies of wood biodegradation suggests that it is largely an oxidative process since the oxygen content of the biodegraded lignin increases (Eriksson *et al.*, 1990). There is also a significant demethylation of the lignin (Chen and Chang, 1985). Nagieb *et al.* (1988) found that the phenolic hydroxyl content per C_9 unit of lignin was increased during biodegradation. I. Reid (Pers. commun.) has found that the MW of the water soluble and alkali extractable lignin is relatively unchanged from that of the residual lignin in hardwood kraft pulp.

1.4.2. Enzymology of Lignin Degradation by White-rot Fungi

Though the three oxidative enzymes most commonly associated with delignification are discussed in terms of the reactions which they mediate, it is clear that the reaction specificities are overlapping. In fact, the generic term phenoloxidase collectively describes all three. The presence of different reactants in fungal cultures or *in vivo* systems greatly influences the nature of the reactions which occur. A brief

summary of each of the three oxidative enzymes, and the reactions mediated by each which are thought to be important in delignification are outlined.

1.4.2.1. Laccase

The role of laccases (phenoloxidases) (EC 1.10.3.2) in lignin biodegradation has been extensively studied (Kirk, 1971; Kirk and Farrell, 1987: Eriksson *et al.*, 1990). Unlike the lignin peroxidases, the laccases typically oxidize aromatic compounds having phenolic hydroxyl or amino groups, concommitantly reducing O_2 tetravalently to form two water molecules. Laccase, in the presence of O_2, will catalyze the single-electron oxidation of its substrate to a reactive intermediate having an unpaired electron (a free radical). This intermediate can then undergo many different non-enzymatic reactions such as coupling and polymerization, alkyl-phenyl cleavage, and demethylation before becoming a stable product (Kirk and Shimada, 1985).

Lignin contains phenolic hydroxyl groups, and most white-rot fungi produce laccase. The fact that lignin degradation is oxidative provides circumstantial evidence that laccase may play a role (Eriksson *et al.*, 1990). In *T. versicolor*, two laccases (A and B) were isolated, purified and characterized as copper proteins (4 atoms/laccase molecule)(Fahraeus and Reinhammar, 1967). Studies of *T. versicolor* laccase by Kaplan (1979) found that it did not depolymerize a number of lignin preparations, though Leonowicz *et al.* (1985) found evidence that it both polymerized and depolymerized lignosulphonate. Morohoshi *et al.* (1987) separated three *T. versicolor* laccases and found that laccases "I" and "II" both polymerized and depolymerized lignin, whereas

laccase "III" depolymerized milled wood lignin and a water soluble lignin fraction. Clearly, laccase can attack exposed phenolic units in lignin, however non-phenolic structures may not be affected (Eriksson *et al.*, 1990).

Recently, Bourbonnais and Paice (1990) have shown that in the presence of ABTS (2,2'-azino-di-[3-ethylbenzthiazoline sulfonate (6)]) laccase catalyzed the oxidation of a nonphenolic substrate, veratryl alcohol, to veratraldehyde in quantitative yield. ABTS was oxidized by laccase to form a relatively stable cation radical with a long half life which in some fashion promoted the oxidation of compounds not normally oxidized by this enzyme. Sariaslani *et al.* (1985) reported that in the presence, but not in the absence of another phenathiazine, chlorpromazine, that laccase oxidized rotenone. In a subsequent study it was found that incubation of kraft pulp with laccase and ABTS led to substantial demethylation and delignification of the pulp (Bourbonnais and Paice, 1992). Clearly then, laccase can indirectly initiate reactions in substances which are not normally or directly substrates for the enzyme. This implies that the true substrate range *in vivo* where "physiological" cofactors (analogues of ABTS perhaps) are present, is undoubtably much greater than what *in vitro* assays of laccase *per se* would suggest.

1.4.2.2 Lignin Peroxidase

In 1983, two groups simultaneously published their finding of a new enzyme produced by *Phanerochaete chrysosporium* (Tien and Kirk, 1983; Glenn *et al.*, 1983). The enzyme was identified by its ability to oxidize veratryl alcohol to veratraldehyde in the presence of H_2O_2 and was given the trivial name ligninase (Tien and Kirk, 1984). The

enzyme is unique among the fungal oxidative enzymes characterized to date in its ability to directly oxidize non-phenolic aromatic compounds.

The lignin peroxidase (LP) gene is widely distributed among the white-rot fungi and is produced as multiple isozymes in most of strains (Gold *et al.*, 1993). In fact the number of isozymes produced by *P. chrysosporium* and *T. versicolor* is quite striking, the former producing 6 LPs (Kirk *et al.*, 1986) and 4 MnPs (Pease and Tien, 1992) and the latter 16 LPs and 5 MnPs (Johansson and Nyman, 1993; Johansson *et al.*, 1993). The enzyme was isolated from ligninolytic culture supernatnats of *P. chrysosporium*, purified and characterized as a glycoprotein (15% carbohydrate) with a molecular weight of 41-42000 and a heme prosthetic group (protoporphyrin IX (Gold *et al.*, 1984; Tien and Kirk, 1984). The crystal structure of LP has been solved and overall is most similar to cytochrome c peroxidase (Edwards *et al.*, 1993; Piontek *et al.*, 1993; Poulos *et al.*, 1993).

LP has a typical peroxidase catalytic cycle (Renganathan and Gold, 1986) and the resting enzyme is oxidized by a one molar equivalent of H_2O_2 to form compound I ($2e^-$) which can then be reduced in two steps (with compound II as an intermediate) to reform the native enzyme in the resting state (Renganathan and Gold, 1986). The sequential one-electron oxidations suggests that a veratric radical is formed as an intermediate; however this radical was not detected using EPR (Tien *et al.*, 1986). LP can apparently oxidize methoxybenzenes having a redox potential too high for them to be susceptible to oxidation by horseradish peroxidase (Kersten *et al.*, 1987). Using proton nuclear magnetic resonance spectroscopy, Banci *et al.* (1991) observed slight differences in the ligands in the heme pocket of LP, horseradish peroxidase, and cytochrome c peroxidase.

They have suggested that this might account for the higher redox potential of the LP enzyme. Unfortunately, the behavior of the many small lignin model compounds whose reactions with LP have been extensively characterized do not appear very relevant to the action of LP on kraft lignin. Therefore they will not be extensively discussed.

Following the initial LP-catalyzed oxidation of non-phenolic aromatic nuclei to their corresponding cation radicals, many reactions can follow. Some that have been reported include β-O-4, C_α-C_β, and aromatic ring cleavage, though the latter will only occur in the presence of O_2 (Miki *et al.*, 1987; Eriksson *et al.*, 1990).

Evidence of ^{14}C-methylated birch lignin depolymerization has been reported, showing that approximately 6% of the total radioactivity incubated with LP was recovered as a low molecular weight fraction (Kirk *et al.*, 1984). However, it was suggested that the methylation of the birch lignin may have blocked free hydroxyl groups, thereby decreasing polymerization and favoring depolymerization reactions (Haemmerli *et al.*, 1986).

LP is also produced by *T. versicolor* (Evans *et al.*, 1984; Dodson *et al.*, 1987; Jönsson *et al.*, 1987; Waldner *et al.*, 1988; Archibald, 1992; Johansson and Nyman, 1993). The enzyme molecular weight is reported at values between 43-53000 with a pI between 3.7 and 3.1. Like the LP isolated from *P. chrysosporium*, the enzyme from *T. versicolor* has a heme cofactor (Evans *et al.*, 1984; Dodson *et al.*, 1987; Jönsson *et al.*, 1987; Archibald, 1992; Johansson and Nyman, 1993).

Though the *T. versicolor* strain (52J) employed in this work synthesized and secreted LP under certain conditions, Archibald (1992) did not detect LP activity in

biobleaching cultures. One reason was the finding that biobleaching supernatants inhibited the LP enzyme in the three different assay systems tested (Archibald, 1992). It was also shown that the high N, high C, short growth, shaken conditions found in the biobleaching system completely repressed the expression of LP in *T. versicolor* 52J (Archibald, 1992). Recently, it has been shown that oxalic acid, a common metabolite produced by many white-rot fungi, inculding *T. versicolor* (Dutton *et al.*, 1993) can non-competitively inhibit the activity of LP (Ma *et al.*, 1993). Furthermore, the metavanadate ion, which was a potent inhibitor of *T. versicolor* LP, had no significant effect on biobleaching (Archibald, 1992). Wariishi and Gold (1989) demonstrated that veratryl alcohol could prevent the formation of the inactive compound III form of LP by reducing the enzyme back to the native state, and a lack of veratryl alcohol in biobleaching *T. versicolor* cultures may have increased LP inactivation (Archibald, 1992).

1.4.2.3 Manganese Peroxidase

Soon after the discovery of LP, a distinctly different class of manganese-dependent peroxidases (MnP) was reported in *P. chrysosporium* by Kuwahara *et al.* (1984). Four heme co-factored isozymes of MnP were isolated from *P. chrysosporium* cultures which were dependent on H_2O_2 and Mn(II) for activity (Glenn *et al.*, 1986). The primary activity of the MnP was catalyzing the oxidation of Mn(II) to Mn(III), though a limited number of phenolic substrates could be oxidized directly by the enzyme (Glenn *et al.*, 1986). Unlike LP, the enzyme normally oxidizes only phenolic aromatic compounds (Wariishi *et al.*, 1989). In the presence of thiol compounds, non-phenolic lignin model

compounds were oxidized by MnP-formed Mn(III)(Forrester *et al.*,1988; Wariishi *et al.*, 1989). MnP is also produced by *T. versicolor* (eg. Johansson and Nyman, 1987; 1993; Johansson *et al.*,1993; Paice *et al.*, 1993).

The catalytic cycle of *P. chrysosporium* MnP has been characterized. The resting state of MnP forms compound I (in the presence of H_2O_2) which is then reduced in two steps with an intermediate, compound II (Wariishi *et al.*, 1988). It was suggested that Mn(II) is an obligatory substrate for the reduction of MnP from compound II to the resting enzyme (Wariishi *et al.*, 1988). In the presence of excess peroxide, MnP forms inactive compound III (Wariishi *et al.*, 1988).

Datta *et al.* (1991) have identified glyoxal oxidase, LP, and a relatively large production of MnP protein among extracellular proteins present during the degradation of aspen wood pulp by *P. chrysosporium.* Limited depolymerization of four different lignin preparations by MnP *in vitro* has been reported (Wariishi *et al.*, 1991). MnP was produced by *T. versicolor* during biobleaching of kraft pulp, and the maximum level of MnP activity in the culture supernatant was concurrent with the maximum rate of pulp bleaching (Paice *et al.*, 1993). Purified MnP from *T. versicolor* cultures was responsible for most of the observed demethylation and delignification exhibited by the cell-free culture supernatants (Paice *et al.*, 1993). The MnP *per se* did not account for the whole of the substantial brightening normally observed with the whole fungal system (Paice *et al.*, 1993).

Like LP, MnP is produced during secondary metabolism in nitrogen-limited cultures of *P. chrysosporium* (Kirk and Farrell, 1987). In *P. chrysosporium*, MnP

secretion is promoted by the presence of Mn(II) ions in the culture medium (Brown *et al.*, 1990). A similar induction of MnP production in response to manganese was noted in *T. versicolor* (Paice *et al.*, 1993) and *Dichomitus squalens* (Perie and Gold, 1991). Van der Woude *et al.* (1993) found that nitrogen regulation of *P. chrysosporium* MnP is independent of the effects of carbon and manganese on the expression of the enzyme. The presence of solid MnO_2 was found to improve the production of extracellular peroxidases by *P. chrysosporium* (Kern, 1989; 1990).

It now appears that Mn(III) ions produced by MnP are important in lignin degradation. Forrester *et al.* (1988) demonstrated that suitably chelated Mn(III) alone was capable of oxidizing veratryl alcohol, lignin model compounds and lignin. Considerable attention has therefore focused on the role of chelators in the biochemistry of Mn(III)-complex formation by MnP from *P. chrysosporium* (Kuan *et al.*, 1993; Wariishi *et al.*, 1992). Kuan *et al.* (1993) suggested that MnP requires a chelated form of Mn(II) for reduction of compound II to the resting enzyme, whereas Wariishi *et al.*(1992) believe that uncomplexed Mn(II) is the substrate for the enzyme. In the *T. versicolor* kraft pulp biobleaching system, added manganese (30-100 μM) beyond what is absolutely required for normal fungal growth is essential for kraft pulp biobleaching by *T. versicolor* (F. Archibald, pers. comm.).

Most ligninolytic fungi produce small acidic metabolites which can form complexes with Mn(III) (Wariishi *et al.*, 1992; Kuan and Tien, 1993a; 1993b; Perez and Jefferies, 1992; 1993). The regulation of MnP expression by Mn ions is reported to be greatly modulated by the presence of organic acids and that removal of soluble Mn causes

substantial induction of LP (Perez and Jefferies, 1993). It was found that low levels of MnP (high LP) favored the depolymerization of DHP lignins and that soluble Mn(II)/Mn(III) organic acid complexes repress LP synthesis in *P. chrysosporium* (Perez and Jefferies, 1992). Both oxalate and glyoxalate, effective chelators of Mn(III), are produced by *P. chrysosporium* and support the activity of MnP even in the absence of an exogenous source of H_2O_2 (Kuan and Tien, 1993a; 1993b).

1.4.3. Reductive Enzymes

In addition to the redox enzymes described in the previous sections whose oxidative half reactions are considered to be important for driving fungal delignification, there are other redox enzymes whose reductive half reactions have been implicated in lignin biodegradation. These enzymes are described in sections 1.4.3.1 and 1.4.3.2.

1.4.3.1 H_2O_2-generating Enzymes

The discovery of LP in 1983 (Tien and Kirk, 1983; Glenn *et al.*, 1983) and MnP in 1984 (Kuwahara *et al.*, 1984) has led to considerable effort to identify potential sources of the H_2O_2 required for the secreted peroxidases. Koenigs (1972) first reported the extracellular production of H_2O_2 by several white-rot fungi, including *T. versicolor*. In *T. versicolor* H_2O_2 was produced within the hyphal sheath and the cytoplasm (Highley and Murmanis, 1985). Numerous enzymes have been suggested as potential sources of H_2O_2, however the two which have been extensively characterized are glucose and glyoxal oxidases.

Two different intracellular glucose-oxidizing enzymes have been isolated from ligninolytic cultures of *P. chrysosporium*, a glucose-1-oxidase (Kelley and Reddy, 1986), and a glucose-2-oxidase (Eriksson *et al.*,1986). However, for driving secreted peroxidase extracellular H_2O_2 production is probably required, since at least in *P. chrysosporium* (Green and Gould, 1983) and in *T. versicolor* (Archibald, 1992) significant levels of intracellular catalase are produced.

Another enzyme which could potentially supply H_2O_2 to extracellular peroxidases is glyoxal oxidase (Kersten and Kirk, 1987). This extracellular enzyme, is produced by *P. chrysosporium* during secondary metabolism and oxidizes a number of low molecular weight compounds, such as glyoxal, acetaldehyde, and methylglyoxal also found under similar conditions (Kersten and Kirk, 1987). It has recently been cloned and mRNA expression studies show that it is produced under ligninolytic conditions (Kersten and Cullen, 1993).

In addition to the enzymes described above, *P. chrysosporium* produces intracellular alcohol oxidases which catalyze the oxidation of methanol to formaldehyde and produce H_2O_2 in the presence of O_2 (Nishida and Eriksson, 1987; Kuwahara *et al.*,1987). The veratryl alcohol oxidase isozymes secreted by *Pleurotus sajor-caju* efficiently generate H_2O_2 in the presence of O_2 with a concommitant oxidation of a range of aromatic alcohols (Bourbonnais and Paice, 1988). Veratryl alcohol oxidase has not been found in cultures of ligninolytic *T. versicolor* (Robert Bourbonnais, pers. comm.). Extracellular Mn-dependent peroxidases will also reduce O_2, in the presence of electron donors such as NADH, NADPH or glutathione. However the extracellular concentrations

of these cofactors may not be sufficient to produce significant amounts of H_2O_2 (Paszcynski *et al.*, 1985; Glenn and Gold, 1985) i.e. this may not be a significant reaction *in vivo*. It has also been demonstrated that in the presence of certain phenolic compounds and manganese, that peroxidases can oxidize oxalic acid and form H_2O_2 (Kenten and Mann, 1953). Finally, the ability of cellobiose:quinone oxidoreductase (CBQase) to produce H_2O_2 has been demonstrated for the CBQase and cellobiose oxidase (CBO) isolated from *P. chrysosporium*. This will be discussed in detail in section 1.4.3.2.

1.4.3.2 Cellobiose:quinone Oxidoreductase

The idea that reductive enzymes may play a role in the degradation of lignin was first put forward by Westermark and Eriksson (1974a, 1974b). Law (1959) had reported that the white-rot fungus *Polystictus sanguineus* could decolorize phenolic oxidation products and reduce preformed quinones. An enzymatic activity that could decolorize the oxidation product of guaiacol (2-methoxyphenol) formed by growing cultures of *Phaerochaete chrysosporium* and *Trametes versicolor* in the presence of cellobiose was isolated and purified from the former (Westermark and Eriksson, 1974b).

Since the initial report approximately 40 papers have been published on a variety of possible roles for cellobiose-dependant quinone reducing activity. Unfortunately, the nomenclature for this enzyme has been anything but uniform, and proteins sharing many of the same catalytic activities have been variously described as cellobiose oxidase (Ayers *et al.*, 1978), cellobiose dehydrogenase (Bao *et al.*, 1992), cellobiose:quinone

oxidoreductase (Westermark and Eriksson, 1974), cellodextrin:Fe(III) oxidoreductase (Kremer and Wood, 1992) and cytochrome c:oxidoreductase (Samejima and Eriksson, 1992). In this work the term CBQase has been used throughout to describe this enzymatic activity in *T. versicolor* and *Sporotrichum thermophile*. Glucose oxidase, a flavin - cofactored enzyme with a number of properties (including quinone reduction) similar to those of CBQase is produced by many non-ligninolytic fungi (e.g. *Aspergillus niger*). This class of enzymes may differ from CBQases only in their preferred electron-donating substrate (Szklarz and Leonowicz, 1986).

A survey of a number of white-rot (Ander and Eriksson, 1977) and brown-rot (Ander and Eriksson, 1978) fungi suggested that CBQase was produced only by former. Subsequently, CBQase was found in many non-lignin degrading cellulolytic fungi (Table 1.1), including *Coniophora puteana* (Schmidhalter and Canevascini, 1993) initially reported as a non-producer of this enzyme (Ander and Eriksson, 1978). CBQases may represent a retained component of the ligninolytic system of white-rot fungi if the hypothesis of brown-rot fungal evolution is correct (Gilbertson, 1980; page 21, this thesis).

Those CBQases which have been purified and characterized are shown in Table 1.2. Two key properties are shared by most CBQases: (1) oxidation of cellobiose to cellobionolactone; (2) reduction of quinones to hydroquinones. The isolations of the enzyme have been based on the ability of the enzyme to reduce quinones, or in the case of Ayers *et al.* (1978) the production of aldonic acids from lactose was measured using lactate dehydrogenase. CBQase, like many of the other ligninolytic enzymes described

Table 1.1. Fungi in which CBQase activity has been reported.

Species	Culture medium	Detection method	References
A: White-rot Fungi[1]			
Sporotrichum pulverulentum (Phaerochaete chrysosporium)	cel[2] +lig[3]	TBBQ[4]	Ander and Eriksson (1978)
Phanerochaete (3 strains)[5]	"	"	ibid.
Polyporus (Gloeoporus) dichrous	"	"	ibid.
Phlebia radiata	"	"	ibid.
Merulius (Phlebia) tremellosus (tremollosa)	"	"	ibid.
Pycnosporus cinnabarinus	"	"	ibid.
Pleurotus ostreatus	"	"	ibid.
Fomes annosus	cb[6] + lig	DCIP[7]	Hütterman *et al.* (1982)
Ischnoderma (Polyporus) resinosus (benzoinus)	cel + lig	clear zone[8]	Ander and Eriksson (1978)
Poria ambigua	"	"	ibid.
BP5-1 (not identified)	"	"	ibid.
Phlebia (Peniophora)gigantia	"	"	ibid.
Phanerochaete velutina	"	"	ibid.
Bjerkandera (Polyporus) adusta	"	"	ibid.
Trametes (Polyporus) hirsuta	"	"	ibid.
Trametes (Polyporus/Coriolus) versicolor	"	"	ibid.
Trametes (Polyporus) zonata (zonatus)	"	"	ibid.
Lycoperdon pyriforme	"	"	ibid.
Pholiota mutabilis	"	"	ibid.
Gymnopilus (Pholiota) spectabilis	"	"	ibid.
Cerrena unicolor	"	"	ibid.
Fomes ulmarius	"	"	ibid.
Phellinus isabellinus	"	"	ibid.
B: Other Fungi[9]			
Sporotrichum (Myceliophthora) thermophile	cel.	DCIP	Coudray *et al.* (1982)
Trichoderma reesei	"	"	Dekker (1988)[10]

Table 1.1 (continued)

Species	Culture medium	Detection method	References
Cladosporium spp.	cel.	DCIP	Dekker (1988)
Sclerotium rolfsii	"	"	Dekker (1988); Sadana and Patil (1988)
Stachybotrys spp.	"	"	Dekker (1988)
Chaetomium cellulolyticum	"	"	Dekker (1988); Fähnrich and Irrgang (1982)
Monilia sitophila	"	"	Dekker (1988)
Coniophora puteana	"	"	Schmidhalter and Canevascini. (1993)

[1] Synonyms for genus and species names are given in parentheses.

[2] cel = cellulose.

[3] lig = lignin.

[4] TBBQ = 3,5-di-*tert*-butylbenzoquinone(1,2).

[5] *Phanerochaete* strains PB-1, 74084-11, and L-1.

[6] cb = cellobiose.

[7] DCIP = dichlorophenolindophenol.

[8] CBQase was reported as the formation of clearance zones around colonies on agar plates containing kraft lignin ± cellulose.

[9] *C. puteana* is a brown-rot fungus, *M. sitophila* (anamorph of *Neurospora spp.),* S. *thermophile* (anamorph of *Thielavia heterothallica)* are ascomycetes. *T. reesei* is a soft rot fungus.

[10] CBQase was detected as zones of clearing in non-denaturing polyacrylamide activity gels stained with DCIP in the presence of cellobiose.

Table 1.2. CBQases which have been isolated.

Organism	Characteristics				Reference
	Isozymes[1]	MW (kDa)	pI	Cofactor[2]	
Phanerochaete chrysosporium	2;1	57-75;90-98[3]	6.4;4.2	f;h,f	Westermark and Eriksson(1974a; 1975); Ayers et al. (1978); Morpeth (1985); Morpeth and Jones (1986);Bao et al. (1993)
Fomes annosus	2	70,t(300)[4]	7.0	nr	Hüttermann and Noelle (1982)
Coniophora puteana	1	100,d(192)[5]	3.9	h,f	Schmidhalter and Canevascini (1993)
Monilia sitophilia	1	48	5.3-5.5	nr	Dekker (1980)
Sclerotium rolfsii	1	62-5-64.5	5.18	none	Sadana and Patil (1985)
Sporotrichum thermophile	2	89.6,d;91	3.45;4.1	h,f;h,f	Coudray et al. (1982); Canevascini et al. (1991)

[1] Isozymes having distinct characteristics. A semi-colon (;) separates the properties for the different forms of the enzyme which have been identified.
[2] f= Flavin cofactor and h= heme cofactor.
[3] Shown to have a MW of 89,170 by laser induced desorption mass spectroscopy (Perera *et al.*, 1990)
[4] t= tetramer native form (MW of complex in parentheses).
[5] d= homodimeric native form (MW of complex in parentheses).

earlier, is produced by *P. chrysosporium* and the enzyme has been most extensively studied in this fungus. Approximately 70% of the publications describe the CBQase produced by this fungus.

In *P. chrysosporium*, CBQase and CBO are produced most abundantly in nitrogen-sufficient medium containing cellulosic substrates (Westermark and Eriksson, 1974; Ayers *et al.*, 1978; Ander and Eriksson, 1977; Costa-Ferreira *et al.*, 1992). Production of CBQase and CBO is very low in non-cellulose media containing glucose (Westermark and Eriksson, 1974a; 1974b). Kelleher *et al.*,(1987) found that CBQase production was highest in the presence of 4% cellulose and 0.15% acid precipitated lignin. However, ligninolytic enzymes of *P. chrysosporium* are usually produced under nitrogen-limited conditions (Fenn and Kirk, 1981; Kirk and Farrell, 1987), though lignin and Mn peroxidases are produced by *P. chrysosporium* under high nitrogen conditions in the presence of cellulosic substrates (Costa-Ferreira *et al.*, 1992). Thus both CBQase and CBO will likely influence the activity of these oxidative enzymes in *P. chrysosporium* cultures (Ander, 1993).

In *P. chrysosporium*, two enzymes can catalyze quinone reduction, an enzyme with haem and flavin cofactors, also known as cellobiose oxidase (Ayers *et al.*, 1978), and a second enzyme which has only a flavin (CBQase)(Westermark and Eriksson, 1974). Both enzymes use cellobiose as the preferred reductant for the enzyme, however lactose, higher cello-oligosaccharides (up to n=6) and even glucose will reduce the enzyme (Ayers *et al.*, 1978; Morpeth and Jones, 1986; Bao *et al.*, 1993). The CBQase enzyme can reduce both *ortho*- and *para* quinones (Westermark and Eriksson, 1974) and

subequently it was demonstrated that CBO also reduced quinones (eg. Morpeth, 1985). The reduction of quinones such as dichlorophenolindophenol (DCIP) and 3,5-di-*tert*-butyl-1,2-benzoquinone (TBBQ) are used routinely to assay for these enzymes. The catalytic constants for CBO and CBQase are comparable (Samejima and Eriksson, 1992).

Ayers *et al.* (1978) suggested that a primary function of CBO was the oxidation of cellobiose with the reduction of oxygen (which was measured with a polarographic oxygen cell) to water. Though the production of H_2O_2 from the oxidation of cellobiose was originally postulated, no H_2O_2 production could be detected (Ayers *et al.*, 1978). The choice of a coupled assay to measure H_2O_2 formation using horseradish peroxidase and o-dianisidine oxidation (to form colored products detected spectrophotmetrically) was unfortunate since any peroxidase-*o*-dianisidine reaction intermediates (radicals) would have been susceptible to reduction by CBO (Samejima and Eriksson, 1992). This led to the conclusion that H_2O_2 was not formed by CBO (Ayers *et al.*, 1978). However in a subsequent study, Morpeth (1985) demonstrated H_2O_2 formation by CBO. The importance of the oxygen-reducing activity of CBO has since been questioned in light of the observation that in fully reduced CBO, only the *b*-type haem is reduced by O_2, whereas both the flavin and the haem are reduced by DCIP, a quinone substrate (Wilson *et al.*, 1990). In a study of the CBO protein using several analytical techniques, it has been shown that the heme ligands in this protein are histidine and methionine (Cox *et al.*, 1992). The CBO enzyme cytochrome *b* cofactor in both its redox states is low spin suggesting that O_2 is not bound and therefore is an unlikely substrate for the enzyme (Cox

et al., 1992). In *S. thermophile*, a much lower relative rate of O_2 reduction compared to quinone substrates was also observed (Canevascini *et al.*, 1991).

In a mechanistic study of CBO, using stopped flow kinetics, it was found that the flavin cofactor was reduced first, followed by reduction of the heme (Jones and Wilson, 1988). Furthemore, the heme centers were more likely to be reduced by the flavin moiety of a different protein molecule rather than by its partner flavin within the same polypeptide (Jones and Wilson, 1988). In other studies it was found that the pH dependences of the reduction of the heme and flavin cofactors differed depending on the electron acceptor used in the reaction. At pH 4.2, both DCIP and cytochrome c were efficient electron acceptors, but at pH 5.9 DCIP was while cytochrome c was not (Samejima *et al.*, 1992). When the redox states of the heme and flavin cofactors were monitored by stopped-flow spectroscopy, it was found that at pH 4.2, both heme and flavin were rapidly reduced, while at pH 5.9, FAD reduction was much faster than heme reduction (Samejima *et al.*, 1992). It was concluded that cytochrome c was reduced by the heme ligand, and that this reaction occurs at low pH values (Samejima *et al.*, 1992).

Numerous other compounds can act as electron acceptors for CBQase including Mn(III) (Bao *et al.*, 1993), Fe(III) complexed with phenanthroline (4 mM) (Coudray *et al.*, 1982) or Fe(III) complexed with acetate (Kremer and Wood, 1992), and cytochrome c (Coudray *et al.*, 1982; Dekker, 1988; Samejima and Eriksson, 1992). Using aerobic incubations at pH 6, both cytochrome c and O_2 were reduced by CBO and CBQase with a concommitant production of superoxide and H_2O_2 (Morpeth, 1985; Morpeth and Jones, 1986). However Samejima and Eriksson (1992) have concluded that CBQase does not

reduce cytochrome c, and that its reduction by CBO was not oxygen dependent. This observation has been used to distinguish between CBQase and CBO (Habu *et al.*, 1993; Costa-Ferreira *et al.*, 1993).

Kremer and Wood (1992) have suggested that the physiological function of CBO in *P. chrysosporium* is primarily that of an Fe(III) reductase. They also suggested that the Fe(II) formed by CBO might serve as a component of Fenton's reagent ($Fe(II)/H_2O_2$). Crystalline cellulose is only very slowly hydrolyzed by hydrolytic enzymes (cellulases), however it is susceptible to disruption by hydroxyl radicals (von Sonntag, 1979). Such radicals can be formed by the Fenton reaction (Eq. 2):

$$H_2O_2 + Fe^{2+} \rightarrow HO^{\bullet} + HO^{-} + Fe^{3+} \qquad (2)$$

The structure of cellulose fibers can be disrupted by Fenton's reagent (Koenigs, 1974). These authors chose to use acetate as the buffer system, although, acetate was not reported to be a major metabolite of *P. chrysosporium* (Kuan and Tien,1993a; 1993b; Wariishi *et al.*, 1992). Earlier work had demonstrated Fe(III) reduction by a CBQase from *S. thermophile* (Coudray *et al.*, 1982) though these authors had not looked for the production of hyrdroxyl radicals. It was found that most of the electrons in an aerobic reaction mixture were accepted by Fe(III)-acetate (Kremer and Wood, 1992).

The Fe(III) reduction rate was about ten-fold higher than that observed for O_2 (Kremer and Wood, 1992) and similar to the ratio for quinone versus O_2 reduction observed with both CBO (Morpeth, 1985) and CBQase (Morpeth and Jones, 1986). CBO catalyzed the formation of hydroxyl radicals in the presence of Fe(III), cellobiose, acetate

buffer (pH 4) and O_2. The formation of hydroxyl radicals by CBO was detected using the oxidation of deoxyribose to malondialdehyde and the hydroxylation of salicylic acid to 2,3- and 2,5-dihydroxybenzoic acid (Kremer and Wood, 1992b). It was also found that cellulose (Avicel) could drive the reduction of CBO (Kremer and Wood, 1992c). A flux of oxygen consumption by the reaction system which was inhibited by the presence of catalase (which destroyed any H_2O_2 in the reaction system) was taken as evidence that the Avicel was being oxidized by the flux of hydroxyl radicals (Kremer and Wood, 1992b).

The interaction of CBQase and CBO from *P. chrysosporium* with cellulose occurs on another level as well. Both of the enzymes were very effectively bound to cellulose at acidic to neutral pH values (Renganathan *et al.*, 1990; Henriksson *et al.*, 1991).

The CBO from *P. chrysosporium* can be cleaved by the protease papain into two domains, one of which binds to cellulose, and the other which contains the flavin cofactor (Henriksson *et al.*, 1991). Using digestions with staphylococcal V8 proteinase and cyanogen bromide as a peptide fragment fingerprinting tool, it was shown that CBQase and CBO digestion products when examined by polyacrylamide gel electrophoresis, showed many common bands (Wood and Wood, 1992). Polyclonal antibodies to either CBO or CBQase proteins cross-reacted. Western blots of whole protein gels (i.e. that contained all of the proteins secreted by *P. chrysosporium*) revealed a 31 kDa haem protein which cross-reacted with antibodies produced to the CBO enzyme. It was suggested that CBQase and this fragment were breakdown products of CBO (Wood and Wood, 1992). Habu *et al.* (1993) have shown that proteases present in cellulolytic cultures of *P. chrysosporium* contain a protease which cleaves CBO, but only when CBO

is bound to cellulose. Two acidic proteases have previously been purified from cellulolytic cultures of *P. chrysosporium* and were implicated in the regulation of endo-1,4-β-glucanases (Eriksson and Pettersson, 1982).

Bao and Renganathan (1992) have reported that the degradation of crystalline cellulose was enhanced by the presence of CBO. The cellulolytic system which they used came from *Trichoderma reesei*, an organism which the authors suggested did not produce CBO. However Dekker (1988; Table 1.1, this thesis) has shown that CBQase is produced by this fungus, and this may have influenced the results of Bao and Renganathan. In any case, moderate improvements in cellulose solubilization were observed when the *T. reesei* cellulases were augmented with *P. chrysosporium* CBO (Bao and Renganathan, 1992). Clearly, the induction, localization, activity and proteolytic degradation of CBQase and CBO are greatly influenced by the presence of cellulose in the culture. There remain many questions concerning how CBQase and CBO influence both the rate and extent of cellulose catabolism by white-rot fungi.

A role for CBQases in lignin degradation has been suggested since the first report of the enzyme (Westermark and Eriksson, 1974a). The original report of decolorization of the colored quinones formed on guaiacol-containing agar plates suggested that CBQase could interact with the oxidation products formed by the laccases and peroxidases secreted by white-rot fungi (Westermark and Eriksson, 1974a). That lignin degradation is a net oxidative process is widely accepted (Kirk and Farrell, 1987; Eriksson *et al.*, 1990) and with the discovery of LP and MnP , it was felt by many workers that all of the essential components of the delignification system of white-rot fungi were known. However a

common observation was that there was more synthesis of higher molecular weight material observed than depolymerization when lignin was incubated with purified LP or MnP (Haemmerli *et al.*, 1986).

In 1977, Hütterman *et al.* reported that in *Fomes annosus* cultures, the polymerization of lignosulfonates was decreased in the presence of cellobiose relative to what was observed with glucose as the carbon source. This was the first circumstantial evidence that CBQase could be important in lignin biodegradation. The authors were subsequently able to isolate CBQase from their cellobiose-grown cultures (Hütterman and Noelle, 1982).

In a further test of the original hypothesis of Westermark and Eriksson (1974a), the effect of CBQase on the reaction of LP with a number of different substrates was assessed (Odier *et al.*, 1988). It was reported that CBQase did not prevent or even affect the polymerization of guaiacol and of a synthetic lignin by LP (Odier *et al.*, 1988). Furthermore, the formation of the acetosyringone radical was also unaffected by CBQase (Odier *et al.*, 1988).

Ander *et al.* (1990) showed that lignin polymerization was decreased in the presence of CBQase. The decarboxylation of vanillic acid by laccase, MnP and LP as well as veratryl alcohol oxidation by LP, were inhibited by CBQase and cellobiose (Ander *et al.*, 1990). Samejima and Eriksson (1991) showed that the formation of free radicals during the LP-mediated oxidation of tetramethoxybenzene was inhibited, or the radical was reduced by either CBQase or CBO.

CBO may serve another role in the catalytic cycles of MnP and LP, namely the reduction of the compound II form of these enzymes to the resting state (Ander *et al.*, 1993). The rate of CBO-mediated reduction of compound II of LP was 40-fold lower than the rate of cytochrome c reduction by this enzyme. The reduction by CBQase of the compound II forms of both MnP and LP was considerably more efficient with either Mn(II) or veratryl alcohol, respectively (Ander *et al.*, 1993). CBO did not interfere with the formation of compound I from resting MnP or LP (Ander *et al.*, 1993).

1.4.4 Other Enzymes

In the lignin-degrading basidiomycete *Pleurotus sajor-caju*, veratryl alcohol oxidase (VAO) has been isolated, purified and partially characterized (Bourbonnais and Paice, 1988). VAO, like LP, oxidizes veratryl alcohol and reduces O_2 to form H_2O_2 (Bourbonnais and Paice, 1988). Unlike LP, VAO is induced, and lignin degraded during primary metabolism by *P. sajor-caju*. Despite a careful search, VAO activity has not been demonstrated in *T. versicolor* (R. Bourbonnais, pers. comm.).

Recently it has been suggested that the formation of lignin-glycosides may be an important reaction in delignification. Kondo *et al.* (1988;1989;1990) have shown that the β-glucosidases and β-xylosidases can covalently attach veratryl alcohol to glucose and xylose, respectively. The proposed mechanism for the formation of these compounds is a transglycosylation (or xylosylation) reaction. The authors suggest that xylosylation is an important mechanism which prevents repolymerization of lignin fragments in *T. versicolor*, thereby favoring depolymerization. However, the formation of these

glycosides was only demonstrated under either anaerobic conditions or in the presence of reducing agents such as cysteine and ascorbate (Kondo *et al.*, 1993). Furthermore, the xylosylation was not mediated by extracellular enzymes requiring an intracellular extract; thus phenolic radicals produced distant from the fungal hyphae could still undergo coupling reactions (Kondo *et al.*, 1993).

PREFACE TO CHAPTER 2.

Prior the present studies, the biobleaching system of *T. versicolor* had been optimized to obtain a maximum bleaching effect (Paice *et al.*, 1989). It was demonstrated that intimate hyphal-fiber contact was not necessary (Archibald, 1992), suggesting that culture supernatants contain all of the essential components of the biobleaching system. Work aimed at improving the efficacy of the fungal bleaching system was being carried out by a number of workers within the group (Ziomek *et al.*, 1991; Ho *et al.*, 1990; Addleman and Archibald, 1992). Other studies were aimed at defining the role(s), if any, of various enzymes implicated in lignin biodegradation by *T. versicolor* and other fungi. Enzymes such as LP (Archibald, 1992) and laccase (Bourbonnais and Paice, 1991; 1992) were being isolated from *T. versicolor* to see whether they might contribute to the biobleaching produced by the fungus. The objectives for this part of the thesis were as follows: (1) to determine whether or not CBQase was secreted by *T. versicolor* 52J during biobleaching and how it might interact with other secreted enzymes; (2) to determine what enzymes and low molecular weight metabolites were produced during biobleaching; (3) to evaluate the efficacy of these low molecular weight species as chelators of Mn(III); (4) to determine the effects of kraft pulp on the overall carbon metabolism of *T. versicolor* 52J in terms of growth and the metabolites secreted into the culture medium; and (5) to determine whether there is any interaction between those oxidative enzymes implicated in biobleaching and CBQase.

CHAPTER 2. THE EFFECTS OF KRAFT PULP AND LIGNIN ON *TRAMETES VERSICOLOR* CARBON METABOLISM

2.1. INTRODUCTION

The white-rot fungi are the only microorganisms known to completely degrade wood. It has been recognized for some time that certain of these fungi can also partially delignify unbleached kraft pulp (wood fibers delignified using NaOH and NaSH) (Kirk and Yang, 1979). Work in this laboratory has demonstrated that strains of the white-rot fungus *Trametes* (*Coriolus*) *versicolor* can decrease both residual lignin color (brightness) and concentration (kappa number) in hardwood (Paice *et al.*, 1989) and softwood (Reid *et al.*, 1990) kraft pulps. Thus, fungal treatments yield pulps with substantially increased brightness (biobleaching). To date, only *T. versicolor* (Paice *et al.*, 1989; Reid *et al.*, 1990), *Coriolus hirsutus*, *Phanerochaete chrysosporium* and the fungus IZU-154 (Fujita *et al.*, 1991) have been shown to brighten hardwood kraft pulps (HWKP).

The mechanisms by which these fungi brighten pulp are not known. An obvious candidate for part of the mechanism is the "lignin" peroxidase (LP) reported in *T. versicolor* (Archibald, 1992; Dodson *et al.*, 1987; Evans *et al.*, 1984) and other ligninolytic fungi (Galliano *et al.*, 1991; Tien and Kirk, 1983; Waldner *et al.*, 1988) but all assays for LP in *T. versicolor* 52 under the high nitrogen, agitated, and short growth period conditions used for pulp biobleaching have been negative (Archibald, 1992a; 1992b). *T. versicolor* has been shown to secrete several enzymes in addition to LP that are suspected of being important in delignification and bleaching of kraft pulp, including

Mn-peroxidase (MnP)(Johansson and Nyman, 1987; Paice *et al.*, 1993), laccases (phenoloxidases) (Fåhraeus and Reinhammar,1967; Morohoshi *et al.*, 1987l; Paice *et al.*, 1989), and cellobiose-quinone oxidoreductases (CBQase)(this thesis; Westermark and Eriksson, 1974). It has also been shown that direct fungal-fibre contact is not essential (Archibald, 1992), i.e., the *T. versicolor* culture liquor contains everything necessary for biobleaching and delignification. Nevertheless, incubation of unbleached HWKP with supernatants from actively biobleaching cultures did not increase pulp brightness (Archibald, 1992) indicating that the fungus needs to restore, renew, or recycle certain liquor-borne components for effective biobleaching and delignification. To date, these essential components have not been identified.

In the *T. versicolor* system employed here (Addleman and Archibald, 1993; Paice *et al.*, 1989; 1993), MnP has been shown to be present at substantial levels during biobleaching (Paice *et al.*, 1993). Using a fungus-free system, *T. versicolor* 52 MnP has been shown, with the addition of hydrogen peroxide, Mn(II), and a chelator, to generate Mn(III) complexes which can delignify and bleach hardwood kraft pulp, although to a much smaller extent than the complete fungal system (Paice *et al.*, 1989; 1993). Both MnP and laccase proteins from this fungus can also demethoxylate kraft lignin, in the presence of appropriate accessory substances. White-rot fungi also secrete a variety of heme- and flavin-dependent oxidases evolving hydrogen peroxide upon the oxidation of veratryl alcohol (Bourbonnais and Paice, 1988), glyoxal, methylglyoxal, glycolaldehyde, glyoxylic acid, glyceraldehyde (Kersten and Kirk, 1987), glucose (Eriksson *et al.*, 1986; Kelley and Reddy, 1986), oxalate (Koenigs, 1972), cellobiose (Morpeth, 1985; Morpeth

and Jones, 1986; Roy and Archibald, 1991), and methanol (Buswell and Eriksson, 1986). Clearly, if any of these enzymes are important in the *T. versicolor* HWKP biobleaching and delignification system, not only must they be present, but the complementary low molecular weight compounds must be as well, including substrates for the peroxide generating enzymes, cellobiose and quinones for CBQase, hydrogen peroxide and veratryl alcohol (or an analog) for LP, and hydrogen peroxide, Mn(II) ions, and appropriate Mn(III)-chelating anionic species for Mn-peroxidase. Aside from their enzyme-related functions, low molecular weight fungal metabolites may be directly involved in delignification, and be the vital renewed or replaced functions in biobleaching liquor (Archibald, 1992). Direct delignifying activity by low molecular weight species like Mn(III)-carboxylic acid chelates would also explain fungal delignification in regions of the woody cell wall apparently inaccessible to enzyme-sized molecules (Strebotnik and Messner, 1990). The establishment of a catalytic redox cycle to allow repeated rounds of lignin oxidation has been proposed as an important feature of fungal delignification systems (e.g. Green, 1972; Westermark and Eriksson, 1974; Westermark and Eriksson, 1974a; Ander *et al.*, 1990). Both MnP and laccase were known to be produced by *T. versicolor* under biobleaching conditions (Paice *et al.*, 1989; Paice *et al.*, 1993) and could oxidize phenolic substrates. In *P. chrysosporium* various glucose oxidases (Kelley and Reddy, 1986; Eriksson *et al.*, 1986; Szklarz and Leonowicz, 1986), CBQase (Westermark and Eriksson, 1974; Ander *et al.*, 1990; Samejima and Eriksson, 1991) or CBO (Ayers *et al.*, 1978) could all fulfill this role. To establish whether such a redox cycle might be important in *T. versicolor*-mediated biobleaching, the extracellular reductive enzymes were

also sought. Though the production of a cellobiose-dependent reductive activity by *T. versicolor* had been reported earlier (Westermark and Eriksson, 1974), it was not known whether this enzyme was present under biobleaching conditions. Therefore, a comprehensive study of the effects of kraft pulp and pulp bleaching on the overall metabolic carbon balance as well as the production of minor metabolites by *T. versicolor* was undertaken. Biobleaching and pulp-related metabolic changes were observed and their possible role in delignification is discussed.

2.2. MATERIALS AND METHODS

2.2.1. Strain and Culture Conditions

A monokaryotic strain of *Trametes versicolor*, 52J, derived from the parent dikaryon 52 (ATCC 20869)(Addleman and Archibald, 1993) was used throughout. The fungus was maintained on mycological broth (MB) agar plates (glucose, 40 g/l; soytone, 10 g/l) or plates of Trametes Defined Medium (TDM) containing: glucose, 83 mM; glutamine, 5 mM; NaCl 5 mM; KH_2PO_4, 5 mM; $MgSO_4$, 1 mM; $CaCl_2$, 0.1 mM; 2,2 dimethyl succinate, 10 mM; thiamine, 2.4 μM; trace metals ($FeSO_4 \cdot 7H_2O$, 20 μM; $CuSO_4 \cdot 5H_2O$, 2 μM; $ZnCl_2$, 5 μM; $MnSO_4 \cdot H_2O$, 20 μM; $CoCl_2 \cdot 6H_2O$, 6 μM; $NiCl_2 \cdot 6H_2O$, 0.1 μM; $(NH_4)_6MO_7O_{24} \cdot 4H_2O$, 0.5 μM), 1 ml/l, solidified with agar (15 g/l). The pH of all growth media was adjusted to pH 5.0 before autoclaving. For metabolic studies 200 ml aliquots of sterile TDM in 500-ml flasks were inoculated with six 12-mm diam. plugs of mycelium and agar from the growing periphery of a 5-7 d colony on TDM-agar. This inoculum culture was incubated for 5 d, 27°C, 200 rpm (r=10 mm), harvested (10,000 xg;

10 min), washed 3x with one volume of sterile TDM salts (TDM less glucose and glutamine), and resuspended in 200 ml TDM salts. Liquid cultures were monitored for contamination by microscopy and viable counts on both MB and nutrient agar plates.

Aliquots (20 ml) (0.010 ± 0.003 g oven dry weight) of washed mycelium were added to 1 liter bottles containing 180 ml of growth medium. The bottles were sealed with rubber plugs each having two flush lines for gas sampling. These culture bottles contained:(a) fungus and TDM;(b) fungus, TDM and 2% w/v unbleached hardwood kraft pulp (HWKP); (c) TDM and 2% w/v HWKP(no fungus); (d) fungus, TDM, and 2% w/v chemically bleached HWKP from the same mill as the unbleached (brownstock) HWKP. All bottles also contained 0.5 g/l Tween 80. HWKP was from a kraft mill in eastern Canada using: maple (44.1%), beech (7.3%), elm (1.7%), poplar (23.8%), basswood (5.5%), birch (9.2%), softwood (8.3%). The culture bottles containing pulp had additional amounts of Fe (32 μM), Mn (14 μM), Ca (880 μM) and Cu (<63 nM) determined from ashed samples of pulp by atomic absorption spectroscopy. Initially, ISO brightness was 30.5%, the kappa 14.8, and viscosity 24.6 centipoises. Where desired, 6.6 x 10^6 dpm of uniformly labelled(UL) ^{14}C-glucose (7.3 mCi/mmole) (270 MBq/mmole) was added to the 100 mmoles of free glucose in each bottle. Inoculated bottles were incubated (200 rpm, r=6mm) at 27°C with daily headspace gas flushing and sampling. After 7 days incubation the cultures were harvested. All chemicals were obtained from Sigma or Aldrich.

2.2.2. Headspace Gas Analysis

Triplicate aliquots (1 ml) of headspace gas were taken from each bottle and analyzed for CO_2, O_2,and N_2 using a gas partitioner (Fisher-Hamilton) run at 25°C and equipped with sequential DEHS and molecular sieve columns and a thermal conductivity detector. Sample peak areas and retention times were calibrated against gas standards. The dissolved O_2 in each culture bottle was measured at day 7 using a polarographic oxygen sensor.

Bottle headspaces were flushed with O_2 daily to 95% replacement and the evolved CO_2 trapped in 10 ml of CO_2-trapping scintillant (Carbon 14 Cocktail; R.J. Harvey Instrument Corp., Hillsdale, N.J., USA) and counted.

2.2.3. ^{14}C-label Incorporation

Incorporation of ^{14}C label into culture biomass was determined using a OX-400 Biological Oxidizer (R.J. Harvey Instrument Corp.) to mineralize total biomass carbon from day 7 cultures to CO_2, subsequently trapped in 20 ml of scintillant. The efficiency of ^{14}C recovery was 95%.

2.2.4. Measurement of Metabolites

Glucose was determined using a glucose oxidase-based assay kit (DMA Inc., Arlington, TX, USA). Citric, oxalic and lactic acids were also determined using commercial enzyme assay kits (citrate from Boehringer Mannheim, oxalate and lactate from Sigma). Non-volatile organic acids were resolved by high pressure liquid

chromatography (HPLC) with an Aminex HPX-87H column (7.8 x 300 mm) using 0.008 M H_2SO_4 at a flow rate of 0.6 ml min^{-1} and a column temperature of 35°C. Eluates were monitored at 210 nm using a Hewlett Packard 1040A diode array detector. Peaks were identified and quantitated by retention time and peak area units against internal standards, as well as their UV-vis absorption spectra.

Samples assayed for volatile metabolites were initially clarified with activated charcoal followed by 0.45 μm filtration and resolved by gas chromatography (GC) as described (Ni *et al.*, 1990). An HP 5890A GC with a Chromosorb 102 packed column (1.85 m x 3.2 mm; 80/100 mesh) was used with injector, oven, and FID detector at 130,120, and 150°C, respectively, and (30 ml/min) helium carrier. There was a linear relationship between peak area and concentration for methanol (5-50 mg/l) and ethanol (2-50 mg/l). For determination of ^{14}C ethanol, 5 ml culture liquor and 2 ml unlabelled ethanol were distilled and the distillate counted. A Chromasorb 101 packed column (1.85 m x 3.2 mm; 80/100 mesh) (as well as GC-MS) was used for determinations of 2,3-butanediol as previously described (Yu and Saddler, 1982).

2.2.5. Enzyme Assays

Laccase activity was quantified by the oxidation of 2,2'-azino-di-(3-ethylbenzthiazoline)-6'-sulfonate (ABTS) measured at 420 nm in 100 mM Na-acetate, pH 5.0 (Wolfenden and Willson, 1982). Cellobiose quinone oxidoreductase (CBQase) was assayed by following the reduction of 3,5-di-*tert*-butylbenzoquinone at 420 nm (Westermark and Eriksson, 1974). Assays contained 100 mM sodium acetate (pH 4.5),

0.33 mM TBBQ, and 0.67 mM cellobiose and sample. Lignin peroxidase (LP) was measured by the oxidation of veratryl alcohol to veratraldehyde at 310 nm (Tien and Kirk, 1988). Assays contained 2.5 mM veratryl alcohol, 0.1 mM H_2O_2, and sample in 20 mM Na-succinate, pH 3.0. Manganese peroxidase (MnP) activity was determined by the formation of Mn^{+3}-malonate chelates at 270 nm (Wariishi *et al.*, 1989) or by measuring the rate of phenol red oxidation at 431 nm. All assays contained 0.1 mM H_2O_2, 0.2 mM $MnSO_4$ and sample in 50 mM Na-malonate, pH 4.5. Phenol red (0.067 mM) oxidation was used to compare the chelating activities of a buffer composed of organic acids induced in the culture supernatants by pulp (pH 4.5, 4.3 mM) with the rate obtained using the standard 50 mM malonate buffer. Purified *T. versicolor* 52J MnP was produced in 14-liter aerated carboys of MB with 0.2 mM $MnSO_4$, and the 8-day culture supernatant concentrated, dialyzed, and microfiltered (0.45 μM). MnP was purified by conventional column chromatography and the resultant enzyme preparation contained two isozymes with a MW of 42,000 (SDS-PAGE), an $OD_{280/407}$ ratio of 0.26 and a specific activity in the phenol red assay of 89.8 U·mg protein^{-1}. Cellulase activity was determined by reducing sugar formation from 1% (w/v) carboxymethylcellulose incubated with the sample (30°C, 10 min), quantified using dinitrosalicylic acid measured at 570 nm (Miller, 1959). Activities of cellulase, CBQase, LP, and laccase are in: Unit=1μmole indicator substrate·min^{-1} converted. MnP activities are in: Unit=1 ΔODU·min^{-1}. Glucose, xylose, cellobiose, glyoxylate, glycolate, glyoxal and veratryl alcohol oxidase activities were determined by detecting H_2O_2 with a coupled assay using *P. chrysosporium* MnP (Tienzyme Inc., State College, PA.) and the formation of Mn^{+3}-malonate an complex as

the detector. Glucose, xylose, cellobiose, glyoxylate, glycolate, or glyoxal were added at 1.5 mM (veratryl alcohol, 0.5 mM) to 50 mM Na-malonate, pH 4.5 with 0.2 units of MnP and 0.2 mM $MnSO_4$.

2.2.6. Dry weight and Ergosterol Determinations

In the absence of pulp, the growth of *T. versicolor* was determined by measuring triplicate oven dry weights from a known volume of culture retained on a 0.45 μm filter. In the presence of pulp, biomass was estimated by measuring ergosterol content (Seitz *et al.*, 1977). Ergosterol was extracted from pulp and biomass with methanol (2x), saponified by refluxing for 1 h in methanol:ethanol (4:1) containing 7.5% (w/v) KOH and extracted 2 times with petroleum ether. This extract was evaporated to dryness, redissolved in methanol, fractionated on a C-18 reverse phase column (Waters μBondpak™) and then eluted with methanol (100%). Identification and quantitation of ergosterol used its retention time, peak height at 280 nm and its complex UV absorbance spectrum.

The amount of glucose-derived ethanol percipitable material in the culture supernatant was determined by addition of 2 volumes of cold ethanol, centrifugation (60,000 x g, 60 min) and ^{14}C counting.

2.2.7. Gas Chromatography-pyrolysis Mass Spectroscopy (GC-MS)

Culture liquors were analysed by GC-MS for acidic metabolites. Samples (5 ml) were saturated with NaCl, adjusted with NaOH (approx. 10% w/v) to pH>12, then

extracted twice with 2 ml of diethyl ether (basic extracts) and the organic phase removed. The aqueous phase was acidified (pH<2) with dilute HCl then extracted twice with diethyl ether (acid extract). Acid extracts were pooled, treated with anhydrous $MgSO_4$ to remove water and then concentrated in a stream of N_2. The concentrated sample was dehydrated with Na_2SO_4, the organic phase transferred and evaporated to dryness. Sialylation was done (15 min, 60°C) in excess TRI-SIL/BSA (Pierce Chemical Co.). Samples were analyzed in an LKB 9000 GC-MS with a DB-1 capillary column (30 m long, 5 μm film) with a temperature ramp of 70-280°C at 4°C/min. Injector and separator were at 280°C, and the electron impact ion source at 290°C, 70 eV at 60μA. A mass spectrum for each of the peaks detected was obtained. The concentrations of sample compounds were quantified using capric acid as an internal standard. Peaks were identified by comparison of their mass spectra and retention times to standards.

2.2.8. Pulp Properties

Handsheets (2.5 x 4.0 cm) were formed using a miniature apparatus with a 150-mesh screen and 20 ml samples of 2% w/v pulp suspension. After 24 h drying, sheet reflectance was measured at 457 nm (Kirkpatrick *et al.*, 1989). Viscosity was determined using CPPA method G.24P, and kappa numbers were determined using a micro method (Kirkpatrick *et al.*, 1989). Zero-span breaking length was determined with a tensiometer equipped with zero span jaws using a standard method proposed by the CPPA (method number pending).

2.3. RESULTS

2.3.1. Fungal effects on the pulp

In 7-day incubations, 200-ml cultures of *T. versicolor* 52J in sealed 1 liter bottles were monitored to determine both the effects of the fungus on the pulp, and the effects of the pulp on the growth and metabolism of the fungus. Figure 2.1 and Table 2.1 show that in this bottle format 52J cultures biobleached and delignified, as reported previously for the parent dikaryon strain 52 in slightly different systems (Paice *et al.*, 1989; Reid *et al.*, 1990). As before, incubation with the fungus decreased the pulp viscosity, presumably via its hemicellulase and cellulase activities, but Z-span breaking length was less affected, showing that fiber strength was maintained. Earlier work had demonstrated that both biobleaching *T. versicolor* 52 biomass (Paice *et al.*, 1989; Reid *et al.*, 1990) and its secreted laccase and the indicator ABTS alone (Bourbonnais and Paice, 1992) could demethoxylate the residual lignin in unbleached HWKP. Likewise here, only the lignin-containing cultures produced significant methanol (Table 2.1). The methoxyl content of the residual kraft lignin present in the unbleached pulp was calculated to be 15% of total residual lignin carbon which in turn constituted ≈2.5% of the oven dry pulp weight, thus, about 29% of the lignin methoxyl groups were cleaved during biobleaching (Paice *et al.*, 1993).

2.3.2. Pulp effects on the fungus; overall nutrient carbon balance

One of the ways in which HWKP effects on the fungus were measured was via a balance of the carbon from the principal nutrient present, 83 mM Ul-^{14}C-D-glucose

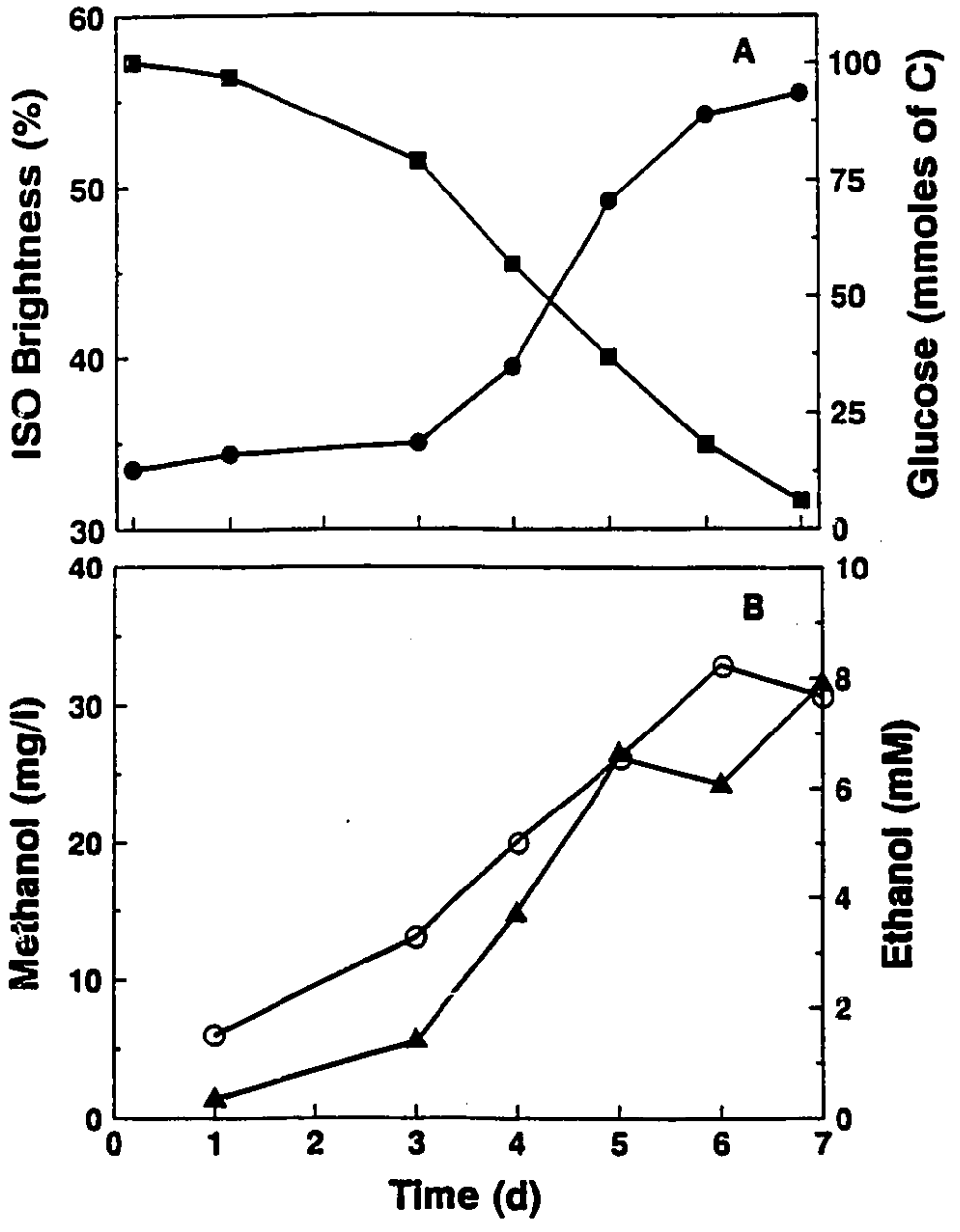


Figure 2.1. Time course of *T. versicolor* 52J mediated biobleaching. A biobleaching culture was sampled daily and analysed for pulp brightness (●), glucose (■), methanol (○), and ethanol (▲) concentrations.

Table 2.1. Effect of *T.versicolor* 52J on HWKP and bleached HWKP.

Culture Condition	ISO Brightness	Kappa Number	Z-Span[1] (km)	Viscosity (Mpa.s)	Methanol (mg/l)
Fungus + HWKP	46.9 ± 2.0	11.9 ± 0.7	10.6 ± 0.6	17.6 ± 0.3	21.9
HWKP Alone	34.6 ± 0.1	15.4 ± 0.3	12.3 ± 0.1	29.2	1.3
Fungus + Bleached HWKP	78.9 ± 0.0	2.6 ± 0.0	11.8	8.7	2.7
Bleached HWKP Alone	87.8 ± 0.0	<0.5	10.6	12.9	0.0

[1] Z-span = zero span fibre breaking length. *Z*-span is a measure of the strength of individual pulp fibres and is determined by applying a force parallel to the formation axis of a pulp sheet of precisely measured dimensionsand basis weight (g/cm^3) produced under controlled conditions (50% relative humidity and 23°C). The unit is the length of a pulp sheet prepared using these standard conditions that will just support its' own weight without breaking. Higher values correspond to greater fibre strength.

(Table 2.2). Comparison of total and ^{14}C-CO_2 production indicated that 92.3% (fungus alone), 81.7% (fungus and unbleached pulp), and 72.7% (fungus and bleached pulp) of the total CO_2 generated was derived from the metabolism of the 83 mM glucose, with the remainder derived from biomass (endogenous C), the medium glutamine (5mM), or pulp (Table 2.2). The rates and total amounts of CO_2 evolved and the O_2:glucose C consumed and CO_2 evolved:glucose C consumed ratios were similar with and without unbleached pulp. A delay in CO_2 evolution occurred with both pulps (Figure 2.2). This was independent of the presence of lignin, and not due to a preferential use of pulp-derived unlabelled carbohydrate (Table 2.2). In the absence of fungal activity, 10 mmoles of glucose C leached from the unbleached pulp in 7 days (Table 2.2). In the presence of fungal cellulolytic and ligninolytic activity glucose release was probably higher. The lower biomass ^{14}C content and higher residual glucose seen with bleached compared to unbleached pulp (Table 2.2) was probably due to dilution of the added labelled glucose with this pulp-derived glucose, not altered fungal metabolism as total evolved CO_2, consumed O_2 and culture pO_2 were comparable for both pulp-containing cultures (Tables 2.2,2.3). The presence of pulp did not greatly affect two other important bulk parameters, culture pH and pO_2 (Table 2.3). This was fortunate, because had either changed in the presence of pulp, then other observed metabolic changes could as easily be attributed to them as to the presence or absence of pulp.

Did pulp affect fungal growth? Because the presence of pulp precluded dry weight as a measure of growth, and glucosamine assays lacked sensitivity in pulp, the ergosterol content of washed culture solids was measured. Ergosterol, the dominant membrane sterol

Table 2.2. Basic carbon balance of *T. versicolor* 52J after seven days incubation with and without HWKP.[1]

Culture Fraction (Total mmole C flask^{-1})	Fungus alone	Fungus + HWKP	HWKP alone	Fungus + bleached HWKP
Initial Glucose (labelled)	100	100	100	100
Residual Glucose[2]	5.4	3.6	110	24.7
CO_2 (^{14}C-Glucose Derived)	40.8	35.3	0.1	33.0
CO_2 (Total Produced)	44.2 ± 2.7	43.2 ± 2.1	0	45.4 ± 2.4
Biomass[3]	33.7 ± 2.0	28.1 ± 0.4	0.9 ± 0.4	14.3 ± 3.7
Soluble Metabolites[4]	20.2	28.1	0	14.9
Glucose ^{14}C Recovery	100.1	95.1	91.3	86.9
O_2/glucose ratio[5]	0.44	0.50	-	0.44
CO_2/glucose ratio[6]	0.46	0.45	-	0.60

[1] Determined using [^{14}C]-U-D-glucose and specific assays and reported as total C in each 200 ml culture.[2] Total labelled (added) and unlabelled (pulp-derived) D-glucose remaining.

[3] Controls (7-d) of labelled glucose in sterile pulp indicated that ≈1% of the labelled glucose became pulp-associated and that some unlabelled glucose is leached from the pulp.

[4] Comprised of compounds described in Tables 4 and 5 and unknowns.

[5] Total mmoles O_2 consumed/mmole of ^{14}C-glucose C consumed.

[6] Total mmoles CO_2 evolved/mmole of ^{14}C-glucose C consumed.

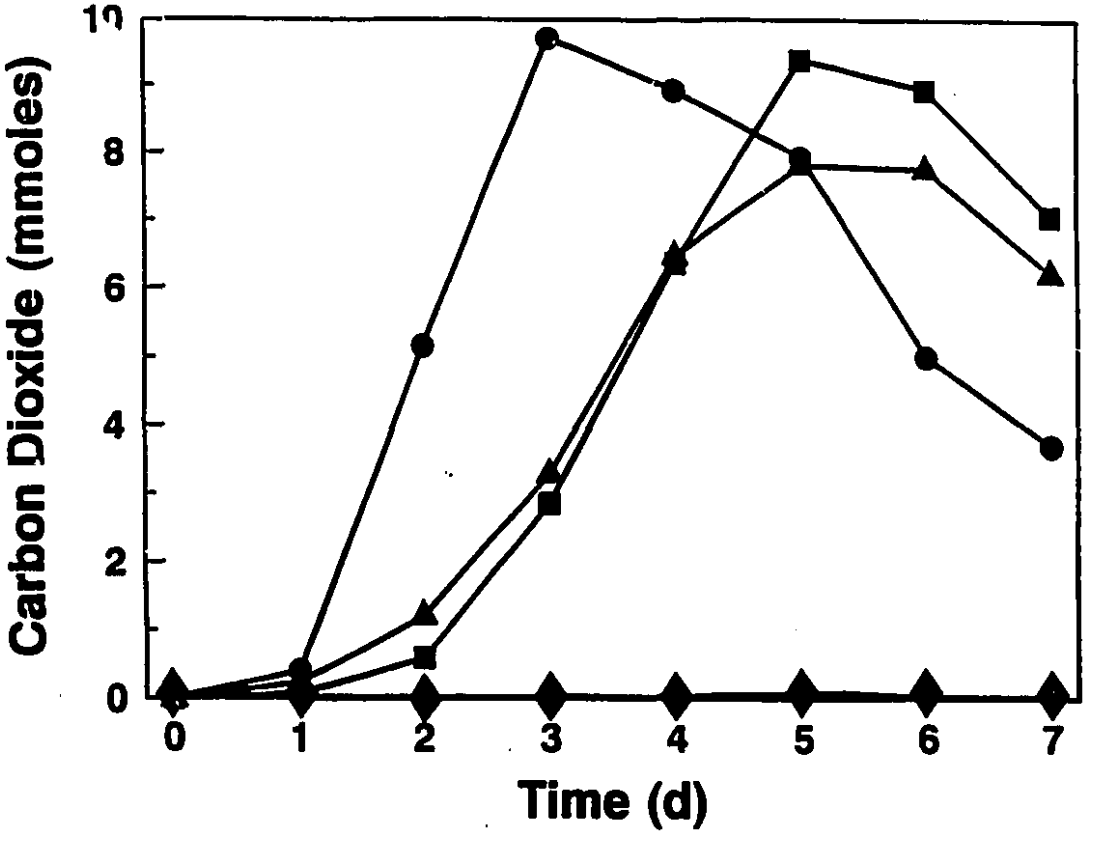


Figure 2.2. Time course of ^{14}C-CO_2 production by *T. versicolor* 52J. Cultures were as follows: fungus alone (•), HWKP alone (♦), fungus + HWKP (■), and fungus + bleached HWKP (▲). Culture bottles were flushed daily as described in Materials and Methods.

Table 2.3. Comparison of physical conditions, biomass and ergosterol in the culture bottles after 7-day incubation

Culture Condition	pH	pO_2 (mg/l)	Ergosterol (mg)	Dry Weight (g)
Fungus Alone	4.1	5.8	3.6	0.7
Fungus + HWKP	4.5	5.9	1.3	4.2[1]
HWKP Alone	5.7	8.0	0.0	3.5[1]
Fungus + Bleached HWKP	4.6	5.7	1.0	4.0

[1]Sum of fungus and pulp weights

of most fungi, was not detectable in HWKP (Table 2.3). Since ergosterol content has been shown to be a reasonable measure of fungal biomass in some fungi (Martin *et al.*, 1990; Matcham *et al.*, 1985; Seitz *et al.*, 1977), including *T. versicolor* 52J (data not shown), ectomycorrhizas (Martin *et al.*, 1990), and conifer needles (Osswald *et al.*, 1986), the ergosterol content of the pulp-fungus mixture was determined. Results indicated that, as pulp-containing cultures had about 3-fold less ergosterol, either there was only one-third as much growth in the presence of pulp or the mycelium had only one third as much ergosterol per unit of mass (Table 2.3). The former implies three-fold greater specific metabolic rates in the pulp-containing flasks, as their total O_2 and glucose consumption and CO_2 evolution were close to those from pulp-free cultures. Alternatively, since ergosterol-spiked pulp and fungus controls showed efficient, quantitative recovery of the sterol, the presence of pulp reduced fungal cell membrane specific ergosterol content, not growth. Since the fungal biomass increased many fold during the 7-day incubation (from 10 mg in the inoculum to 700 mg d.w. in the pulp-free flask) at the expense of ^{14}C-glucose, and the biomass ^{14}C was comparable with or without unbleached HWKP, the latter explanation would seem to be correct. Thus, ergosterol cannot be used as a valid measure of biomass when comparing growth of *T. versicolor* in the presence and absence of HWKP.

2.3.3. Culture Liquor Metabolite Analysis

After 7 days, 21.4% of the initial glucose C in cultures without pulp and 29.1% in cultures containing unbleached pulp was present as soluble secreted metabolites (Table

2.2). These liquors were analyzed by specific enzyme assays, GC, GC-MS, and HPLC for a wide range of possible metabolites. The results (Tables 2.4,2.5) show that, next to CO_2, ethanol was the metabolite produced in the largest quantities. Comparison of total culture liquor ethanol to volatile labelled C, distilled using unlabelled ethanol as a carrier, indicated that most or all of the ethanol came from the labelled glucose, both in the presence and absence of pulp. Somewhat unexpected were the millimolar quantities of 2,3-butanediol detected, which decreased in the presence of pulp (Tables 2.4 and 2.5). Butanediol was identified by two independent GC methods as well as the mass spectrum of its pyrolyzed sialylated derivative. The quantity of 2,3-butanediol measured by GC-MS was much lower than that obtained by direct GC, likely a consequence of the low extraction efficiency for this compound using a GC-MS method optimized for the recovery of acidic and not neutral metabolites. Methanol was also identified, but as it was apparently lignin-derived (Bourbonnais and Paice, 1992), it did not contribute to the volatile radiolabel total (Tables 2.1,2.4).

Although oxalic and glycolic acids are generated in kraft black liquor by nonbiological processes (Niemelä *et al.*, 1985), oxalate's absence from the uninoculated HWKP and presence in all cultures indicated that it was of fungal origin (Table 2.4). Counting (^{14}C) of the HPLC oxalate peak also indicated that it was derived largely from glucose. The glyoxylate was also glucose-derived and strongly induced by the presence of pulp, whether lignin-containing or not.

The compound(s) accounting for the remaining ^{14}C-label in the soluble fraction of the culture supernatant were not identified. When fractionated on an organic acid HPLC

Table 2.4. Metabolites detected by HPLC, GC, and enzymatic assay in *T. versicolor* 52J culture supernatants after 7 days of growth.

Culture liquor component (Total mmole C·flask^{-1})	Fungus alone	Fungus + HWKP	HWKP alone	Fungus + Bleached HWKP
Ethanol	8.60	12.50	0.02	4.80
2,3-butanediol	0.48	0.09	0.00	0.06
Glyoxylate	0.14	1.61	0.00	1.83
Oxalate	0.04	0.11	0.00	0.12
Lactate	0.0039	0.0039	0.0042	0.0036
Polysaccharide[1]	1.70	1.60	1.50	0.90
Unknown[2]	9.72	12.3	0.00	7.32

[1] Measured as ethanol precipitable material

[2] Other compounds for which standards were run and which were not detected with their approximate limits of detection: Volatiles (GC); formaldehyde (1 mg/l), acetaldehyde (0.5 mg/l), acetone (1 mg/l), acetoin (1 mg/l), iso-propanol (0.5 mg/l), propanol (0.5 mg/l), butanol (0.2 mg/l). Nonvolatiles (HPLC); glycerol (25 mg/l), mannitol (30 mg/l), sorbitol (30 mg/l), pyruvate (0.13 mg/l), acetate (50 mg/l), glycolate (15 mg/l), glucuronate (625 mg/l), glycerate (200 mg/l), gluconate (600 mg/l), *cis*-aconitate (12.5 mg/l), fumarate (0.8 mg/l) (peak masked by the dimethylsuccinate buffer peak), citrate (12.5 mg/l), propionate (21 mg/l), tartrate (125 mg/l), malonate (10 mg/l), malate (2.5 mg/l), isocitrate (4.2 mg/l), α-ketoglutarate (10 mg/l), oxaloacetate (1.1 mg/l), succinate (40 mg/l), butyrate (300 mg/l) and formate (40 mg/l).

Table 2.5. Acidic metabolites detected by GC-MS in the culture supernatants after 7 days of growth.

Metabolic acid[1]	Fungus Alone μM (μmole C)	Fungus + HWKP μM (μmole C)	HWKP Alone μM (μmole C)	Fungus + Bleached HWKP μM (μmole C)
2,3-butanediol	26.0 (20.8)	3.1 (2.4)	0.0 (0.0)	3.7 (3.2)
lactic	4.85 (3.0)	10.9 (6.6)	17.1 (10.3)	14.7 (8.7)
2-hydroxybutyric	nd[2]	1.45 (1.2)	nd	0.9 (0.8)
3-hydroxyisobutyric	1.35 (1.2)	2.6 (2.0)	nd	5.1 (4.0)
3-hydroxybutyric	nd	0.6 (0.48)	nd	nd
2-hydroxyisovaleric	2.2 (2.2)	nd	nd	3.4 (3.5)
3-hydroxyisovaleric	3.5 (3.5)	2.5 (2.5)	nd	16.0 (16.0)
glycolic	nd	0.4 (0.2)	nd	1.1 (0.4)
fumaric	nd	42.9 (34.4)	nd	29.5 (23.6)
adipic	0.8 (2.0)	3.2 (6.0)	0.3 (1.0)	4.2 (8.0)
α-hydroxyphenylacetic (mandelic)	nd	1.1 (1.6)	nd	2.4 (4.0)
2-phenyllactic	nd	17.2 (31.0)	nd	43.6 (78.3)
4-hydroxybenzoic	nd	0.6 (0.7)	nd	nd
3,4-dihydroxybenzoic	nd	nd	nd	0.9 (1.4)

[1] Other substances detected but not reported above due to the likelihood of their being contaminants or compcnents from the buffer or Tween 80: oleic, pimelic, suberic, azelaic, sebacic, palmitic, stearic, nonanoic, α,α-dimethylhydracrylic, α,α-dimethylsuccinic, α-hydrosuccinic, β-hydroxy-α,α-dimethylsuccinic acids, as well as pthalates, which commonly contaminate GC-MS preparations of this type. The glyoxylate and oxalate detected by HPLC (Table 2.4) were not detected here because they are not efficiently derivatized by the method employed. μmole C is per 200 ml culture (done in duplicate).

[2] nd - below the detection limit of the GC-MS.

column, most of the added radioactivity eluted in the void volume (data not shown). Could mucilagenous polysaccharide secretion account for some of this soluble ^{14}C? A small fraction of the labelled C could be precipitated with cold ethanol (≈1.5%) but similar amounts were removed from both the inoculated and uninoculated HWKP flask (Table 2.4). Standards were run for a wide variety of potential metabolites. It should be noted that detection limits for some of the compounds not detected by HPLC, GC, and GC-MS (see footnote of Table 2.4) were quite high and thus low concentrations of some metabolites may have been missed.

Analysis for acidic metabolites as their sialylated derivatives was performed by GC-MS (Table 2.5). Concentrations were low so determination of whether they were glucose-derived by their ^{14}C content was not possible. Comparing metabolite secretion patterns in high (15.4) and low (0.5) kappa pulp cultures (bleached vs. unbleached, Table 2.5), the lignin in the unbleached pulp appeared to have almost no effect. Fumaric, adipic, and 3-hydroxyisobutyric acids showed substantial increases in response to HWKP (Table 2.5). Others, like 2-hydroxybutyric, 3-hydroxybutyric, glycolic, α-hydroxyphenylacetic, and 4-hydroxybenzoic acid went from undetectable levels in the absence of HWKP to low values in its presence. Only one substance, 2,3-butanediol, showed an apparent dependency on the presence of pulp, i.e. it was significantly lower in pulp-containing cultures (Tables 2.4,2.5).

Phenyllactic acid, which was induced by the presence of pulp, is structurally similar to a fungal growth inhibitor active at micromolar concentrations, phenylethyl alcohol (Lingappa *et al.*, 1969). This suggested that phenyllactate might have interfered

with fungal metabolism. To assess the effect of phenyllactate on respiration, aliquots of fresh fungal cultures (1.0 mg/ml) in TDM and 80 mM glucose were incubated with and without phenyllactic acid. Phenyllactate at both 0.3 and 3 mM the rate of O_2 consumption was increased 1.3 fold when compared to a control culture without added phenyllactic acid. the O_2 consumption rate was increased 1.6 times that of the control rate when HWKP (2% w/v) was substituted for phenyllactate, though the O_2 consumption measured over 7 d was only slightly affected (Table 2.2). When an ultrafiltered (MW <10,000) fresh pulp biobleaching culture supernatant was added to *T. versicolor* biomass no change in the instantaneous O_2 consumption rate was observed showing that the metabolites at their *in vivo* concentrations, did not greatly affect O_2 consumption consistent with the O_2 and CO_2 to glucose ratios (Table 2.2). Though both phenylethyl alcohol and phenyllactate are often produced simultaneously in many *Candida* species (Narayaman and Rao, 1974; 1976), the former was not detected in any of the *T. versicolor* cultures.

2.3.4. Enzyme Levels

The culture liquors were assayed for secreted enzymes implicated in lignin biodegradation (Table 2.6). As reported previously (Archibald, 1992a; 1992b), no LP was detectable under *T. versicolor* biobleaching conditions, but CBQase, cellulase and MnP were strongly induced by the presence of HWKP, with either high or low lignin content (Table 2.6)(Paice *et al.*, 1993). Phenyllactate, one of the metabolites present had no effect on LP, MnP, or laccase when tested at 50 μM. At 0.6 and 6 mM concentrations, phenyllactate inhibited the formation of Mn(III)-malonate, probably by competing for

Table 2.6. Levels of some fungal enzymes in the culture supernatants after 7 days of growth.

Culture Condition	Enzyme Activity ($U \cdot L^{-1}$)				
	Mn Peroxidase[1]	Laccase	CBQase	Cellulase	Lignin Peroxidase
Fungus alone	<1	110 ± 10	<2	30 ± 20	<0.2
Fungus + HWKP	490 ± 40	130 ± 40	15 ± 0.0	110 ± 50	<0.2
HWKP alone	<1	<2	<2	<5	<0.2
Fungus + bleached HWKP	1030 ± 100	160 ± 0	30 ± 10	390 ± 90	<0.2

[1] Activity reported as change in absorbance per min at 270 nm.

Mn(III) ions with malonate, since it functioned effectively as a Mn(III) chelator (see below). LP and laccase were not affected even at the higher concentrations.

Peroxide-generating enzymes have been linked to lignin biodegradation. Using a sensitive MnP-coupled assay to detect H_2O_2 (see Materials and Methods), no enzymes capable of using glucose, xylose, cellobiose, glycolate, glyoxylate, glyoxal or veratryl alcohol as a source of reductant for H_2O_2 production were detected in any of the culture liquors.

A comparison of MnP-mediated oxidation of phenol red using either the standard assay buffer-chelator malonate (50 mM; pH 4.5) or a mixture of metabolic acids (glyoxalate, oxalate, 2-phenyllactate, and fumarate) in the proportions and concentrations induced in *T. versicolor* cultures by the presence of pulp (total 4.3 mM; pH 4.5) was made. Using equal amounts of purified *T. versicolor* MnP protein, this metabolic acid buffer-chelator increased the rate of MnP-mediated phenol red oxidation 2.4-fold from 0.52 to 1.26 μmoles$\cdot$min^{-1} (Figure 2.3).

2.4. DISCUSSION

The results showed that if free glucose was available, the presence of pulp or ligninolytic activity had little effect on overall carbon metabolism. The *T. versicolor* 52J biobleaching cultures were grown under conditions of nitrogen sufficiency (initial N was 10 mM as glutamine), with agitation, and a relatively short 7-day growth period. Such conditions are consistent with lignin degradation and biobleaching occurring during primary metabolism (Fenn and Kirk, 1981; Kirk *et al.*, 1978; Kirkpatrick *et al.*, 1989).

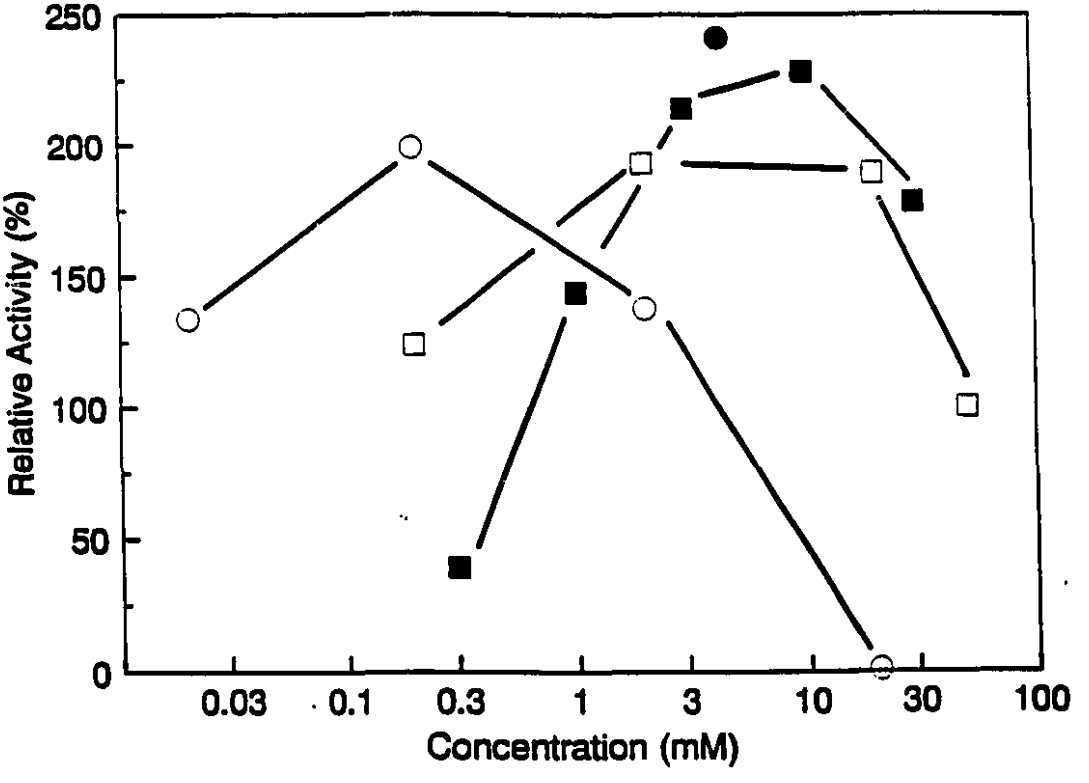


Figure 2.3. Comparison of the ability of three Mn-complexing metabolic acids to promote phenol red oxidation by MnP in the standard assay (which employs 50 mM malonate [see Materials and Methods]) at 23°C, pH 4.5. Legend: (■) glyoxalate; (○) oxalate; (□) malonate; and (●) mixture of glyoxalate (4.0 mM), oxalate (0.28 mM), fumarate (0.04 mM), and 2-phenyllactate (0.02 mM).

Secondary, N-limited metabolism has been shown to stimulate or be essential for both LP production and lignin degrading activity for several other white-rot fungi including *P. chrysosporium* (Keyser *et al.*, 1978; Kirk *et al.*, 1978), *Phlebia brevispora* and *Pholiota mutabilis* (Leatham and Kirk, 1983). The 5 mM glutamine in the TDM medium employed is inhibitory to *P. chrysosporium*-mediated lignin degradation (Fenn and Kirk, 1981), but did not inhibit the biobleaching activity of *T. versicolor* 52J (Table 2.1).

Increases in metabolic acid production by microorganisms are often responses to decreased oxygen. However, the presence of pulp had a negligible effect on *T. versicolor* 52J culture pO_2 (Table 2.3). Interestingly, comparing the organic acids secreted in the four parallel cultures (Tables 2.4,2.5) suggests that pulp, but not the presence of lignin in the pulp, greatly affected the levels of many of the acids secreted by *T. versicolor* 52J. Two metabolites found here, phenyllactate, α-hydroxyphenylacetate, are intermediates in phenylethyl alcohol biosynthesis in *Candida albicans* (Narayanan and Rao, 1974; 1976). Phenylethyl alchohol is both a metabolite and growth inhibitor of this fungus (Lingappa *et al.*, 1969). Many similar compounds are also produced by various species of *Ceratocystis*, a fungus associated with the blue stain disease in pine (Ayer *et al.*, 1986). However, at physiological concentrations, these metabolites did not affect O_2 consumption rate though both HWKP and phenyllactate increased the specific O_2 consumption rate at higher (non-physiological) concentrations (see Results). Phenyllactate also inhibits stearoyl-coenzyme A desaturase, an enzyme of sterol metabolism (Scott and Foote, 1979) and this may explain the differences seen in ergosterol content of *T. versicolor* cultures grown in the presence or absence of pulp (Table 2.3). Glycolate, glyoxylate, and oxalate

(Tables 2.4 and 2.5), three metabolites of the glyoxylate pathway separated by single oxidation steps, were all induced in the presence of pulp. However, at least in bacteria, the glyoxylate pathway normally functions as part of gluconeogensis, an unlikely response in the presence of high medium glucose. Most studies of the glyoxylate cycle in fungi have demonstrated that in the presence of sucrose repression of the cycle occurs (Casselton, 1975). However, with *Coprinus* some expression of the pathway enzymes was detected in the presence of low levels of glucose as seen here (Casselton, 1975).

Anaplerotic reactions to maintain levels of tricarboxylic acid cycle intermediates (Casselton, 1975) are possible sources of glyoxylic and oxalic acids, though oxalate biosynthesis in *Sclerotium rolfsii* was apparently not anaplerotic (Maxwell and Bateman, 1968). In *S. rolfsii*, glyoxylate is reportedly oxidized by glyoxylate dehydrogenase to oxalate and secreted when the glyoxylate cycle activity is low (Kritzman *et al.*, 1977). In *S. rolfsii*, carbon dioxide (1%) significantly decreased both glyoxylate production and increased glyoxylate cycle activity (Kritzman *et al.*, 1977a). Similar levels of CO_2 (0.2%) were observed in our sealed culture bottles when maximum glucose catabolism was occuring (days 4-6) and may explain the differences in biobleaching seen with foam-stoppered culture bottles (Figure 2.1) compared to the sealed system used for the carbon balance (Table 2.1).

Glyoxylate and other 1-3 C compounds are substrates for an H_2O_2-generating glyoxal oxidase found in ligninolytic *P. chrysosporium* cultures (Kersten *et al.*, 1988). Such glyoxal oxidase activity was not detectable in our culture liquors, although it might have been inhibited by the liquor, like LP (Archibald, 1992a), present only transiently

(Kersten and Kirk, 1987), or have required an initial aliquot of added H_2O_2 for activation (Kersten and Kirk, 1987).

Manganese peroxidase and Mn(III)-chelates mediate oxidative reactions thought to be important in delignification (Glenn *et al.*, 1983; Lackner *et al.*, 1991; Paice *et al.*, 1993; Popp *et al.*, 1990; Wariishi *et al.*, 1989), and biobleaching (Paice *et al.*, 1993). An attractive explanation for the pulp-induced secretion of 10 organic acids (Table 2.4,2.5) and MnP (Table 2.6) is that *T. versicolor* is employing one or more of them as the Mn(III) ion complexing agent(s) required for MnP to function. When a buffer composed of the principal metabolic acids induced by pulp at their induced levels (0.28 mM oxalic, 4.0 mM glyoxylic, 0.04 mM fumaric, and 0.02 mM 2-phenyllactic) replaced the 50 mM malonate buffer in the standard phenol red MnP assay, the enzyme's specific activity was enhanced 2.4-fold (Fig. 2.3). MnP was 215% more active when 3.0 mM glyoxylic acid *per se* was used in place of 50 mM malonate. Comparison of oxalate, glyoxalate, and malonate showed that these secreted chelators were very effective stimulators of MnP-mediated phenol red oxidation at their "natural" concentrations in pulp-containing cultures (Fig. 2.3). Since the substrate ranges and reactivity of MnP-generated Mn(III) complexes are highly dependent on the nature and concentration of the Mn(III)-complexing agent employed, identification of glyoxylate and oxalate as likely Mn-ligands in this biobleaching system is not trivial. Of course, more than two Mn-complexing organic acids (or other compounds) may be involved, yielding Mn(III)-complexes with a range of specificities and reactivities. Oxalic acid is a well-known Mn(III)-complexing agent functioning with MnP (Wariishi *et al.*, 1989; 1989a; 1989b). In the presence of oxalate,

Mn(II) and a simple phenol, *T. versicolor* laccase can also generate Mn(III)-oxalate complexes (Archibald and Roy, 1992; Appendix A, this thesis). Some of these pulp-induced carboxylic acids or their Mn(III) complexes may be the factors which must be renewed in the culture liquor for it to continually biobleach and delignify (Archibald, 1992). It should be noted that the ability of a particular Mn(III)-complexing agent to promote optimal oxidation of phenol red (Figures 2.3) may not be indicative of its relative performance on true lignin substrates.

The presence of MnP and laccase in biobleaching cultures suggests that both enzymes have a role. However when HWKP was treated with purified preparations of either or both of these enzymes, only a small part of the biobleaching attained with a complete *T. versicolor* culture was observed (Bourbonnais and Paice, 1992; Paice *et al.*, 1993). Though phenyllactate has been reported as being an inhibitor of oxidative enzymes (Ayer *et al.*, 1986), such an inhibitory effect was not observed with LP, MnP, or laccase. It is reported that CBQase can complement the oxidative activities of laccase, MnP, and LP by (re)generating phenolic substrates for them (Ander *et al.*, 1990; Morpeth and Jones, 1986; Samejima and Eriksson, 1991; Westermark and Eriksson, 1974). CBQase has been shown to reduce (and decolorize) quinones to hydroquinones in the presence of cellobiose, and may mediate reactions which lead to a net depolymerization of lignin (Ander *et al.*, 1990; Westermark and Eriksson, 1974). Thus the fungal biobleaching observed here (Table 2.1) and in earlier work (Addleman and Archibald, 1993; Archibald, 1992; 1992a; Paice *et al.*, 1989; Reid *et al.*, 1990) may require pulp-induced CBQase (Table 2.6) since both *T. versicolor* MnP and laccases can delignify and demethoxylate

HWKP (Bourbonnais and Paice, 1992; Paice *et al.*, 1993) but do not substantially increase pulp brightness, as does the complete fungal system. While pulp-mediated cellulase induction renders the prime carbon and energy source in wood available to *T. versicolor*, we have shown that cellulase secretion is not highly repressed by free glucose (Tables 2.2,2.6). *T. versicolor* 52J may therefore be producing cellulase under glucose-sufficient conditions primarily to furnish CBQase with substrate (cellobiose) or to open the lignocellulosic cell wall structure, rendering CBQase, MnP, and laccase substrates more accessible. Such cooperativity is suggested by the induction of MnP, CBQase and cellulase in the presence of pulp. Therefore, we have observed in the *T. versicolor* biobleaching system all the components (except hydrogen peroxide) necessary for complementarity between MnP- and/or laccase-mediated oxidation and the CBQase-mediated reduction of lignin, which we suspect is essential to the biobleaching process.

Preface to CHAPTER 3.

Pascynski *et al.* (1986) proposed that a redox cycle could be established between oxidative enzymes such as laccase and MnP or LP and quinone-reducing enzymes such as glucose oxidase (Pascynski *et al.*, 1986) or CBQase (Westermark and Eriksson, 1974; Ander *et al.*, 1990). As shown in Chapter 2, many possible reductive enzymes were assayed for in *T. versicolor* 52J biobleaching cultures, but only CBQase was detected extracellularly. Since Archibald (1992) had demonstrated that biobleaching can occur without hyphal-fibre contact, it was presumed that if such a redox cycle was important for lignin degradation, it must occur outside the fungal hyphae and probably involved CBQase. Since the purification and characterization of CBQase from *T. versicolor* has never been reported and it was necessary to obtain purified CBQase to study what role, if any, that it might play in biobleaching, a purification and characterization of the enzyme was performed.

CHAPTER 3. PURIFICATION AND CHARACTERIZATION OF THE MAJOR CELLOBIOSE:QUINONE OXIDOREDUCTASE PROTEINS

3.1. INTRODUCTION

The white rot fungus *Trametes versicolor* 52J can biobleach and delignify both hardwood and softwood kraft pulps (Paice *et al.*, 1989; Reid *et al.*, 1990, Reid and Paice, 1994; Roy and Archibald, 1993, Addleman and Archibald, 1993). Intimate contact between the fungus and pulp fibers is not required for delignification (Archibald, 1992). *T. versicolor* secretes both low molecular weight metabolites and a number of lytic enzymes (Roy and Archibald, 1993). Two of these enzymes, manganese peroxidase (MnP) (Paice *et al.*, 1993) and laccase (Bourbonnais and Paice, 1992) can, in the presence of appropriate cofactors, demethylate and delignify kraft pulps, although not nearly as extensively as a complete *T. versicolor* culture.

Cellobiose:quinone oxidoreductase (CBQase) has been proposed as an enzyme important in both lignin (Eriksson *et al.*, 1974; Eriksson *et al.*, 1990; Westermark and Eriksson, 1974) and cellulose biodegradation (Kremer and Wood, 1992; Bao and Renganathan, 1992). CBQase is produced by many white-rot fungi (Ander and Eriksson, 1977) including *T. versicolor* (Westermark and Eriksson, 1974a). CBQase is also secreted by a number of non white-rot fungi (Sadana and Patil, 1988; Dekker, 1980; Coudray *et al.*, 1982; Schmidhalter and Canevascini, 1993). CBQase has reportedly been isolated from only two ligninolytic white-rot fungi, *Phanerochaete chrysosporium* (Westermark and Eriksson, 1974b), and *Fomes annosus* (Hüttermann and Noelle, 1982) (see Table 1.2).

It should be noted that CBQase activity may be partially or completely missed as laccases can mask its presence in culture supernatants by rapid re-oxidation of the hydroquinones produced by CBQases from quinone assay substrates (Roy and Archibald, 1994). CBQases use electrons from the oxidation of cello-oligosaccharides (Bao and Renganathan, 1992; Kremer and Wood, 1992; Roy *et al.*, 1994) to reduce free radicals (Westermark and Eriksson, 1974a; Ander *et al.*, 1990; Roy and Archibald, 1993; Samejima and Eriksson, 1993), quinones (Westermark and Eriksson, 1974a; Coudray *et al.*, 1982; Dekker, 1980), Fe(III) (Coudray *et al.*, 1982; Kremer and Wood, 1992; 1992a), Mn(III) (Bao *et al.*, 1993), and Mn(IV) (Roy *et al.*, 1994)(Chapter 4, this thesis). Thus, CBQase-mediated reduction produces many species which are good substrates for the laccases and peroxidases commonly secreted by white-rot fungi during delignification. The reduction of O_2 to H_2O_2 via a superoxide intermediate is also catalyzed by *P. chrysosporium* CBQase *in vitro* (Ayers *et al.*, 1982; Coudray *et al.*, 1982; Morpeth and Jones, 1988); however O_2 reduction reaction rates are low when compared to those of many other CBQase substrates (Wilson *et al.*, 1990).

A number of schemes have been proposed to show the possible role(s) of CBQase in wood and pulp delignification (Roy *et al.*, 1994; Eriksson *et al.*, 1990). These findings suggest that CBQase may be an important component of the delignification system in white-rot fungi. The objective of this study was to determine some of the structural and catalytic properties of the *T. versicolor* CBQases to better understand their role in *T. versicolor*-mediated kraft pulp biobleaching.

3.2. MATERIALS AND METHODS

3.2.1. Fungal Cultures

T. versicolor strain 52J was kept at -80°C and cultured on mycological broth (MB) plates as previously described (Addleman and Archibald, 1993). For the production of CBQase, the complex spore germination medium described by Canevascini (1988) (Medium A; 0.5 g/l KCl, 0.1 g/l $MgSO_4 \cdot 7H_2O$, 0.1 g/l yeast extract, 1 g/l KH_2PO_4, 0.4 g/l NH_4Cl, and 1 ml of a trace metals solution (Roy and Archibald, 1993) was inoculated with 4 agar plugs (1 cm dia.) taken from the periphery of a growing colony of *T. versicolor*. Inoculum for production cultures were prepared in a 500 ml plastic Erlenmeyer flask containing 200 ml of Medium A supplemented with 5 g/l glucose and incubated at 27°C for 48 h with shaking (r=4.5 mm) at 200 rpm, followed by transfer to 800 ml of medium A plus 5 g/l glucose to a 3.8 litre Fernbach flask that was in turn incubated for 48 h agitated at 100 rpm (r=4.5). CBQase production was done in aerated 20 litre Nalgene carboys containing 14 litres of Medium A supplemented with 5 g/l cellulose (Solka Floc) inoculated with 1 litre of a growing *T. versicolor* culture and incubated on a platform shaker at 75 rpm (r=4.5) at 27°C . The culture was sampled every 12 h, clarified by centrifugation (10,000 x g; 5 min) and CBQase activity assayed.

3.2.2. Assays

CBQase activity was determined by monitoring the cellobiose-dependent reduction of 3,5-di-tert-butylbenzoquinone(1,2) (TBBQ) at 420 nm (Westermark and Eriksson, 1974a). The assay mixture contained sodium acetate (100 mM; pH 4.5), ethanol (20%

v/v), cellobiose (2 mM), and TBBQ (0.33 mM) in a total assay volume of 1.5 ml. To inhibit interference in the assay caused by laccase-mediated re-oxidation of the hydroquinone formed from TBBQ by CBQase, the reaction mixture was made anoxic by bubbling 1.5 ml of N_2 through the reaction mixture in 3 min. One unit of enzyme was defined as the amount of enzyme that reduced 1 μmole TBBQ $min^{-1}ml^{-1}$. The reduction of other substrates was measured in the sodium acetate-ethanol buffer mixture as for the standard assay and monitored at the appropriate wavelength (λ_{max}) of the substrate and the activity calculated using the experimentally determined extinction coefficients of the quinones. The experimentally determined extinction coefficients for quinones synthesized according to Teuber and Staiger (1955) and used as CBQase substrates were measured in 100 mM acetate buffer (pH 4.5) containing 20% v/v ethanol.

Cellobiose-dependent O_2 uptake by CBQase was measured with a Clark oxygen electrode in a water jacketed cell (Rank Brothers, Cambridge, U.K.) at 25°C in a total volume of 3 ml. The reaction mixture consisted of 10 U/ml CBQase and 2 mM cellobiose in 100 mM sodium acetate buffer. H_2O_2 production in the reaction mixture was determined by the addition of 600 U/ml catalase to the reaction mixture and measuring the flux of O_2 produced.

3.2.3. Purification Procedure

When maximum CBQase activity was obtained (72-96 h), the culture supernatant was separated from the residual cellulose and fungal biomass by filtration through cheesecloth and Whatman filter No. 4 paper (yielding >90% recovery of TBBQ-reducing

activity). The clarified supernatant was concentrated 15-fold using a Pellicon ultrafiltration unit (Millipore, Danvers, MA.) and 10,000 MW cutoff polysulphone membranes. The concentrated supernatants were dialyzed first against distilled water (10 x 1 liter), then against buffer B (10 mM Bis-tris, pH 6.5; 4 x 1 liter). The sample was further concentrated to 200 ml in an membrane ultrafiltration unit (Amicon, Beverly, MA.) again using 10,000 MW cutoff membranes. The concentrated crude enzyme was applied to a DEAE-sephacryl gel column (5 cm x 30 cm; bed volume 250 ml) previously equilibrated with buffer B. The column washed with 500 ml of buffer B then eluted with a linear salt gradient from 0-500 mM NaCl in a total volume of 2 liters at a flow rate of 80 ml/h. Fractions containing CBQase activity were pooled, dialyzed (5 x buffer B) and concentrated using an Amicon ultrafiltration cell (10,000 MW cutoff). The sample was further fractionated on a Sephacryl S-300 (Pharmacia) column (2.5 cm x 92 cm) equilibrated in 5 x buffer B at a flow rate of 22 ml/h. Active fractions were pooled, dialyzed (10 mM Bis-tris propane buffer, pH 6.4), and concentrated (as above). The final purification steps consisted of fractionating the pooled active fractions on a Mono-Q HR 5/5 column (Pharmacia) equilibrated with Bis-tris propane buffer (pH 6.4; 20 mM) twice with an intermediate step of pooling and dialyzing the CBQase containing fractions. The column was washed with 2 ml buffer, and retained proteins were eluted with a linear salt gradient (0-400 mM NaCl) in starting buffer in a total volume of 40 ml. Active fractions were pooled, dialyzed (dH_2O) and concentrated. Enzyme was stored at 4°C at a concentration of 1 mg ml^{-1}.

3.2.4. Protein concentration and cofactor identification

Protein content was measured spectrophotometrically (595 nm) using Coomassie blue (Bio-Rad Protein Assay) (Bradford, 1976), with bovine serum albumin as the standard. The flavin coenzyme was released from homogeneous CBQase by precipitating the protein with 5% w/v trichloroacetic acid (Morpeth, 1985). The protein was removed by centrifugation (10,000 x g; 10 min.) and the supernatant extracted four times with diethyl ether (4°C) and the aqueous phase lyophilized. The residue was reconstituted in H_2O (100 µl) and separated by thin layer chromatography (TLC) on silica gel plates with Na_2HPO_4 (5% w/v) in water as solvent (Fazekas and Kokai, 1971) and the resolved flavin bands were observed using ultraviolet light. The R_f of the dissolved material resolved by TLC was obtained and compared to that of genuine FAD (Sigma Chemical Co.). The amount of FAD released from CBQase was quantified spectrophotometrically using an extinction coefficient (450 nm) of 11,300 mM^{-1} cm^{-1} for FAD (Fazekas and Kokai, 1971). The nature of the heme group was identified by its Soret peak in the spectrum of the CBQase with a pI 4.2. The heme was extracted from CBQase with acidified (0.1 M HCl) acetone and quantified using the pyridine-hemochromogen method (Appleby and Morton, 1959; Morpeth, 1985).

3.2.5. Enzyme kinetics

The K_m and V_{max} were determined for cellobiose and a number of quinone substrates of CBQase 4.2. TBBQ was used as the electron acceptor when various electron donors were used, while cellobiose was used to compare the different electron acceptors.

All determinations were at pH 4.5 in 100 mM sodium acetate buffer. Temperature optima of the two CBQase enzymes were determined using a jacketed cuvette holder with a Häake circulating waterbath using TBBQ and cellobiose as substrates at pH 5 in 100 mM sodium acetate buffer. The optimal pH of the two CBQase enzymes were determined using sodium acetate (pH 4.0, 4.5, 5.0), sodium succinate (pH 5.5), potassium phosphate (pH 6.0, 6.5, 7.0) and Tris-HCl (pH 7.5, 8.0, 8.5) buffers.

3.2.6. Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed using the Pharmacia Phast Gel system with a 10-25% gradient acrylamide against molecular weight standards. Isoelectric focusing was performed with the Phast Gel IEF apparatus with pH ranges of 3-9 and 2.5-6 in conjunction with appropriate IEF isoelectric point (pI) standards (Pharmacia). Protein bands were visualized by staining with Coomassie blue and CBQase activity detected using dichlorophenolindophenol (DCIP) (pH 6) and cellobiose. Schiff staining was used to identify glycoproteins following SDS-PAGE (Glossmann and Neville, 1971).

3.2.7. Chemicals

The quinone substrates were synthesized using Fremy's salt (potasssium nitrosodisulfonate) oxidation of the corresponding phenols as follows: 2-methoxy-4-methyl-1,2-benzoquinone from 2-methoxy-4-methyphenol, 4-*tert*-octyl-1,2-benzoquinone from 4-tert-octylphenol, 4-*tert*-butyl-1,2-benzoquinone from 4-tert-butylphenol, 4-methyl-

1,2-benzoquinone from 4-cresol, 3,4,5-trimethyl-1,2-benzoquinone from 3,4,5-trimethylphenol and 3,4-dimethyl-1,2-benzoquinone from 3,4-dimethylphenol (Teuber and Staiger, 1955) (see Appendix C). Melting point and UV-visible absorbance spectra were compared to published standards to confirm purity of the quinones (Teuber and Staiger, 1955). Other quinone substrates used to test CBQase activity were from Aldrich Chemical Co. Cellobiose, lactose, glucose, galactose, xylose and were from Sigma Chemical Co. Oligomers of glucose (n=3-8) were synthesized as described (Miller, 1963).

3.2.8. Spectroscopy

Absorption spectra were recorded on a Perkin-Elmer Lambda 3 spectrophotometer at room temperature in a cuvette with a 1 cm lightpath. Enzyme spectra were recorded in 20 mM sodium acetate buffer (pH 4.5). Enzymes were reduced using cellobiose (10-fold excess) or sodium dithionite (several grains).

The EPR spectra of frozen samples of CBQase were measured using a X-band Bruker ER 200D-SRC spectrometer using 100 kHz modulation. The flavin semiquinone spectra were detected with the following conditions: temperature 120 K; microwave power, 5 mW; microwave frequency, 9.3 GHz; modulation amplitude, 0.16 mT (Morpeth, 1985). Diphenyl-picryl-hydrazyl (DPPH) was used to measure the exact microwave frequency.

3.2.9. Amino Terminal Sequence

The amino terminal sequence of purified CBQase 4.2 was determined using a Porton protein sequencer by Dr A. Bell of the Sheldon Biotechnology Center, McGill University, Montreal, Canada. The terminal methionine was blocked and therefore the CBQase protein (100 μg) was treated with CNBr to remove the modified terminal methionine. The resulting protein was then subjected to standard Edman degradation sequencing and the identity of the cleaved amino acid derivatives separated and identified by HPLC.

3.3. RESULTS

3.3.1. Purification of CBQase

The production of CBQases by *T. versicolor* 52J in several growth media was investigated and it was concluded that the best medium was one substituting a cellulosic carbon source for glucose (data not shown). Though a kraft lignin (Indulin) apparently greatly increased the yield of CBQase, its presence interfered with subsequent isolation and purification of the enzyme. Nearly all of the CBQase enzyme activity was found extracellularly (Table 3.1) as two proteins which could be easily separated on non-denaturing polyacrylamide gels (Figure 3.1, lane B). The CBQases secreted by *T. versicolor* 52J during growth on Solka Floc cellulose were isolated and purified from the culture medium in four steps. The initial separation on DEAE-Sephacryl gave three activity peaks (Figure 3.2).

Table 3.1. Localization of total CBQase activity.[1]

Enzyme	Enzyme Activity (U liter^{-1})[2]			
	Extracellular		Intracellular	
	TDM	TDM + Indulin	TDM	TDM + Indulin
CBQase	50 ± 10	270 ± 80	0	0
Laccase	3710 ± 230	900 ± 50	75 ± 0	196 ± 10
Catalase	0	0	789 ± 73	2300 ± 41

[1] Intra- and extracellular CBQase activity in *T. versicolor* 52J was measured to find the primary site of reductive activity in biobleaching cultures. Cultures were grown in a defined medium (Chapter 2) with cellobiose as carbon source ± Indulin (0.05 g ml^{-1} soluble kraft lignin). Values are the mean of three determinations ± standard deviations.

[2] Enzyme activity was calculated as the total units of enzyme recovered divided by the culture volume.

[3] Intracellular enzymes were released using a French pressure cell.

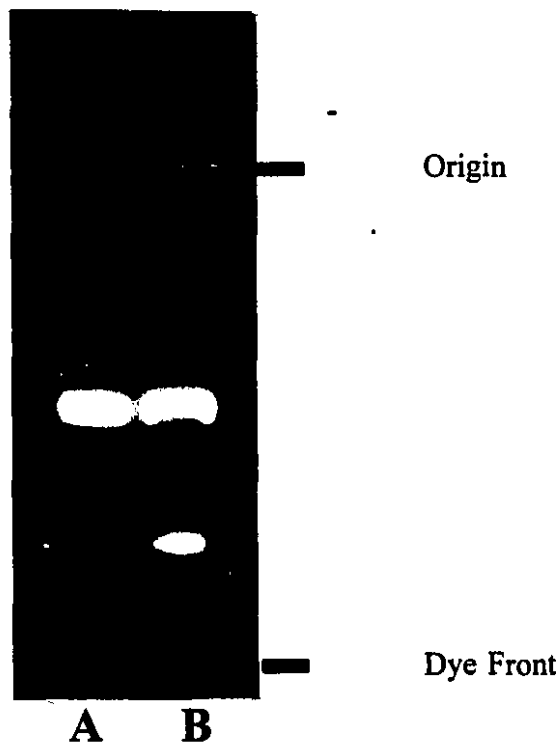


Figure 3.1. Resolution of concentrated (MW >10,000) supernatant proteins by nondenaturing polyacrylamide gel electrophoresis (PAGE). Proteins were separated (400 volt-hours) on 8-25% gradient Phast gels. The gel was stained with 2 mM DCIP in 20 mM phosphate buffer (pH 6.5), and proteins with CBQase activity were detected as clear bands appearing in the blue-stained gel. These bands only appeared after the addition of cellobiose (2 mM) to the gel. Lane A was a purified preparation of CBQase 4.2 and lane B was a concentrated extracellular supernatant from *T. versicolor*.

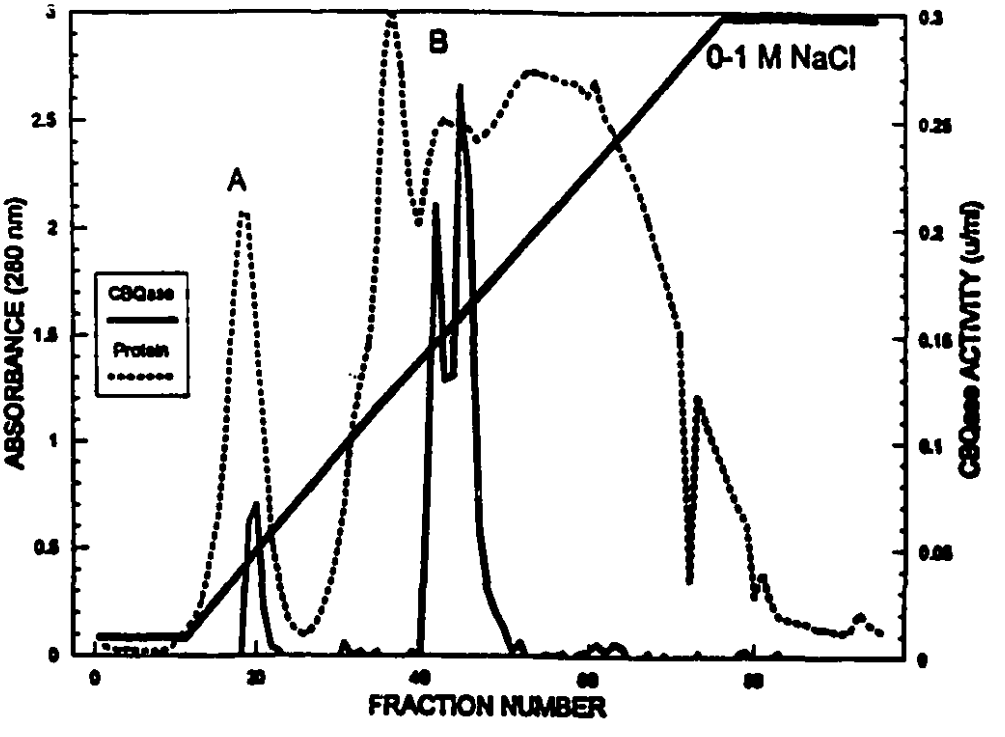


Figure 3.2. Purification of CBQase by ion exchange chromatography. Concentrated and dialysed culture supernatant was passed through DEAE-Sephacryl equilibrated with Bis-Tris buffer (20 mM, pH 6.5), washed with 1 liter of the same buffer and eluted with a linear gradient of NaCl (0-1 M in 2 liters). Eluate was assayed for protein and CBQase activity.

Fractions containing the first activity peak (Figure 3.2, peak A) were pooled and subsequently purified. CBQase A varied from 0-10% of the total TBBQ-reducing activity in different concentrated unfractionated culture supernatants, and co-eluted with >90% of the laccase activity in the crude preparation. The fractions containing the second and third (Figure 3.2, peak B) CBQase activity peaks were pooled and further purified. Table 3.2 shows the results of a typical purification procedure and gives the yield of each of the enzymes from a typical culture liquor preparation.

Purification of CBQase A activity increased the specific activity of the enzyme five-fold. The protein was obtained as a pale yellow solution. The molecular weight of the protein was estimated to be 48 kDa by SDS-PAGE (Figure 3.3 lane A). Gel permeation chromatography gave a similar estimate of size, suggesting that the enzyme occurs as a monomer. By isoelectric focusing using ampholytes in the pH 3 to 9 range, the pI of the 48kDa CBQase was determined to be 6.4. Therefore CBQase A was designated CBQase 6.4.

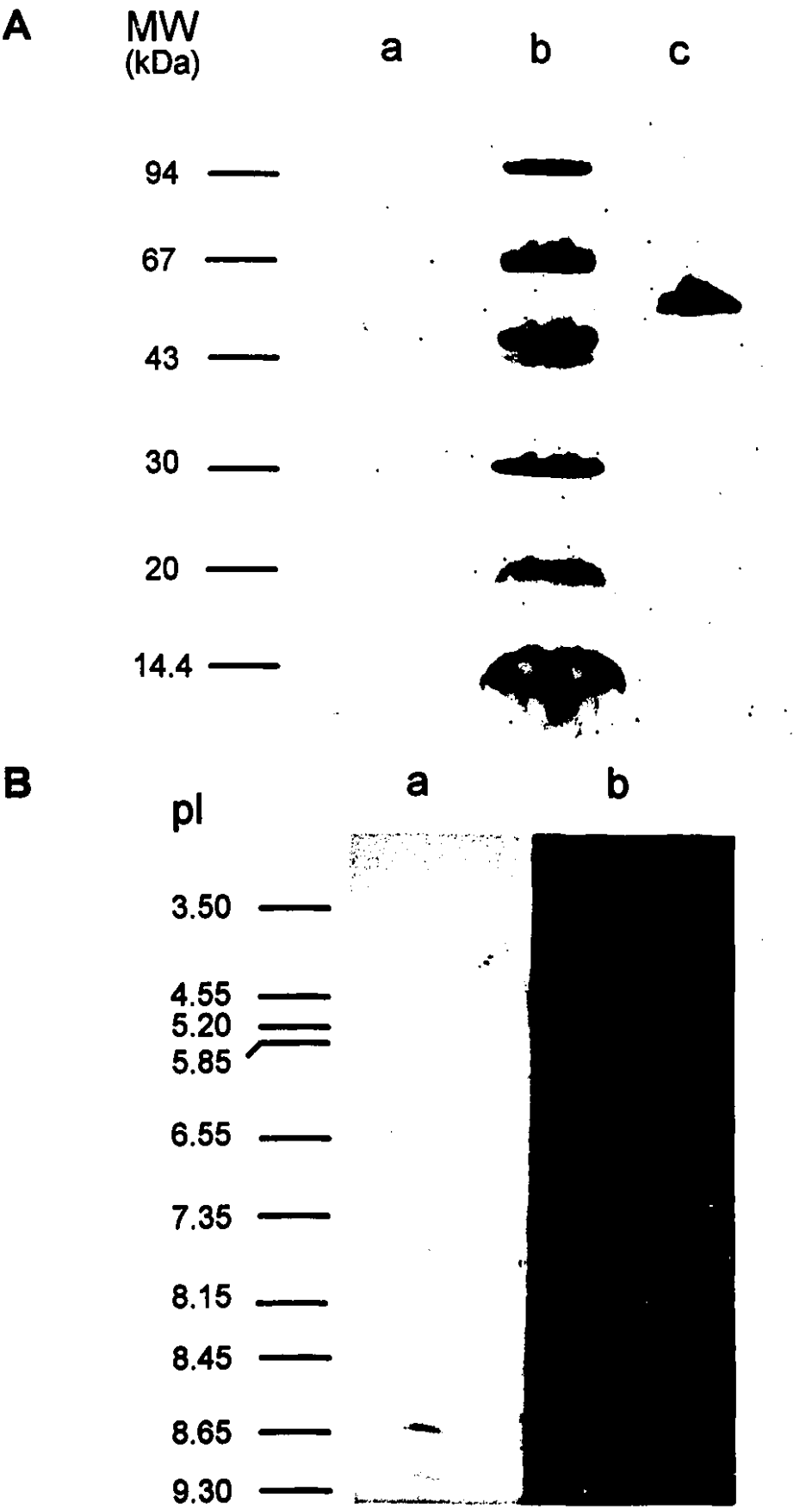
Purification of CBQase B yielded a homogenous red-brown protein which formed a single band with a relative molecular weight of 58 kDa when resolved by SDS-PAGE (Figure 3.3 lane C). The turnover number (k_{cat}) of the purified CBQase preparation was 24 s^{-1} and its specific activity was 25 μmole min^{-1} mg^{-1} protein, with TBBQ as substrate for the enzyme. When a non-denaturing PAGE gradient gel (10-25%) was stained for activity using dichlorophenolindophenol (DCIP) or with Coomassie blue, a single diffuse band was observed with an apparent molecular weight of 110 kDa. This suggested that the enzyme forms a homodimer in its native state. A molecular weight of 113 kDa was

Table 3.2. Purification of *T. versicolor* CBQase pI 4.2 and pI 6.4.[1]

Purification Step	Total Protein (mg)[2]	Enzyme Activity (U)[3]	Specific Activity (U/mg)	Yield (%)	Purification (fold)
Supernatant	-	2000	-	100	-
Ultrafiltration[4]	2544	1774	0.70	88.7	1
DEAE-Sephacryl	141 (17)	900 (44)	6.38 (2.59)	45.0 (2.2)	9.1 (3.7)
Sephacryl S300	67 (11)	840 (32)	12.5 (2.91)	42.0 (1.6)	17.9 (4.2)
Mono-Q (FPLC)	24 (5)	590 (17)	24.6 (3.4)	29.5 (0.9)	35.1 (4.9)

[1] Values in parentheses are for the flavin-cofactored CBQase (pI = 6.4)
[2] Protein determined using BioRad dye-binding protein assay.
[3] Activity was measured using TBBQ and cellobiose as substrates.
[4] Total TBBQ reducing activity of two CBQases (pI's = 4.2, 6.4).

Figure 3.3. Electropherograms of purified CBQase 4.2 and 6.4. Panel A is a Coomassie blue stained SDS-PAGE gradient gel of purified preparations of CBQase 6.4 (lane a), CBQase 4.2 (lane c) and molecular weight standards (lane b). The molecular weight of the protein standards are shown on the left. Panel B shows an IEF gel (pH range 3-9) with coomassie blue stained IEF standards (lane a) with their respective pI's indicated on the left and a purified preparation of CBQase 4.2 (lane b) stained with DCIP.


A
MW
(kDa)
a
b
c
94
67
43
30
20
14.4
B
a
b
pI
3.50
4.55
5.20
5.85
6.55
7.35
8.15
8.45
8.65
9.30

estimated using gel permeation chromatography on both Sephacryl S300 and Superose 12 FPLC columns. Isoelectric focusing gave a pI of CBQase B of 4.2 and so in subsequent discussion, it is designated CBQase 4.2. The amino terminal sequence of the purified CBQase 4.2 was determined and compared to proteins catalogued in the EMBL protein database and to the sequences of *T. versicolor* laccases and peroxidases (Figure 3.4). The N-terminal of the protein was found to be blocked. However a mild treatment with cyanogen bromide allowed for the removal of the blocked methionine and the sequence was obtained for the protein. The enzyme shares very little relationship to sequences of known proteins, being most closely related to xylanase proteins. This is the first report describing the N-terminal amino acid sequence for a CBQase protein.

CBQase 4.2 has maximal activity with TBBQ in acetate buffer at a pH of 5.0 (Figure 3.5) and a temperature of 55°C (Figure 3.6). The enzyme is rapidly inactivated at temperatures >60°C (50% inactivation in 30 s at 60°C).

The purified CBQases (3 mg ml^{-1}) could be stored at 4°C in water for several months without loss of enzymatic activity, but lost 30-50% of their initial activity when frozen and thawed. Lyophilization resulted in complete inactivation of the CBQase proteins.

3.3.2. Cofactors of CBQase 4.2 and 6.4

Comparison of the cofactor released by 5% TCA from both CBQase 4.2 and CBQase 6.4 yielded flourescent products with R_f values identical to that of purified FAD. The cofactor contents were approximately 0.8 and 0.9 FAD molecules per monomeric

	1	11
TvCBQase4.2	IGNLLLVAMP	NGDWIVSSxx
TvLP7	VACPDGKNTA	TNAACCSLFA
TvLP12	VACPDGVNTA	TNAACCQLFA
TvMnP1	VACPDGVNTA	SNAACCQLFx
TvMnP5	VACPDGVNTA	SNAACCAxFP
TvLaccI	AIGPVASLVV	ANAPVSPDGQ
TvLaccIII	GIGPVADLT I	TNAGVSPDGL

Figure 3.4. N-terminal amino acid sequences of CBQase 4.2 and several secreted laccases and peroxidases from *T. versicolor*. Proteins are aligned and identical(homologous) residues are highlighted in bold typeface. Lacc = laccase, MnP = manganese peroxidase, LP = lignin peroxidase, and CBQase = cellobiose:quinone oxidoreductase 4.2 (heme-flavoprotein) purified from *T. versicolor* 52J. Laccase sequences are from Limura *et al.* (1992) and MnP and LP amino terminal sequences are from Johansson *et al.* (1993). Positions identified with an "x" are for residues which could not be identified during sequencing.

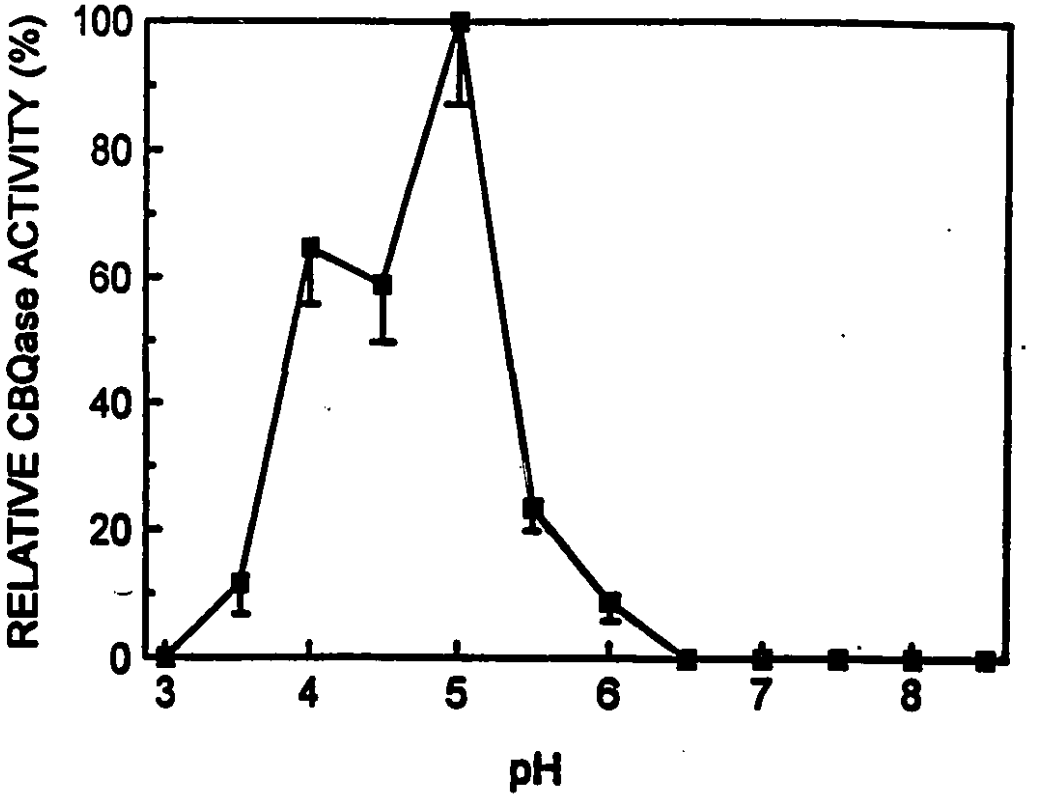


Figure 3.5. pH profile of combined CBQase 4.2 and 6.4 activity. CBQase activity was determined using TBBQ (0.33 mM) as substrate and cellobiose (2mM) in sodium citrate (pH 3.0, 3.5), sodium acetate (pH 4.0,4.5, 5.0), sodium succinate (pH 5.5), potassium phosphate (pH 6.0, 6.5, 7.0, 7.5) and Tris-HCl (pH 8.0, 8.5) buffers as described in Materials and Methods. Values shown are the means of triplicate determinations ± standard deviations.

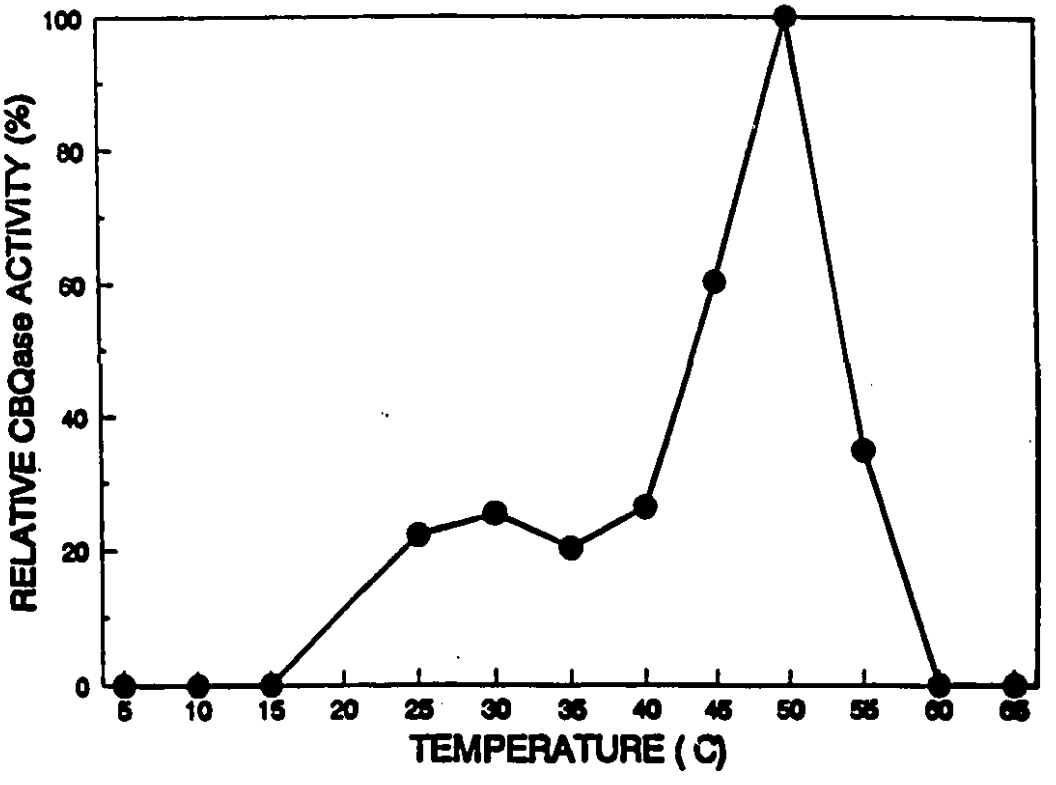


Figure 3.6. TBBQ-reducing activity of CBQase 4.2 at various temperatures. The enzyme activity was determined in 50 mM sodium acetate buffer (pH 5.0) with TBBQ (0.33 mM) and cellobiose (2 mM) as substrates. Values shown are the means of duplicate determinations.

CBQase 4.2 and CBQase 6.4 molecule, respectively. The less than 1:1 ratio may reflect the presence of modified flavins in the enzyme preparation (Morpeth and Jones, 1986), less than quantitative flavin recovery, or the presence of deflavinated protein in the enzyme preparations. Subsequent extraction of CBQase 4.2 with acidified acetone and preparation of a pyridine hemochromogen yielded a strong absorbance at 555 nm and demonstrated that the enzyme heme was of the cytochrome *b* type similar to the *P. chrysosporium* cellobiose oxidase (CBO) enzyme (Morpeth, 1985; Bao *et al.*, 1993).

3.3.3. Spectrophotometric analysis

The spectrum of CBQase 6.4 (Figure 3.7) had absorption peaks at 457 and 496 nm which are similar to the CBQase from *P. chrysosporium* (Westermark and Eriksson, 1975; Morpeth and Jones, 1986). The visible wavelength absorbance peaks were bleached with the addition of cellobiose or Na_2SO_3. Unlike the *P. chrysosporium* CBQase activity (Morpeth and Jones, 1986), only a single isozyme of CBQase 6.4 was obtained from cultures of *T. versicolor* 52. as determined by IEF and nondenaturing polyacrylamide gels, and protein separations on Mono Q columns. Homogeneous enzyme preparations had a A_{457}/A_{276} ratio of approximately 0.09.

Oxidized CBQase 4.2 was quite different from CBQase 6.4 with absorption peaks at 419 nm (ϵ = 56.2 $mM^{-1}cm^{-1}$), 535 nm (ϵ = 5.2 $mM^{-1}cm^{-1}$), and 564 nm (ϵ = 4.7 $mM^{-1}cm^{-1}$). (Figure 3.8). When CBQase 4.2 is reduced to the ferrous form with cellobiose, the absorption maxima are shifted to 425, 538 and 570 nm and the molar absorptivities of the peaks at 538 nm (ϵ = 6.7 $mM^{-1}cm^{-1}$) and 570 nm (ϵ = 7.2 $mM^{-1}cm^{-1}$) are increased

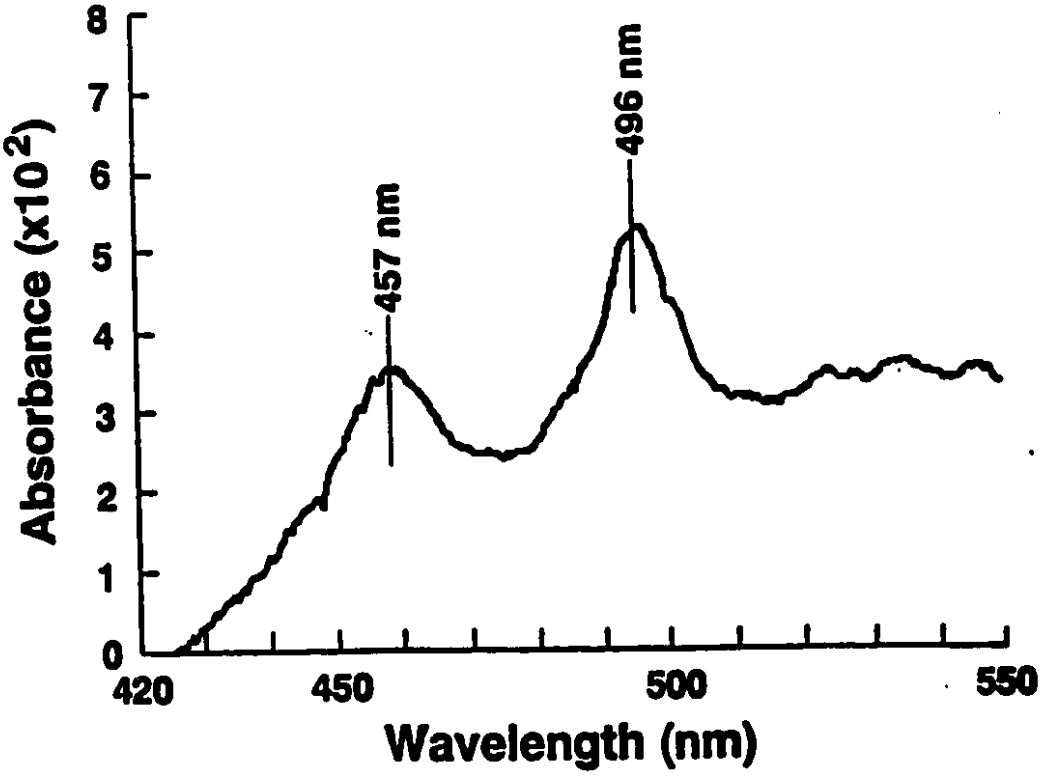


Figure 3.7. Visible spectrum of a purified preparation of CBQase 6.4 in 50 mM sodium acetate buffer (pH 4.5). The enzyme was purified as described in Table 3.2 and the absorbance profile of the enzyme recorded.

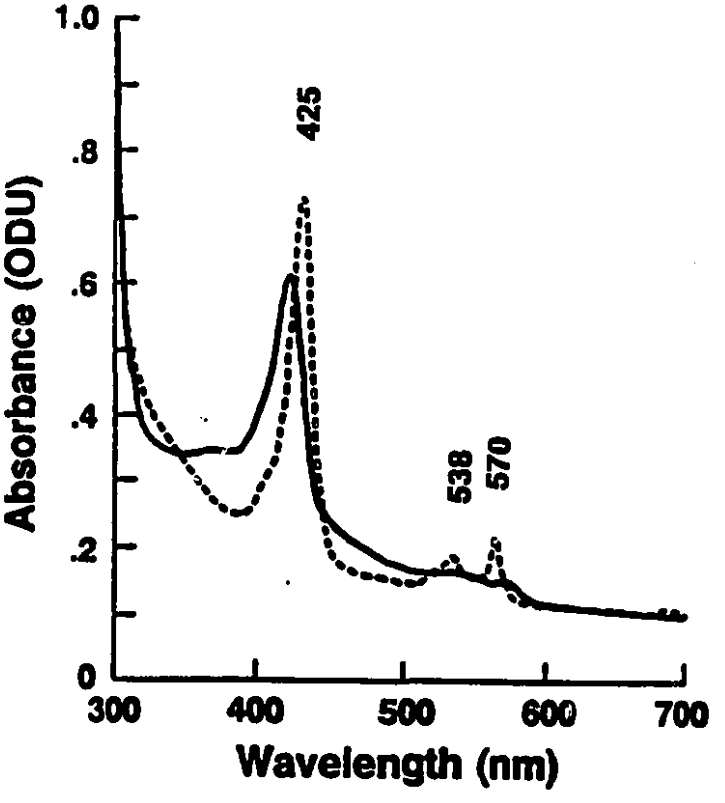


Figure 3.8. Visible spectrum of oxidized (——) and reduced (----) CBQase 4.2 (8 μM) in 50 mM sodium acetate buffer (pH 4.5). The enzyme was purified as described in Table 3.2 and the absorbance profile recorded. The enzyme was reduced by adding cellobiose (2mM) to the enzyme preparation.

(Figure 3.8). The observed decrease in absorption between 500 and 450 nm upon reduction by cellobiose (Figure 3.8) has been associated with the reduction of the flavin cofactor in *P. chrysosporium* CBO (Morpeth, 1985; Bao *et al.*, 1993). FAD was confirmed as the cofactor by isolation of a fluorescent material from the purified CBQase 4.2 which co-migrated with genuine FAD on silica gel TLC plates eluted with sodium phosphate (Fazekas and Kokai, 1971). The spectral properties of the eluted material was also identical to those of genuine FAD.

At 120° K, an EPR signal appeared in preparations of CBQase 4.2 (8 μM) reduced with 50 μM cellobiose not seen with the oxidized form of the enzyme. The EPR signal was centered on a field strength of 357.9 mT and had a calculated g value of 1.947 with a linewidth of approximately 6 mT, similar to that reported for the red (anionic) flavin semiquinone (Massey and Palmer, 1966). At 120° K, no EPR signal was detected in preparations of oxidized CBQase 6.4, however reduction of the enzyme with cellobiose produced a signal. This EPR signal is similar to that of reduced *P. chrysosporium* CBO (Morpeth, 1985).

3.3.4. Catalytic properties of the enzymes

When TBBQ was used as the electron acceptor, both CBQases oxidized cellobiose as their preferred substrate (Table 3.3), though cellotriose and lactose were also used at a lower rate. Though the cello-oligosaccharides did not reduce detectable amounts of TBBQ in a short 5-10 min assay, TBBQ reduction by CBQase was detectable with longer incubation periods (6-24 h).

Table 3.3. CBQase activity using various sugar substrates.[1]

Substrate[2]	CBQase 6.4 activity (Umg^{-1})	CBQase 4.2 activity (Umg^{-1})
Cellobiose	3.6 ± 0.51[3]	25 ± 0.4
Cellotriose	1.6 ± 0.14	17 ± 0.4
Glucose	0.13 ± 0.06	9 ± 0.1
Lactose	1.8 ± 0.17	3.1 ± 0.0
Mannitol	0.34 ± 0.03	0
Sorbose	0.36 ± 0.06	0.1 ± 0.004
Raffinose	0.49 ± 0.11	0
Bacterial cellulose	0	0
Whatman cellulose	0	0
Solka Floc cellulose	0	0
Cellotetraose	0	-[4]
Cellopentaose	0	-
Cellohexaose	0	-
Celloheptaose	0	-
Xylose	0	0
Sorbitol	0	-
Glycerol	0	-
Glucuronate	0	0
Gluconate	0	0
Gentobiose	0	-
Galactose	0	0
Fructose	0	0
Arabinose	0	0

[1] Electron acceptor was TBBQ in each case.

[2] The cellulose preparations (Solka floc, bacterial cellulose, Whatman cellulose) and cellulo-oligosaccharides were tested for their ability to reduce CBQase 4.2. Though none of these cellooligosaccharides gave significant amounts of reduction of TBBQ reduction in a 5-10 min assay, TBBQ was reduced by CBQase incubated with them over a longer time period (6-24 h).

[3] Results are the mean of duplicate determinations ± standard deviations.

[4] not determined.

Three types of electron accepting CBQase substrates were evaluated, Mn(III) complexes (Figure 3.9, group IV), oxidized phenthiazoline-type cation radicals (Figure 3.9, Group II) and a variety of *ortho* and *para* quinones (Figure 3.9, groups I,III and **Appendix C**). Consistent with the properties of similar enzymes isolated from *P. chrysosporium*, CBQase 4.2 from *T. versicolor* 52J reduced a similar wide range of substrates (Tables 3.4, 3.5, 3.6 and Chapter 5). Kinetic parameters for the reduction of various quinone showed that functional group substitution at positions adjacent to the ring oxygens decreased the V_{max} of CBQase catalysis significantly (Table 3.5); however the enzyme reduced most of the quinones tested. The larger *p*-quinone substrates (such as the acid dyes shown in Table 3.4, group III) were not reduced at a detectable rate in a 5-30 minute cuvette assay. In contrast, cation radicals with similar conjugated ring structures (e.g. ABTS•) were readily reduced by CBQase (Figure 3.9, Group II). A comparison of the turnover numbers (Table 3.4) for CBQase with various substrates with reference to their structures (Figure 3.9) shows that overall, CBQase was most effective at reducing Mn(III) complexes, followed by the free radicals forms of chlorpromazine and ABTS and finally reducing quinones. In *P. chrysosporium*, it was suggested that the heme-cofactored CBO (which is similar in its behavior with quinones to the *T. versicolor* CBQase 4.2) functions as a peroxide-generating enzyme (Ayers *et al.*, 1978). The *T. versicolor* CBQases reduce quinones much more rapidly than they form H_2O_2, (Table 3.6). Even in pure oxygen it was ineffective as a peroxide generator.

To better understand the reactions of CBQase with *ortho*quinones, molecular modeling was used to predict the reactivities of the ring oxygens as the functional groups

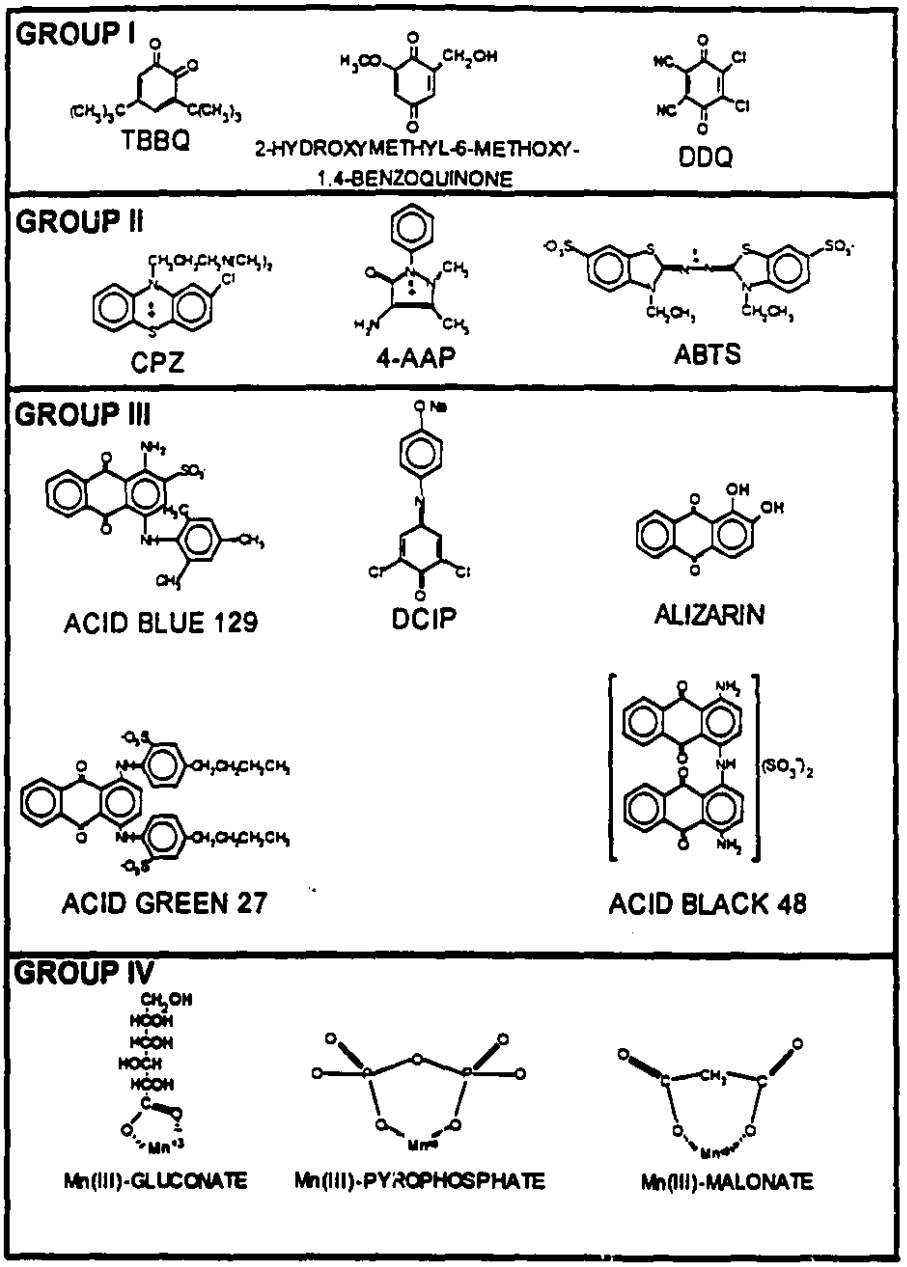


Figure 3.9. Structures of compounds which were used to evaluate the substrate specificity of CBQase. Structures are classified as simple single aromatic ring quinones (Group I), phenthiazoline radicals (Group II), multiple aromatic ring quinones (Group III), and metal-organic acid complexes (Group IV). The free radical forms of the Group II compounds are the substrates for CBQase. See Table 3.4 for the complete names of compounds identified using acronyms (e.g. CPZ).

Table 3.4. Reduction of a number of substrates by CBQase 4.2.

Substrate	Substrate conc.(mM)	Wavelength measured (nm)[1]	Extinction coefficient ($mM^{-1}cm^{-1}$)	Turnover number (s^{-1})
3,5-Di-*tert*-butyl-1,2-benzoquinone [TBBQ]	0.33	420	1.4	25
Dichlorophenolindophenol [DCIP]	0.20	520	2.5	37
4-Aminoantipyrine [4-AAP]	2.0	520	6.4	22
ABTS (ox)	1.0	420	36	54
Acid green 27	0.05-0.10[2]	631	-	0[3]
Acid blue 129	0.15-0.03[2]	629	-	0
Acid black 48	0.03-0.06[2]	666	-	0
2-Hydroxymethyl-6-dimethoxy-1,4-benzoquinone	0.33	430	1	8.1
2,3-Dichloro-5,6-dicyano-1,4 benzoquinone [DDQ]	0.33	440	1.1	0.2
Mn(III)-pyrophosphate	2.0	478	0.104	53
Mn(III)-malonate[4]	≈0.3	270	9.0	87
Mn(III)-gluconate[5]	0.4	265	8.4	80
Chlorpromazine [CPZ]	0.1	520	11.8	48
Alizarin	0.2	420	0.8	0.7

[1] The wavelength at which reduction was measured with the experimentally determined extinction coefficients ($mM^{-1}cm^{-1}$) used to calculate activity.
[2] Extinction coefficients for the acid green, blue, and black dyes were not determined since they were not of uniform composition, nor did they completely dissolve in ethanol. However, estimates of their concentrations in the assay were based on dye contents of 40% (acid black 48), 25% (acid blue 129) and 65% (acid green 27). Sufficient dye was added to give a final concentration of 150 μM of each dye in the assay. Assuming that 50-100% of each of the dyes dissolved, the concentration of the dye in the assays would have been in the range shown.

[3] A zero denotes no activity detected during a standard cuvette assay.
[4] Extinction coefficient from Bodini *et al.* (1976).
[5] Extinction coefficient from Wariishi *et al.* (1992). Some difficulty was encountered in reproducing this value in this laboratory probably due to the formation of mono, di, and tri-malonate-Mn(III) complexes (F. Archibald, pers. commun.).

Table 3.5. Determination of kinetic parameters for a number of CBQase substrates. Determinations were with CBQase 4.2 only.

Substrate[1]	Wavelength monitored (nm)	Extinction coefficient ($mM^{-1}cm^{-1}$)[2]	Apparent K_m (μM)	V_{max} ($\mu mol\ min^{-1}$)	Turnover number (s^{-1})[3]
3,5-Di-*tert*-butyl-1,2-benzoquinone(TBBQ)[4]	418	1.36	26	25	25
3,4-Dimethyl-1,2-benzoquinone	416	0.42	20	26.2	20
3-Methoxy-5-methyl-1,2-benzoquinone	460	0.50	16980	2540	111
4-Methyl-1,2-benzoquinone	380	0.57	nr[5]	nr	0
4-*tert*-Octyl-1,2-benzoquinone	400	1.10	80.7	6.5	13
4-*tert*-Butyl-1,2-benzoquinone	400	0.35	2370	66.9	41
3,4,5-Trimethyl-1,2-benzoquinone	430	0.16	nr	nr	0
Dichlorophenolindophenol (DCIP)	520	2.50	11	36	37
2,3-Dimethoxy-1,4-benzoquinone	410	0.73	1103	9.1	4.1

[1] The structures of all of these quinones can be found in Appendix C.

[2] Extinction coefficients were determined in the assay buffer system (50 mM sodium acetate, pH 4.5 containing 20% v/v ethanol).

[3] Enzyme turnover number at a substrate concentration equal to the K_m for that substrate.

[4] In comparison, a rate of 0.0019 μmoles of O_2 reduced per min was measured at a concentration of 210 μM O_2 (air saturation).

[5] nr= no reaction.

Table 3.6. Direct oxygen reduction by CBQase 4.2 and CBQase 6.4.

Enzyme	Oxygen consumption (nmoles min^{-1})[1]	
Dissolved O_2[2]	1200 μM	240 μM
CBQase 6.4	nd[3]	70.4
CBQase 4.2	0.08	0.07

[1] Oxygen consumption per unit of added CBQase where one unit CBQase = 1 μmole of TBBQ reduced per min. Reaction mixture contained 3 U of CBQase and 2 mM cellobiose in 20 mM acetate buffer (pH 4.5) incubated at 22°C.

[2] Oxygen concentration in the buffer calculated assuming an air pressure of 760 mm of Hg and moderate solute levels.

[3] nd= not determined.

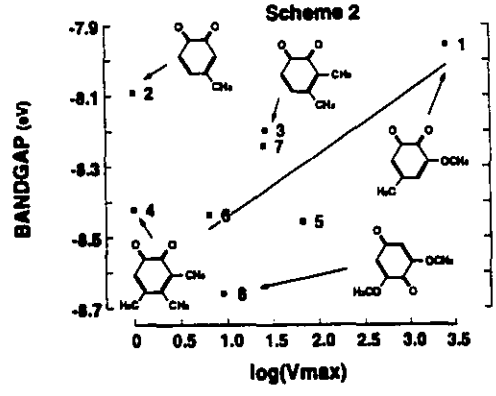
were changed. The effect of these substituents on the reactivity predicted from modeling each of the *ortho*-quinones and their observed reactivity with CBQase were compared. First, the minimum energy conformations of each of these quinones was determined. Then using this conformation, the lowest unoccupied molecular orbital (LUMO) and highest occupied molecular orbital (HOMO) for each of the quinone substrates was calculated. This information was used to determine the following properties of each of the *ortho* quinones: (1) the band gap of the ring oxygens; (2) an electron density map of each quinone; and (3) an estimation of the electrophilicity of each ring oxygen (Scheme 1).

Scheme 1

Modeling of synthesis of *ortho*quinones and estimation of band gap

1. Begin with simplest precursor (e.g. O-benzoquinone).
2. Minimize total energy with molecular mechanics (MM+) using conjugate-gradient method (Fletcher-Reeves).
3. Refine geometry with AM1 (conjugate-gradient method) to obtain lowest energy conformer.
4. Add substituent.
5. Repeat steps 2-3.
6. Repeat steps 4-5 until molecule is completed.
7. Repeat for stereocenters (if present).

Scheme 2



No large differences in the electrophilicity and electron density maps of these *ortho* quinones was predicted by these modeling studies. However a comparison of the experimentally determined reactivities of these substrates with CBQase (measured as the V_{max} values) with the ring oxygen band gap values (calculated from the predicted LUMO and HOMO values) were somewhat correlated (Scheme 2). The correlation between band gap and reactivity was improved when the data for the *para*-quinone and the two *ortho*-quinones which did not react with CBQase were excluded from the data set (Scheme 2). There is a trend that suggests that the reactivity of these quinones with CBQase is dependent on the band gap of the ring oxygens. The large differences between the *para* and *ortho* quinones can be explained by the large differences in their molecular geometry. The unreactive *ortho* quinones may have had geometries which restricted the access of these substrates to the active site of CBQase. Molecular modeling and kinetic studies with model quinones may prove to be a useful tool for predicting the range of substrates that can be reduced by CBQase.

3.3.5. Pulp binding of CBQase

Both CBQase 4.2 and 6.4 bound readily to cellulosic substrates, most importantly, hardwood and softwood kraft pulps (Table 3.7). This binding was reduced by the presence of 0.1 molar NaCl (Table 3.7). Thus it is likely that a significant fraction of the CBQase activity in *T. versicolor* cultures becomes associated with pulp fibers, and is not present in the culture supernatant, i.e.measured supernatant CBQase levels in biobleaching cultures may (or may not) greatly underestimate the amount of CBQase actually secreted and functioning.

3.3.6. Degradation of CBQase 4.2

Recently, Habu *et al.* (1993) have demonstrated that *P. chrysosporium* produces extracellular proteases which can cleave *P. chrysosporium* CBO into separate heme- and flavin- containing domains. The heme domain of purified CBQase 4.2 from *T. versicolor* 52J was separated from the enzyme by binding the protein to a Mono Q column using a pH 7.5 potassium phosphate buffer (20 mM). The proteins were then eluted with a linear gradient of the same buffer (0-0.5 M) which resulted in the elution of two protein peaks (Figure 3.10). Approximately one tenth of the total TBBQ-reducing activity of the native enzyme was recovered in the peak which eluted first from the ion exchange column. When the absorbance profile of each peak was recorded, the later eluting peak had a visible absorbance profile very similar to that of the heme peak of the native enzyme (Figure 3.11) with a much smaller absorbance at 280 nm (418 nm/280 nm ratio of about 6.5 compared to 1.7-1.9 for the intact enzyme). The rest of the enzymatic activity was not recovered in the eluate. The earlier-eluting non-heme protein peak had a specific

Table 3.7. Binding of CBQase 4.2 and 6.4 to cellulose, hardwood kraft pulp, and Softwood kraft pulp.[1]

Substrate Tested	CBQase 6.4[2]		CBQase 4.2	
	Buffer[3]	Buffer + NaCl[4]	Buffer	Buffer + NaCl
Cellulose	0.005	0.021	0.000	0.036
HWKP	0.010	0.018	0.002	0.027
SWKP	0.008	0.035	0.004	0.022

[1] Fibre substrates (0.05 g) were prepared at 10% consistency in 10 mM sodium acetate buffer (pH 4.5), placed in a Pasteur pipet, and 5 ml of buffer used to equilibrate the small column. Numbers give the total enzyme eluted after application of a total of 0.05 U CBQase in each trial. Numbers shown in the table are for single determinations of a representative experiment. Results varied considerably between trials. Cellulose was a highly purified preparation (Solka Floc). The kraft pulps were standard mill preparations, washed well with water before use.

[2] CBQase (0.05 U) was added to each Pasteur pipet (in total volume of 100 µl) and the total amount of enzyme recovered in 2 ml of eluent was determined.

[3] 10 mM sodium acetate (pH 4.5).

[4] 10 mM sodium acetate (pH 4.5) + 100 mM NaCl.

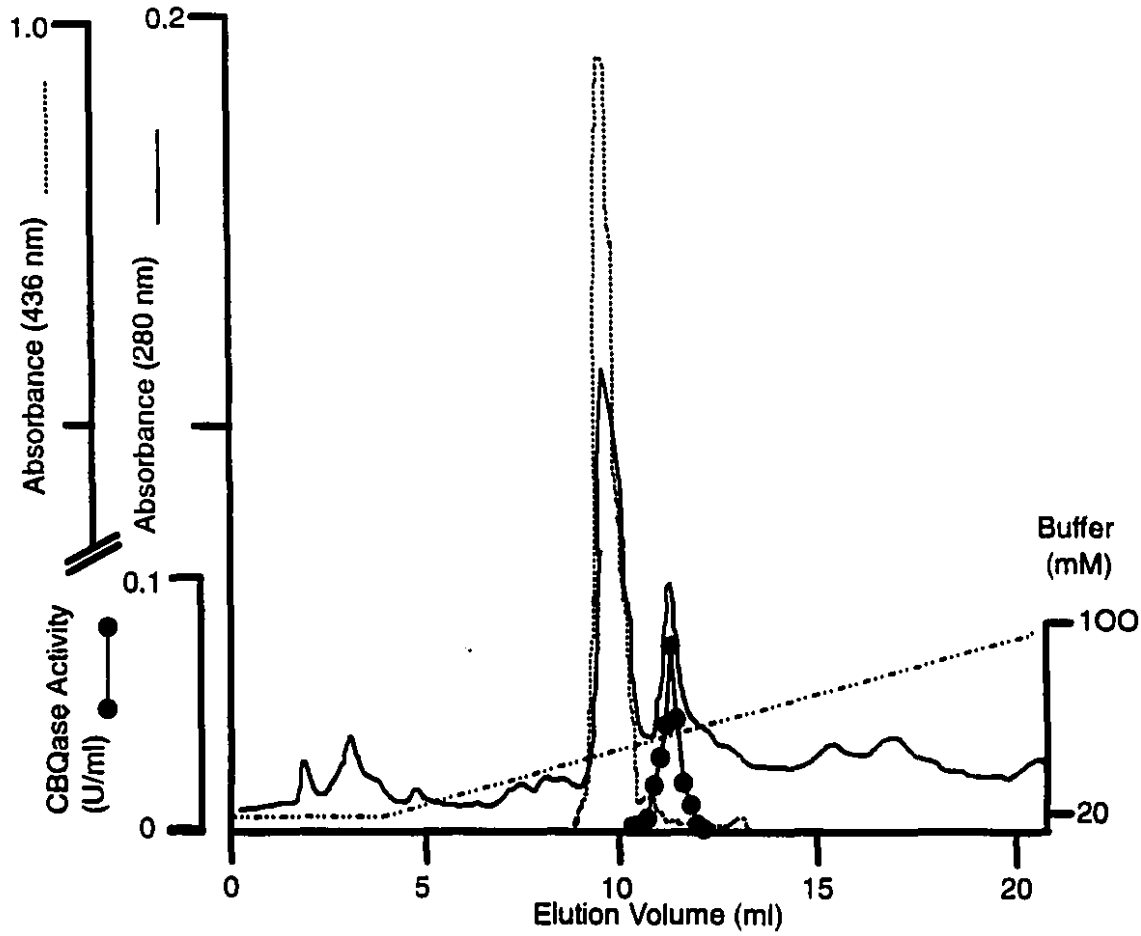


Figure 3.10. Elution profile of CBQase 4.2 from a Mono-Q column. Purified CBQase 4.2 (42 nmoles; 60 U) was loaded on a Mono-Q column equilibrated with phosphate buffer (20 mM; pH 7.5) and eluted with a gradient of the same buffer (20-100 mM). The elution profile measured at 280 nm (——) and at 430 nm (------) are shown. The CBQase activity (●) in fractions eluted from the column are shown.

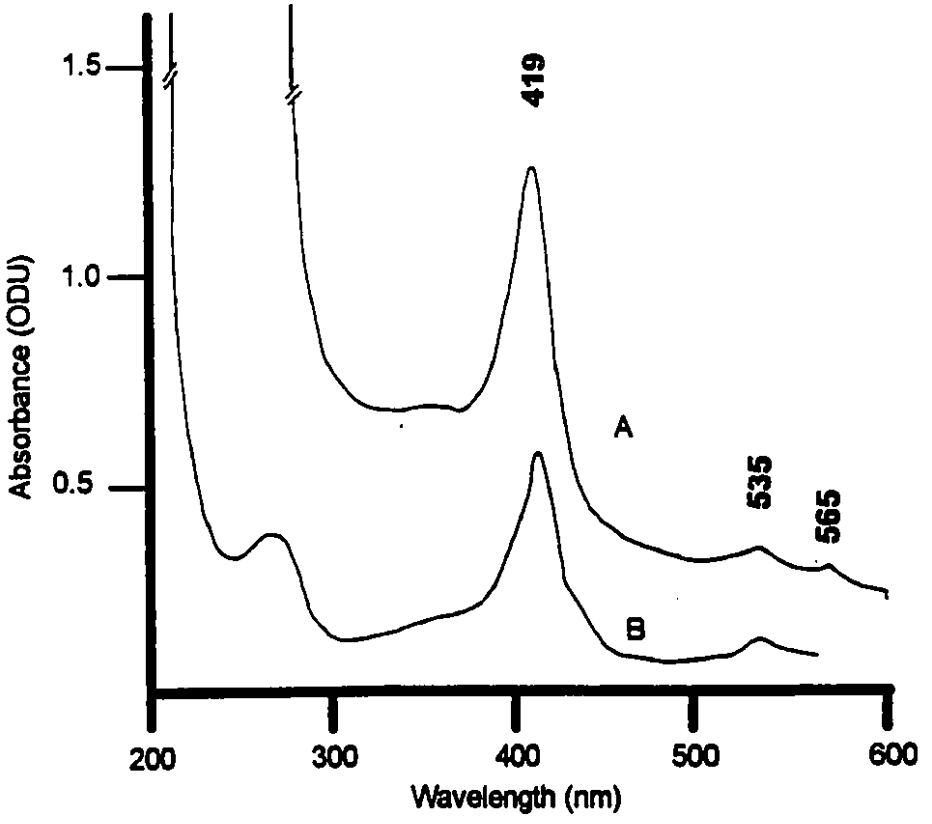


Figure 3.11. UV-visible spectrum of the heme-containing peak eluted from a Mono-Q column equilibrated with phosphate buffer at pH 7.5. The spectral profile of CBQase 4.2 before it was run on the Mono Q column (trace A) and the spectra of the eluted material in the presence and the absence of 2 mM cellobiose (trace B).

activity of 4.2 U mg^{-1} protein. This was very similar to the specific activity of CBQase 6.4 recovered from the *T. versicolor* culture supernatant. Recombining the two eluted protein peaks, both with and without dialysis, did not restore or increase the specific activity of the dissociated CBQase 4.2 protein.

3.4. DISCUSSION

Archibald (1992) has demonstrated that all of the essential components of the *T. versicolor* biobleaching system are present in the extracellular supernatant of actively bleaching cultures. Though oxidative enzymes such as manganese peroxidase and laccase do carry out a limited delignification of hard- and softwood kraft pulps, their effect is not as extensive as that observed when whole fungal cultures are used (Paice *et al.*, 1993; Bourbonnais and Paice, 1992). One plausible hypothesis was developed which supposes that a catalytic oxidation and reduction cycling of lignin substrates is established by ligninolytic cultures of *T. versicolor* which favors a net depolymerization of lignin. Similar catalytic schemes have been proposed by a number of investigators (Green, 1977; Pascynski *et al.*,1985) for the *in vitro* depolymerization of lignosulfonates by peroxidases and glucose oxidase.

CBQase is the only extracellular enzyme that can reduce aromatic substrates and free radicals which has been detected in *T. versicolor* biobleaching cultures. *T. versicolor* 52J induced and secreted CBQase isozymes into the culture medium under nitrogen sufficient conditions when either cellulose or cellobiose were the carbon source, conditions similar to those under which biobleaching is observed (Paice *et al.*, 1989; Reid *et al.*,

1990; Addleman and Archibald, 1993; Roy and Archibald, 1993). The total cellobiose-dependent reductive activity was accounted for by two different proteins. The production of one of these, CBQase 6.4, was not detected in the absence of CBQase 4.2, though the converse was frequently seen. Substantial quantities of CBQase 4.2 were produced in the presence of cellulose. In *P. chrysosporium*, CBQase and not CBO was the major cellobiose-dependent reductive activity detected, especially during the later stages of growth (Habu *et al.*, 1993).

The reductive activities of these two enzymes are very similar to the equivalent enzymes isolated from *Phanerochaete chrysosporium* (Westermark and Eriksson, 1974b; Ayers *et al.*, 1978; Morpeth, 1985; Morpeth and Jones, 1986; Bao *et al.*, 1993; Samejima and Eriksson, 1992). Notable differences include the lower molecular weight (58 kDa versus 90 kDa and 48 KDa versus 60 kDa) of the *T. versicolor* hemeflavo- and flavin enzymes, the inability of the heme enzyme (CBQase 4.2) to produce hydrogen peroxide, and the tendency of the *T. versicolor* CBQase 4.2 enzyme to be present as a dimer in its native state. The latter property is similar to the CBQases isolated from cellulolytic cultures of *S. thermophile* (Canevascini, 1991).

Purified preparations of the two quinone-reducing enzymes (Table 3.2) yielded two proteins with a ten-fold difference in specific activities when TBBQ was used as the quinone substrate. The lower specific activity and the variable yield of CBQase 6.4 produced by *T. versicolor* 52J compared to CBQase 4.2 may be because the former enzyme, like the *P. chrysosporium* CBQase (Habu *et al.*, 1993), may be formed from CBQase 4.2 by proteolysis and heme loss. In *T. versicolor*, possible reasons for the

observed fragmentation (Figures 3.10, 3.11) of the purified CBQase 4.2 may include: (1) the heme domain of the protein is strongly associated with, but not covalently linked to the rest of the protein; (2) the protein has an autoproteolytic activity; or (3) CBQase 4.2 was partially degraded prior to fractionation of the extracellular proteins. The latter is not likely, since during other steps in the purification procedure the reductive activity was associated with the heme absorbance. Furthermore, no sharp decreases in specific activity, as were observed when the heme fragment was separated from the rest of the protein, were seen during the course of the purification of the 4.2 enzyme (Table 3.2). In *P. chrysosporium*, the heme and flavin domains are clearly joined via a protease-sensitive region since not only the proteases from this fungus (Habu *et al.*, 1993), but also staphylococcal V8 protease (Wood and Wood, 1992) and papain (Henriksson *et al.*, 1991) cleave the protein to yield heme- and flavin-containing fragments. Unlike the CBQase isolated from *P. chrysosporium* which has a specific activity similar to CBO, the CBQase 6.4 isolated from *T. versicolor* 52J has a much lower specific activity (as does the CBQase 4.2 flavin-containing fragment) then CBQase 4.2.

The heme containing enzyme in *P. chrysosporium* was thought to serve primarily as a generator of H_2O_2 (Ayers *et al.*, 1978), however our results (Table 3.6) for the *T. versicolor* enzyme and those of others for *P. chrysosporium* (Wilson *et al.*, 1990) are not consistent with this hypothesis. Both CBQase 4.2 and 6.4 have a preference for quinones, organic radicals, and Mn complexes and have very low activity with O_2 (Tables 3.4,3.5). Though the list of quinones reduced by *T. versicolor* CBQase is extensive, many *p*-quinones are only very slowly reduced, unlike most *o*-quinones, cation radicals, and Mn(III)-complexes which were readily attacked (Tables 3.4,3.5; Figure 3.9).

Preface to Chapter 4.

One of the difficulties encountered in working with reductive enzymes in *T. versicolor* is that, in addition to CBQase, this fungus constitutively produces large quantities of laccase. As described in Chapters 2 and 3, this necessitated the use of anaerobic conditions in order to obtain a reasonable estimate of the CBQase activity in a fungal culture. Without strictly anaerobic conditions, the determination of CBQase activities in *T. versicolor* culture supernatants was inaccurate, and the more oxidase and peroxidase activity that were present in the supernatant, the less accurate the CBQase assay became. Also the strict exclusion of oxygen needed to prevent oxidase and peroxidase activity favours the non-enzymatic reduction of CBQase substrates. To overcome these difficulties, a novel assay for CBQase and other enzymes having similar radical reducing activity was developed and a discription of the salient features of the assay are given in Chapter 4.

CHAPTER 4. AN INDIRECT FREE RADICAL BASED ASSAY FOR CELLOBIOSE:QUINONE OXIDOREDUCTASE

4.1. INTRODUCTION

Cellobiose-quinone oxidoreductases (CBQase) are secreted by the ligninolytic "white-rot" basidiomycetes (Westermark and Eriksson, 1974; Ander and Eriksson, 1977) and other fungi (Dekker, 1980; Sadana and Patil, 1985; Canevascini *et al.*, 1991) and have been implicated in both cellulose (Eriksson *et al.*, 1974; Kremer and Wood, 1992; Bao and Renganathan, 1992) and lignin (Ander *et al.*, 1990; Samejima and Eriksson, 1992) degradation. CBQase was first reported in *Polyporus* (*Trametes*) *versicolor* (Westermark and Eriksson, 1974), but the initial isolations and purifications of flavin-cofactored (Westermark and Eriksson, 1975) and flavin-heme cofactored (Ayers *et al.*, 1978) quinone-reducing enzymes were of the *Phanerochaete chrysosporium* proteins. The flavin-heme enzyme was initially described as being a cellobiose oxidase based on its oxygen reducing (hydrogen peroxide evolving) activity (Ayers *et al.*, 1978). Recently the names cellobiose:cytochrome c oxidoreductase (Samejima and Eriksson, 1992), cellodextrin:Fe(III) oxidoreductase (Kremer and Wood, 1992) and cellobiose dehydrogenase (Bao *et al.*, 1992) have all been suggested as more appropriate in light of the enzyme's low rate of oxygen reduction relative to that of other substrates. The CBQase proteins isolated from most species are preferentially reduced by the oxidation of cellobiose and a limited number of other disaccharides (e.g. Coudray *et al.*, 1982) and in turn can reduce quinones (Westermark and Eriksson, 1974), certain organic free-radical

species (Samejima and Eriksson, 1991), cation radicals (Ander *et al.*, 1990) and Fe^{+3} (Coudray *et al.*, 1982; Kremer and Wood, 1992).

The most common method to identify CBQases has been to monitor the disappearance of a quinone substrate spectrophotometrically (eg. Westermark and Eriksson, 1974; Coudray *et al.*, 1982; Canevascini, 1988). However many have low molar extinction coefficients, are only sparingly soluble in water and only a few, such as 3,5-di-*tert*-butylbenzoquinone(1,2)(TBBQ) and dichloroindophenol (DCIP) are commercially available. In addition, assays employing them are prone to two common and important types of interference: (1) the reduced product formed by CBQase can be (and usually is) re-oxidized by one or more of the ubiquitous secreted fungal laccases or peroxidases and; (2) present CBQase assay substrates can also be reduced by a variety of small molecules such as sulfite, ascorbate, glutathione, cysteine, etc. Work in this laboratory on the lignin-degrading fungus *Trametes versicolor* has indicated that the existing CBQase assays are inadequate, especially for measuring these secreted enzymes in fresh culture supernatant.

Though DCIP has a high extinction coefficient and is very soluble in water, and has been used as a CBQase assay substrate (e.g. Bao and Renganathan, 1992), it has a phenolic subunit which makes it susceptible to laccase oxidation which strongly interferes with the quantitation of reductive activity (Figure 3.9). The oxidation of the carbohydrate substrate of CBQase can also be followed by measuring the appearance of the corresponding sugar acids (Ayers *et al.*, 1978), but the method is laborious and other sugar-oxidizing enzymes may interfere.

The behavior of both heme and non-heme containing CBQase proteins suggests that they can directly reduce the phenoxy and other organic radicals which are formed via the one-electron oxidation reactions catalyzed by fungal laccases (Ander *et al.*, 1990; Samejima and Eriksson, 1991; Roy, Misra and Archibald, unpublished). Since phenolic-like substrate oxidizing activities cannot be eliminated from crude samples, can this oxidative activity be incorporated into an assay where it creates the CBQase substrate? To this end, a new CBQase assay was developed containing only a reduced laccase substrate (no CBQase substrate) and in which the sample's oxidizing activity is swamped by sufficient purified laccase in the assay reaction mixture to obtain a standard reduced substrate oxidation rate. CBQase concentrations are then determined as the amount of sample needed to reduce the net measured rate of substrate oxidation by 50%. The report describes the development, advantages and limitations of this assay.

4.2. MATERIALS AND METHODS

4.2.1. Enzymes and chemicals

Pyricularia oryzae laccase (EC 1.10.3.2), and *Aspergillus niger* glucose oxidase (GO) type V-S (EC 1.1.3.4) were from Sigma Chemical Co. and used without further purification. *Trametes versicolor* laccase was partially purified as previously described (Bourbonnais and Paice, 1992). The enzyme preparation was free of CBQase and manganese peroxidase activity. *T. versicolor* flavin and heme-flavin CBQases were purified by column chromatography (Q-Sepharose with 20 mM Bis-Tris (pH 6.4) eluted with a linear gradient of NaCl; Sephacryl S-300 gel filtration, 20 mM phosphate buffer

[pH 7.0]; and a final step in which the enzymes were purified to homogeneity by FPLC with a Mono-Q 5/5 column (20 mM Bis-Tris [pH 6.4] eluted with a linear NaCl gradient). *Sporotrichum thermophile* CBQase (containing both heme and flavin cofactors) was produced as previously described (Canevascini, 1988) and partially purified by column chromatography (DEAE-Sephacryl with 20 mM citrate [pH 6.0]; followed by gel filtration with Sephacryl S-300 eluted with 20 mM phosphate buffer [pH 7.0]).

Chlorpromazine (CPZ), cellobiose (cb), D-glucose, ascorbate, glutathione, and cysteine were purchased from Sigma Chemical Co. Dithionite, dichlorophenolindophenol (DCIP), 3,5-di-*tert*-butyl-1,2-benzoquinone (TBBQ), and 4-aminoantipyrine were from Aldrich Chemical Co. All chemicals were of reagent grade. Oxygen consumption was measured using a polarographic oxygen cell.

4.2.2. Assays

Laccase activity was determined by the oxidation of 2,2'-azino-di-(3-ethylbenzthiazoline)-6'-sulfonate (ABTS) measured as the increase in absorbance at 420 nm in 100 mM Na-acetate buffer, pH 4.5 containing 0.5 mM ABTS and one unit (U) of activity corresponds to 1 μmole of ABTS oxidized per minute. GO activity was measured using a horseradish peroxidase-coupled assay (Worthington, 1972) containing 6 U horseradish peroxidase, 0.3 M glucose, and 2 mM *o*-dianisidine all in 100 mM phosphate buffer (pH 7.0) and activity was measured at 540 nm using *o*-dianisidine oxidation. One unit of activity corresponds to 1 μmole of *o*-dianisidine oxidized per minute.

CBQase activity was determined using the classical quinone assay by following the decrease in absorbance at 420 nm accompanying the reduction of 3,5-di-*tert*-butyl-1,2-benzoquinone (TBBQ) in 100 mM Na-acetate buffer, pH 4.5 containing 0.67 mM TBBQ and 2.0 mM cellobiose. One international unit (IU) of CBQase activity corresponds to 1 μmole of TBBQ reduced per minute per ml (Westermark and Eriksson, 1974). All enzymatic assays were in 100 mM sodium acetate buffer (pH 5.0).

The CPZ CBQase standard assay as developed here contained (final concentrations) 100 mM sodium acetate buffer (pH 5.0), 3 mM chlorpromazine (CPZ), 2 mM cellobiose, 0.03 U of purified laccase and sample, all in a volume of 1.5 ml. The chlorpromazine and laccase were made as 100x stock solutions and 15 μl of each was added to the 1.5 ml assay mixture. Cellobiose was prepared as a 100 mM stock solution and 30 μl was added to the assay. Assay components were added to the cuvette sequentially: first the buffer, followed in order by CPZ, sample (dialyzed), laccase, and cellobiose. Before the cellobiose addition, the oxidation of CPZ to the colored radical cation (CPZ•) was followed at 530 nm and 25°C . This reaction was allowed to proceed for 30-60s until an absorbance of ≈0.1-0.2 ODU (≈8 to 16 μM CPZ•) and a steady CPZ oxidation rate of 0.05-0.1 ODU_{530} per min were obtained. Then 30 μl of cellobiose was added and the net decrease in the rate of the formation of CPZ• radical was followed. Sample additions were always made in a total volume of 0.5 ml, so when CBQase concentrations were high, the sample was first diluted appropriately with water. In those samples which contained substantial levels of endogenous laccase, the amount of purified laccase added was decreased to give a total of 0.03 U. The assay could be run with larger

amounts of laccase, however assay sensitivity was reduced. The assay showed a linear response to CBQase concentration when the amount of enzyme added inhibited (after addition of cellobiose) CPZ• formation by 25-75%. The chart recorder was set to 0.5 absorbance units full scale and a chart speed of 10 mm per min. To follow extended or rapid reactions, the recorder offset was increased as the reaction proceeded or the span was increased to 1.0 OD unit. The CBQase activity in the assay cuvette was calculated using Equation 1:

$$Unit(U)ofCBQase=([\frac{\Delta ODU_{530}\min^{-1}(-cellobiose)}{\Delta ODU_{530}\min^{-1}(+cellobiose)}]-1)(0.67)(df)\,(1)$$

Where df represents the sample dilution factor, and 0.67 adjusts activity to a per milliliter basis. One unit of CBQase as defined in (1) corresponds to 0.04 IU of CBQase when TBBQ is used as the substrate in the classical assay.

Fungal culture supernatants used to determine interferences in the CPZ CBQase assay were from actively biobleaching cultures of *T. versicolor* (Addleman and Archibald. 1993). Cultures were maintained as frozen stocks (-80°C) and cultured on mycological broth plates (Paice *et al.*, 1993). The softwood kraft pulp (SWKP) biobleaching supernatant was from a 7 day culture of *T. versicolor* 52J incubated in a softwood defined medium (SDM) containing 2% (w/v) SWKP as described elsewhere (Addleman and Archibald, 1993). The hardwood kraft pulp (HWKP) biobleaching supernatant were from four day old cultures of *T. versicolor* 52J grown in *Trametes* defined medium (TDM) (Roy and Archibald, 1993), or mycological broth (MB) containing 2% (w/v) HWKP

(Addleman and Archibald, 1993). Where required, supernatants were dialysed overnight at 4°C (12-14 kD cutoff) against two changes of a 20-fold greater volume of distilled water. Heat treatment of culture supernatant involved immersing samples in a boiling water bath for 15 min, then cooling rapidly to room temperature in an ice bath.

4.3 RESULTS AND DISCUSSION

4.3.1. Effect of laccase on direct CBQase assay

The results reported here are for experiments using the heme-flavin CBQase isolated from *T. versicolor*, although similar results were obtained using the flavin cofactored enzyme. The detection of CBQase in fungal cultures using a quinone reduction assay is difficult when there are significant quantities of laccase or peroxidase present in the sample to be tested (Table 4.1). The activity of a preparation of CBQase approximately 10-fold more concentrated than that found in a typical *T. versicolor* culture (0.25 U/ml versus 0.025 U/ml) was 80% masked by laccase at a level (0.15 $U \cdot ml^{-1}$) typically detected in *T. versicolor* cultures (Addleman and Archibald, 1993; Paice *et al.*, 1993; Roy and Archibald, 1993). Though most of the added CBQase activity could usually be detected when the assay was performed under strictly anaerobic conditions (Table 4.1), the method was slow, results showed considerable variability, and hydrogen peroxide and peroxidases interfere (Paice *et al.*, 1993). Furthermore, the complete anaerobiosis necessary for quantitative measurements is very difficult to attain when flushing with nitrogen gas, as can be seen in Table 4.1 where the apparent CBQase level was lower in the presence of laccase than in its absence even after 3 min of flushing with

Table 4.1. Influence of laccase on the measurement of CBQase activity with TBBQ as substrate.

Enzyme(s)	Incubation	TBBQ reduction (μmole/min)[1]
CBQase[2]	Aerobic	2.26 ± 0.23
	Anaerobic[3]	2.20 ± 0.33
CBQase + Laccase[4]	Aerobic	0.35 ± 0.01
	Anaerobic	1.91 ± 0.05

[1] Values are the mean of duplicate determinations ± standard deviation.

[2] Purified *S. thermophile* CBQase (0.23 U) in 100 mM acetate buffer (pH 5.0) with 0.67 mM TBBQ and 2 mM cellobiose in a total vol. 1.0 ml.

[3]Anaerobic incubations were effected by flushing the cuvette containing all assay components (1 ml vol.) with nitrogen for 3 min at a flow rate of 1 $ml \cdot min^{-1}$.

[4] Purified *S. thermophile* CBQase (0.23 U) and laccase (0.15 U) in 100 mM acetate buffer (pH 5.0) with 0.67 mM TBBQ and 2 mM cellobiose in a total vol of 1.0 ml.

nitrogen. To circumvent interference from oxidative enzymes, an assay based on the ability of CBQase to decrease the net rate of organic radical intermediate formation by a standard amount of laccase was conceived and developed.

4.3.2. Indirect assay for CBQase

We sought a commercially available compound forming a stable cation radical when oxidized by laccase but not containing phenolic hydroxyl groups, as such phenolic compounds often undergo coupling reactions. These polymerization reactions will result in a CBQase-independent decrease in the substrate concentration. In developing the inhibition assay for CBQase, a number of substrates were screened. One, 4-aminoantipyrine (4-AAP), a known substrate for peroxidases (Bauminger, 1975), was readily oxidized by laccase to a stable coloured end product (λ max =524nm) that did not polymerize within 60 min and produced an intermediate, presumably a free radical, of laccase-mediated 4-AAP oxidation which was susceptible to reduction by CBQase (data not shown). However, 4-AAP was rejected when it was found that the carbonyl oxygen of the unoxidized substrate (Figure 3.9) was also readily reduced by CBQase. The carbonyl competed with the laccase-generated radical for reduction by CBQase and led to the formation of a second product with a strong absorbance maximum at 430 nm. Therefore a substrate without oxygen substituents on the aromatic ring(s) were sought. The phenothiazines are generally susceptible to laccase oxidation (Sariaslani *et al.*, 1984) and form stable cation radicals, some with visible light absorbtion maxima (Vázquez *et al.*, 1992). One of these, chlorpromazine (CPZ), has neither carbonyl nor hydroxyl ring

substituents (Figure 3.9), is oxidized to form a radical intermediate (CPZ•) with a high extinction coefficient ($\sum_{530}$ =11.9 $mM^{-1}cm^{-1}$) and is readily soluble in water (Vázquez *et al.*, 1992). In this work, *T. versicolor* laccase readily oxidized CPZ to form a cation radical (CPZ•) (Figs. 4.1,4.2,4.3 trace A). The CPZ• radical was most stable when it was generated using low levels of laccase. Low levels of horseradish peroxidase and acidic pH conditions at low buffer concentrations also reduced the enzymatic and non-enzymatic breakdown rates of CPZ• (Escribano *et al.*, 1985). When the CPZ concentration was varied (Table 4.2) a change in the rate of CPZ• formation was observed, with 3 mM substrate giving the maximum oxidation rate with the level of laccase employed. Since saturating substrate levels of CPZ were used, CPZ• formation was first order over a wide range of laccase concentrations (Table 4.3), zero order in CPZ, and constant over time (Figure 4.3, trace A).

The CPZ assay, as described herein, measures the decrease in the net rate of laccase-mediated CPZ oxidation that is observed in the presence of chemical reductants or CBQase-like reductive enzymes. The rate of laccase-mediated CPZ• formation, as measured at 530 nm, was unaffected by the presence of reducing sugars such as glucose (0.1-10 mM) or cellobiose (0.1-10 mM) (Figure 4.3, trace B). CPZ• generated by laccase was quantitatively reduced by low concentrations (1 mM) of reductants such as sodium dithionite, glutathione, ascorbate (reduced form), and to a lesser extent by cysteine (e.g. in Figure 4.3, trace C). However, low levels of chemical reductants will not be mistaken for enzymes as they will not affect the steady-state change in net rate that this assay measures. The laccase-mediated oxidation rate of CPZ, as shown by O_2

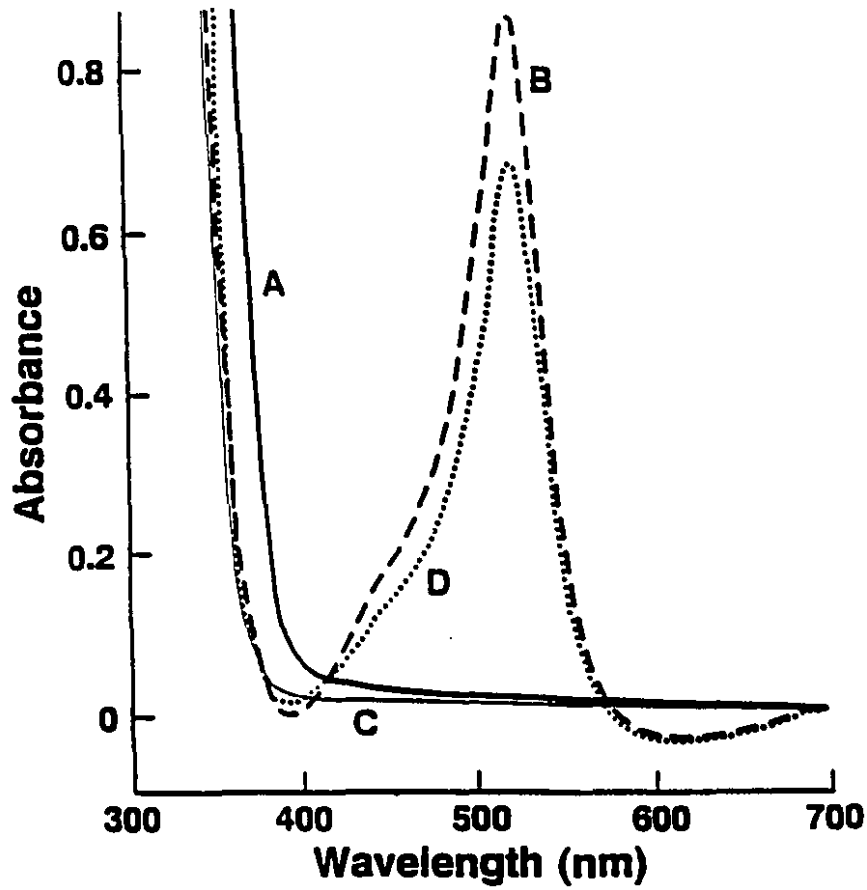


Figure 4.1. Chlorpromazine (CPZ) absorption spectrum before and after laccase oxidation of CPZ to CPZ• and CBQase reduction of CPZ• back to CPZ. Repeated spectrophotometric scans of the CPZ•-CBQase reaction mixture following: Line A; CPZ-containing assay mixture before oxidation by laccase, line B; assay mixture after oxidation with laccase, line C; assay mixture following complete reduction of the CPZ• radical by *T. verscolor* heme-flavin CBQase, and line D; the reaction mixture following re-oxidation of the assay mixture with laccase of the CBQase-reduced reaction mixture.

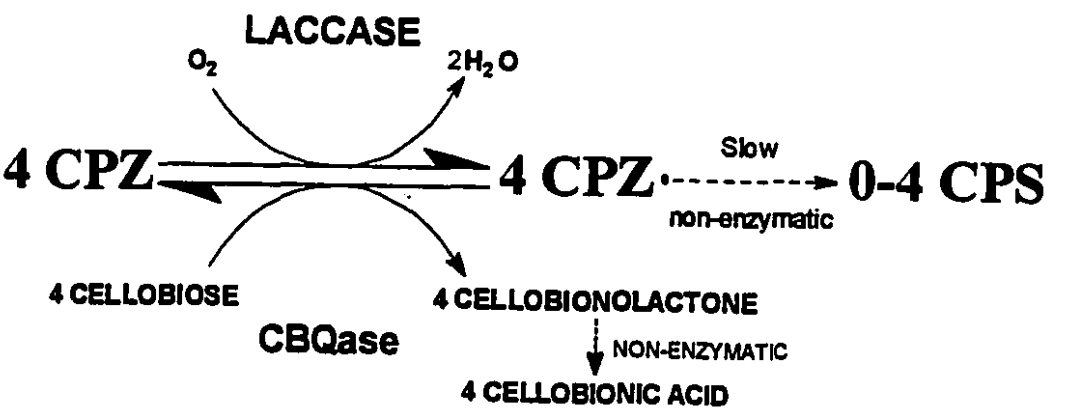


Figure 4.2 Proposed reactions of CPZ mediated by laccase and CBQase.

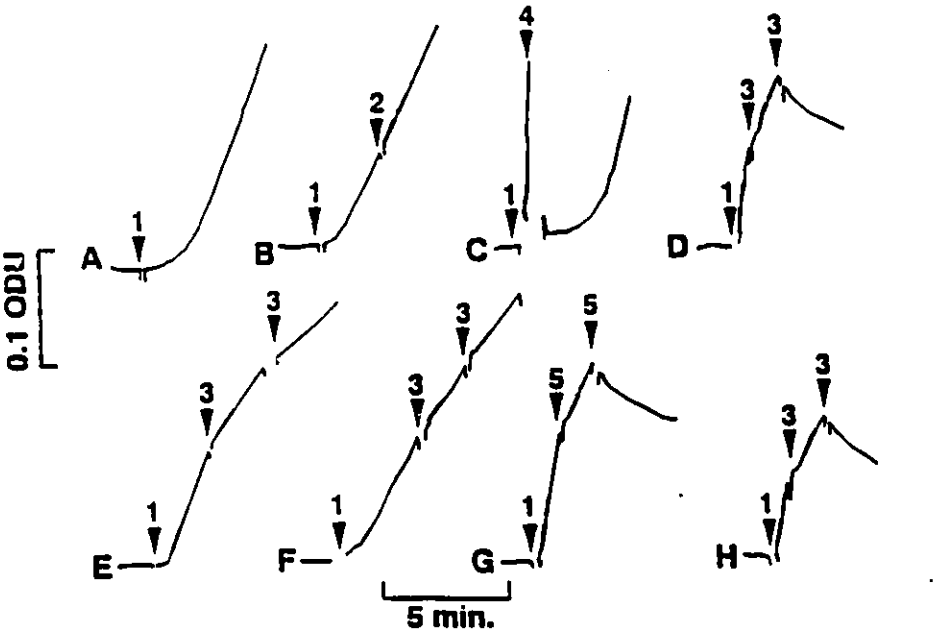


Figure 4.3 Spectrophotometer recorder tracings of CPZ• formation and reduction (530 nm) under a number of different conditions. Numbers on traces indicate additions of laccase (1), cellobiose (2), CBQase (3), glutathione (4), and GO (5). Trace A; After an initial lag, 0.03 U laccase (1) produces CPZ•(CBQase substrate) at a constant rate. Trace B; 2 mM cellobiose (2), used as the CBQase substrate, has no effect on laccase-mediated CPZ• production in the assay. Trace C: 0.1 mM reduced glutathione (4), stoichiometrically reduces CPZ•, but once all of the glutathione is oxidized (2 GSH→ GSSG), the laccase-driven production of CPZ• resumes. Traces D,E,F; *T. verscolor* heme-flavin CBQase catalytically reduces the net rate of CPZ• formation by reducing a fraction of it to CPZ. Each of the two additions contains 0.5 (D), 0.05 (E) and 0.005 (F) mU of CBQase (3). Larger amounts of CBQase reduce pre-existing CPZ• (e.g. traces D,H) . Trace G; additions of 0.005 and 0.05 U of *A. niger* GO (5), can readily reduce CPZ•, both nascent and pre-existing (note drop in absolute absorbance). Trace H; Substitution of *P. oryzae* laccase for the *T. versicolor* laccase.

Table 4.2. The effect of CPZ concentration on the CBQase-mediated inhibition of net laccase-mediated CPZ oxidation.

CPZ (mM)	Rate of CPZ formation ($\Delta ODU_{530} \cdot min^{-1}$)		Inhibition by CBQase (%)
	-CBQase	+CBQase[1]	
0.1	0.008 ± 0.002	0[2]	100[3]
1.0	0.021 ± 0.001	0.009 ± 0.000	57[4]
3.0	0.040 ± 0.005	0.030 ± 0.004	25
10.0	0.027 ± 0.005	0.020 ± 0.004	26

[1] Added 0.008 U (with TBBQ as substrate) *T. versicolor* heme-flavin CBQase per assay.

[2] The rate of CPZ• reduction was greater than the rate of laccase-mediated formation which resulted in a negative slope until all of the CPZ• radical was reduced. Assay conditions were as described in Materials and Methods.

[3] The rate of CPZ• radical reduction was greater than the rate of formation by laccase resulting in reduction of nascent CPZ•.

[4] Despite its apparently greater sensitivity, 1 mM CPZ was not adapted for use in the standard assay because under the standard assay conditions, laccase-mediated generation of CPZ• could deplete 1mM CPZ sufficiently for the reaction to slow and become non-linear with time.

Table 4.3. Effect of laccase concentration on detection of CBQase-mediated CPZ• reduction.

Laccase added ($U \cdot assay^{-1}$)	CPZ oxidation rate ($\Delta ODU \cdot min^{-1}$)		Inhibition by CBQase (%)
	-CBQase	+CBQase[1]	
0.000	0	-	-
0.007	0.009 ± 0.001	0.003 ± 0.001	68
0.013	0.025 ± 0.001	0.017 ± 0.001	34
0.025	0.047 ± 0.001	0.038 ± 0.001	19
0.050	0.118 ± 0.004	0.090 ± 0.006	23
0.075	0.250 ± 0.010	0.210 ± 0.010	16
0.147	0.500 ± 0.020	0.440 ± 0.020	12
0.250	0.899 ± 0.061	0.760 ± 0.040	16
1.000	3.720 ± 0.240	2.356 ± 0.084	37

[1]Added 0.008 U of *T. versicolor* heme-flavin CBQase (TBBQ as substrate) per assay. CPZ assay conditions were as described in Materials and Methods.

consumption, was not affected by CBQase in the presence of cellobiose, nor did CBQase reduce the laccase protein directly since no oxygen consumption occurred in the absence of a laccase substrate when CBQase and cellobiose were present (data not shown). However, the apparent rate of CPZ• formation (ΔOD_{530}) was decreased in the presence of CBQase (Figure 4.3, traces D,E,F). Any of several CBQase enzymes or GO from *A. niger* decreased the net CPZ• formation rates (Table 4.4). The reduction of CPZ• by 0.008 U of *T. versicolor* CBQase was nearly complete when a 100 μM concentration of CPZ was used (Figure 3.9). However, approximately 20% of the CPZ•-forming material was lost when CPZ was subjected to sequential laccase oxidation and reduction by CBQase followed by a second laccase oxidation. This was likely due to the non-enzymatic oxidation of the CPZ• free-radical to a more stable end product, chlorpromazine sulfoxide (CPS) (Figure 4.2) (Escribano *et al.*, 1985). Under the conditions used for the assay, the CPZ• formed by laccase oxidation was stable (absorbance at 530 nm decreased by < 2%) for at least one hour at room temperature, though it was rapidly transformed to a colourless product on heating (100°C, 5 min). The laccase-catalyzed conversion of CPZ to CPZ• was not complete, but the rate of CPZ• formation, as measured spectrophotometrically at 530 nm, was constant when the absorbance was between 0 and 2 ODU (0 -168 μM CPZ•).

This coupled assay provided an advantage, in that reductive activity could be detected and quantified even in the presence of CPZ-oxidizing enzymes, such as are found in the fungal medium under *T. versicolor*-mediated biobleaching conditions (Roy and Archibald, 1993). The CBQase-mediated reduction in the net rate of CPZ• formation by

Table 4.4. Detection of various CBQase and GO proteins using the CPZ assay.

Enzyme	Source	CPZ• formation inhibition (%)[1]
CBQase (heme-flavin)	*T. versicolor*	27 ± 3.3
CBQase (flavin)	*T. versicolor*	31 ± 4.2
CBQase I	*S. thermophile*	24 ± 2.2
Glucose oxidase (GO)	*A. niger*	40 ± 1.6

[1] Inhibition of CPZ• formation by 0.01 U of various CBQase proteins based on TBBQ-reduction assay and by the addition of 0.2 U of GO based on the horseradish peroxidase-dianisidine coupled assay. Results are the mean of triplicate assays ± standard deviations. Assay conditions were as described in Materials and Methods.

laccase was independent of CPZ• concentration over the range of 1.7 to 21 μM CPZ• (0.02 to 0.25 absorbance units). As expected, the fractional inhibition of the oxidation rate by a constant amount of CBQase decreased as the laccase concentration was increased (Table 4.3). However, at the highest concentrations of laccase tried, the apparent inhibition by CBQase was seen to increase. High concentrations of horseradish peroxidase have been found to decrease the stability of CPZ• radicals (Vázquez *et al.*, 1992) and our results suggest that the radical in the presence of laccase behaves in a similar manner. The difficulty of making CBQase additions before the CPZ• concentration exceeded 21 μM made measurement of CBQase activity more difficult at very high laccase concentrations, though this difficulty could be addressed by generating another standard curve for the higher laccase and consequently higher CPZ• radical concentrations. Similarly, at low laccase concentrations (<0.01 U/mL) the rate of CPZ oxidation by laccase rapidly became non-linear, the rate of CPZ• formation decreasing over time whether CBQase was present or not. Within these two extremes, the exact amount of laccase added to the assay did not need to be rigorously controlled and laccase levels between 0.02 and 0.1 U per 1.5 ml assay gave very similar values for CBQase activity. The CBQase-mediated decrease in the rate of laccase-mediated CPZ• formation was linearly correlated with CBQase activity between 0.4 and 12 mU (TBBQ) of enzyme added per ml of assay volume (r^2=0.99; n=8) (Figure 4.4). The CPZ CBQase assay run 10 times with 0.03 U laccase (ABTS as substrate) and 0.008 U CBQase (TBBQ as substrate) had a standard deviation of ±8.5%. The rate of CBQase-mediated reduction was independent of the cellobiose concentration in the assay in the range of 0.1 to 2 mM.

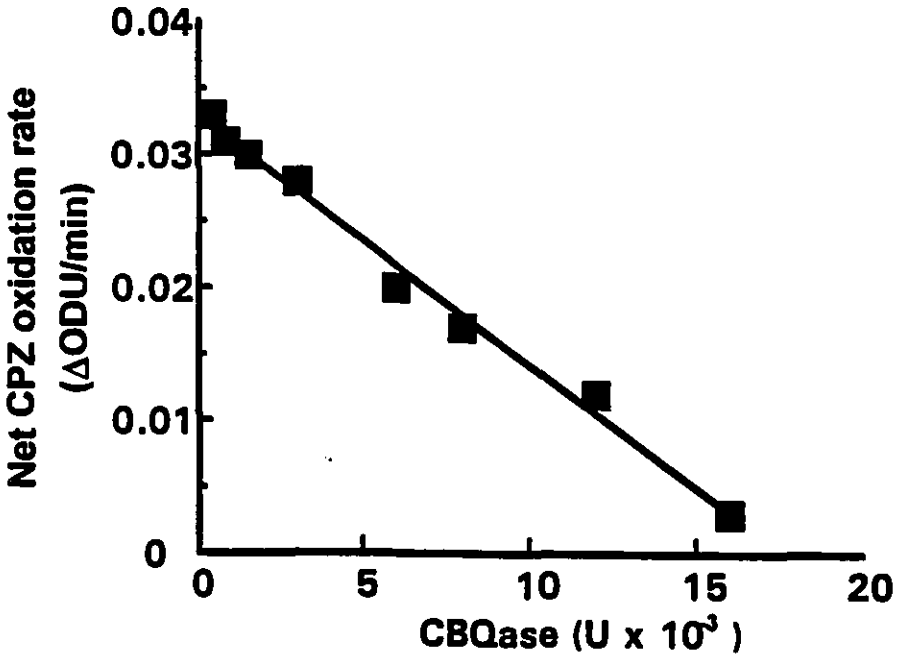


Figure 4.4 Rate of CPZ oxidation as a function of added *T. verscolor* heme-flavin CBQase. Values shown are the means of triplicate determinations. Standard errors are contained within the dimensions of the symbols.

The lowest amount of enzyme which could be reliably detected using the TBBQ assay was about 5 mU per assay, indicating that the CPZ-laccase inhibition assay was about 10-fold more sensitive than the TBBQ-based assay, due to the higher extinction coefficient of CPZ· compared to TBBQ.

4.3.3. Interferences in the assay

An important objective was to determine whether substances interfering with the CPZ CBQase assay were present in the supernatant of *T. versicolor* cultures. Laccase preparations used in the assay should be tested for CBQase activity by measuring the CPZ oxidation rate of the enzyme preparation with and without added cellobiose (or other appropriate substrate). A comparison of several culture supernatants indicated that the reductive activity of CBQase was inhibited by some, but not all, of the complete culture supernatants (Table 4.5). These culture supernatants also inhibited quinone reducing activity by ≈15% when CBQase activity was determined using the classical CBQase assay (data not shown). The inhibitory effect was largely temperature sensitive and non-dialysable (Table 4.5). However, it is suggested that samples to be tested for CBQase activity using this assay should be dialysed before testing. Dialysis times as short as one hour were effective in removing most interfering low molecular weight species (data not shown).

4.3.4. CPZ assay and GO

Since GO has been shown to effectively reduce certain radical intermediates in a manner comparable to CBQase (Szklarz and Leonowicz, 1986), will this assay also

Table 4.5. Effect of kraft pulp biobleaching culture supernatant, before and after heating or dialysis on the proportion of the true CBQase activity detected using the CPZ CBQase assay. All assays are means of two determinations. Standard deviations were less than 10%.

Supernatant pretreatment	CBQase added ($Ux10^{-3}$)[1]	CPZ• formed ($\Delta ODU \cdot min^{-1}$)[2]			
		Control[3]	HWKP (MB)[4]	HWKP (DM)[4]	SWKP (DM)[4]
None	0	0.135	0.130	0.135	0.135
	5	0.075	0.078	0.075	0.090
	10	0.035	0.052	0.035	0.060
	15	0.0	0.017	0.0	0.028
Boiled	0	-	0.133	0.125	0.130
	5	-	0.073	0.075	0.065
	10	-	0.040	0.037	0.015
	15	-	0.0	0.0	nd[5]
Dialysed	0	-	0.130	0.160	0.135
	5	-	0.087	0.104	0.084
	10	-	0.047	0.060	0.051
	15	-	0.018	0.035	0.027

[1] Aliquots of *T. versicolor* heme-flavin CBQase were added to give the number of IU shown per assay (TBBQ as substrate). When sufficient CBQase was added, the CPZ oxidation rates fell to zero, and any nascent CPZ• present was reduced.
[2] An aliquot (100 μl) of fresh biobleaching culture supernatant ± boiling or dialysis added to give the standard 1.5 ml assay volume. Standard assay conditions were used as described in Materials and Methods.
[3] Acetate buffer (pH 5.0; 100 mM) added in place of culture supernatant.
[4] HWKP = hardwood kraft pulp; TM = *Trametes* defined medium; MB = mycological broth at 15% of normal concentration.
[5] nd = not determined.

measure GO activity? It was found that *A. niger* GO does reduce CPZ• in the presence of glucose (Figure 4.3, trace G), and like CBQase, the reduction in CPZ oxidation rate is proportional to the amount of enzyme added in the range of 0.2 to 1.6 mU of GO (data not shown). Because *A. niger* GO is readily available commercially and CBQases are not, this provides a ready source of enzyme for positive assay controls. Furthermore, interferences from HWKP cultures which inhibit CBQase also inhibited GO in a similar fashion (Table 4.6), and thus GO also served as a useful positive control to screen supernatants for inhibitory effects. However, the supernatant from some SWKP cultures inhibited CBQase (Table 4.5) but stimulated GO-mediated CPZ• reduction (Table 4.6), indicating that lack of GO inhibition does **not** ensure lack of CBQase inhibition.

To make the assay dependent only on commercially available materials, the *T. versicolor* laccase was replaced with a *P. oryzae* laccase sold by the Sigma Chemical Co. Similar results were obtained, both in CPZ oxidation and subsequent reduction by CBQase (Figure 4.3, trace H), although the *P. oryzae* preparation had significantly lower specific activity.

4.4. CONCLUSIONS

1. The CPZ CBQase assay as described improves the detection limit for CBQase approximately 10-fold over the TBBQ assay due to the high extinction coefficient of the CPZ•.

2. This assay can be used to quantify CBQase activity aerobically in the presence of oxidative enzymes, substantial advantages over existing quinone-reduction based CBQase assays.

Table 4.6. Effect of *T. versicolor* biobleaching culture supernatant on GO-mediated CPZ• radical reduction.

Supernatant tested[1]	Inhibition by GO of CPZ• formation (%)
Control	43.8 ± 2.9
HWKP (DM)	50.8 ± 2.6
HWKP (MB)	9.7 ± 1.0
SWKP (DM)	71.4 ± 0.0

[1] A 500 µl aliquot of culture supernatant or buffer (Control) was added to the standard assay mixture (to give a total volume of 1.5 ml) containing 0.03 U of laccase and 2 mM glucose and the rate of CPZ• formation was measured. GO (0.2 U) was then added to the cuvette, and the new rate of CPZ• formation determined.

3. The assay does not distinguish among various forms of CBQase and GO, like the cytochrome c assay does (Samejima and Eriksson, 1992).

4. Since the CPZ• radical was reduced by many low molecular weight reductants, samples should be dialysed before assaying for CBQase activity.

5. The analyst should be aware that this assay eliminates only one class of interferences. However, there are other non-dialyzable substance(s) present in at least some culture supernatants which can inhibit or mask the presence of CBQase.

Preface to CHAPTER 5.

In this thesis (Chapter 3) and other studies, it is shown that CBQase can reduce both Fe(III)-acetate complexes (Kremer and Wood, 1992; Coudray *et al.*, 1982) and Mn(III)-malonate complexes (Bao *et al.*, 1993). Paice *et al.* (1993) showed that the MnP produced by *T. versicolor* mediated some of the reactions involved in the delignification of kraft pulps, and both the fungus and MnP could do this in the absence of added manganese. Elemental analysis (Chapter 2) had demonstrated the presence of significant levels of insoluble manganese in most kraft pulps. The most plausible explanation for the presence of this metal in the pulp is that it was precipitated onto the pulp fibers as insoluble MnO_2 during the strongly alkaline kraft pulping process or carried over from the trees from which the pulp was produced. Since Blanchette (1984b) has shown that insoluble MnO_2 deposits are often associated with delignification in wood, a hypothesis that CBQase could catalyze the reduction of insoluble Mn(IV) present as MnO_2 to the freely soluble Mn(II) and Mn(III) ions is suggested.

While trying to identify the nature of the manganese complexing ligands in biobleaching cultures of *T. versicolor* (Chapter 2), it was observed that much of the manganese complexing activity was high molecular weight. Since manganese chemistry was clearly important in biobleaching, both the composition (see Appendix B) and behavior of the high molecular material were studied further. A hypothesis providing a role for CBQase in generating cello-oligosaccharide-derived manganese-complexing activity is proposed.

CHAPTER 5. CREATION OF METAL COMPLEXING AGENTS, REDUCTION OF MANGANESE DIOXIDE AND THE PROMOTION OF MANGANESE PEROXIDASE-MEDIATED Mn(III) PRODUCTION BY CELLOBIOSE:QUINONE OXIDOREDUCTASE FROM *Trametes versicolor*

5.1. INTRODUCTION

Lignin is synthesized in plants by the non-enzymatic polymerization of phenylpropanoid precursors produced by oxidase and peroxidase-mediated one-electron oxidations. Biosynthesis of lignin-like polymers from aromatic alcohol monomers can be catalyzed, by either laccases (Sterjiades *et al.*, 1993) or plant peroxidases (Higuchi and Ito, 1958). The organic free-radicals formed by the enzymes may undergo random free-radical coupling, yielding polymers with a complex non-repeating structure. The white-rot basidiomycete fungi are the most effective lignin-degrading microorganisms known (Eriksson *et al.*, 1990; Kirk, 1971). Many of the white-rot fungi, including *Trametes versicolor*, degrade lignin and cellulose simultaneously (Kirk, 1973). How these two processes are coordinated is unknown, though it has been suggested that cellobiose:quinone oxidoreductase (CBQase) may be important (Eriksson *et al.*, 1990; Ander and Eriksson, 1975; Eriksson *et al.*, 1974; Westermark and Eriksson, 1974).

White-rot fungi produce laccase and peroxidases which can carry out one-electron oxidations of aromatic substrates similar to those catalyzed by the plant lignin biosynthetic enzymes (Eriksson *et al.*, 1990). One class of fungal secreted enzymes, the Mn-dependent peroxidases (MnP) oxidize Mn(II) to Mn(III) when appropriate Mn(III)-complexing agents

are present. Mn(III) complexes can in turn catalyze reactions implicated in biological delignification (Paice *et al.*, 1993; Wariishi *et al.*, 1991). MnP isozymes are secreted by *T. versicolor* (Paice *et al.*, 1993; Johannson and Nyman, 1987). Laccase and other peroxidases can also produce Mn(III)-complexes in the presence of Mn(II), an Mn(III)-complexing agent and phenolic compounds (Archibald and Roy, 1992). This laccase reaction may be important in delignification when H_2O_2 is absent (Archibald and Roy, 1992). Both laccase in the presence of 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate(6)] (ABTS) (Bourbonnais and Paice, 1992) and MnP in the presence of Mn(II) and various Mn-complexing agents (Paice *et al.*, 1993) can attack and partially degrade residual lignin in unbleached kraft pulp. MnP can also depolymerize dehydrogenation polymerizate (DHP) lignin-like polymers (Wariishi *et al.*, 1991). However, the extent of lignin degradation seen with either enzyme alone is only a fraction of that achieved by whole fungal cultures (Paice *et al.*, 1993; Roy and Archibald, 1993; Addleman and Archibald, 1993; Reid *et al.*, 1990).

The stability and reactivity of the Mn(III) ion in water is strongly dependent on the nature and concentrations of the Mn(III)-complexing agents present (Roy and Archibald, 1993; Kuan and Tien, 1993; Wariishi *et al.*, 1992). The small size of simple organic acid Mn(III) complexes also makes them plausible delignifying reagents as they can readily diffuse into lignocellulosic wall regions where protein-size molecules cannot (Strebotnik and Messner, 1990). In fact, Mn(III)-complexing agent(s) such as glyoxalate, oxalate, lactate, and phenyllactate, produced and secreted by *T. versicolor* (Roy and Archibald, 1993) and oxalate and malonate produced by *Phanerochaete chrysosporium*

(Kuan and Tien, 1993; Wariishi *et al.*, 1992) have been shown to be capable of promoting such MnP-mediated oxidations. Cellobiose oxidases and CBQases can oxidize the reducing ends of cellobiose and cellooligosaccharides forming potential Mn-complexing sites while concommitantly reducing oxygen, Fe(III) or quinones (Eriksson *et al.*, 1974; Westermark and Eriksson, 1974; Kremer and Wood, 1992; Kremer and Wood, 1992). Thus both fungal metabolites and wood degradation may be sources of Mn(III) complexing agents.

When wood is degraded by white-rot fungi, dark stains are frequently observed in the delignified tissues (Blanchette, 1984). These stained regions are formed by deposits of insoluble MnO_2 which appear during delignification (Blanchette, 1984a). Glenn *et al.* (1986) showed that under conditions of low Mn-complexing agent concentrations, Mn(II) oxidation by MnP resulted in the formation of insoluble MnO_2 deposits. Since most of the known Mn-complexing agents produced by delignifying cultures of white-rot fungi are present in very low concentrations (Roy and Archibald, 1993; Kuan and Tien, 1993; 1993a; Wariishi *et al.*, 1992; Dutton *et al.*, 1993) manganese oxidation and sequestration as MnO_2 are probably important reactions in natural delignification systems. Blanchette (1984) postulated that in decaying wood a manganese concentration gradient is established, allowing soluble forms of manganese (II and III) to diffuse into regions of low manganese concentration. However, if the manganese in wood is largely present as highly insoluble MnO_2, how does the fungus obtain the soluble Mn(II) necessary for the induction and function of extracellular MnP? One possibility is that some low molecular weight compounds may stoichiometrically reduce MnO_2 (Stone and Morgan, 1984).

However, in this work we show that *T. versicolor* CBQase efficiently catalyzes the reduction of insoluble MnO_2 to soluble Mn(II) and Mn(III) while generating Mn(III)-complexing sugar acids, thus greatly promoting MnP activity.

5.2. MATERIALS AND METHODS

5.2.1. *Trametes versicolor* cultures

Trametes versicolor 52J (Addleman and Archibald, 1993) was cultured using a defined liquid medium (TDM) with glucose (83 mM) and glutamine (5 mM) as carbon and nitrogen sources, respectively (Roy and Archibald, 1993). The fungus was grown in shaking cultures with and without hardwood kraft pulp (2% w/v) at 25°C (Addleman and Archibald, 1993). The cultures were harvested after 7 d, the biomass separated from the culture liquor by centrifugation (10,000 x g; 15 min), and the clarified supernatants filtered (0.45 μm).

5.2.2. Characterization of *T. versicolor* Mn complexation

The 0.45 μm filtered supernatants were separated into high and low molecular weight fractions by overnight dialysis with 1000 and 12-14,000 nominal molecular weight cutoff membranes. For each sample of the retentate, dialysis was against two changes of 40 volumes of distilled water. The Mn-complexing activities of supernatant fractions were determined by adding sodium acetate buffer (5 mM; pH 4.5) (which supports negligible phenol red oxidation MnP activity at this concentration) to the supernatant fractions and substituting these preparations for the usual assay buffer (50 mM malonate;

pH 4.5) using phenol red to measure MnP activity as the H_2O_2-dependant decrease in $OD_{431\ nm}$ (Pick and Keisari, 1980; Kuwahara *et al.*., 1984).

The pKa values for the ionizable groups on the high molecular weight material (HMW) material were determined by titration of individual samples with HCl using an Metrohm autotitrator (636 Titroprocessor controlling an E635 autoburet with a reference electrode). To confirm that the pKa values were independent of ionic effects, samples spiked with $BaCl_2$ were also titrated. Total carbohydrate content of HMW fractions were determined using the anthrone test as described previously using glucose as standard (Hodge and Hofreiter, 1962).

The nature of the HMW material was determined using a number of hydrolytic enzymes and the retained fraction assayed for Mn-complexing activity. The HMW material from 200 mL of culture medium was precipitated with 2 vol of 95% ethanol, and centrifuged (10,000 x g; 30 min)(Buchala and Leisola, 1987). The resulting pellet was resuspended twice in 75% ethanol (40 mL), recovered by centrifugation and lyophilized.

5.2.3. Enzymes and reagents

CBQase was purified from culture supernatants of *Trametes versicolor* (Roy and Archibald, 1993a). Partially purified *Phanerochaete chrysosporium* MnP was obtained from a commercial source (Tienzyme Inc., State College, Pa.). Almond β-glucosidase (E.C. 3.2.1.21) (cat. no. G-8625) and endocellulase (E.C. 3.2.1.4) (cat. no. 7377) were from Sigma Chemical Co. Cellobiose, gluconate, glucuronate, 3,5-di-tert-

butylbenzoquinone(1,2)(TBBQ), phenol red, and $MnSO_4$ were from Sigma Chemical Co. α-D-glucoisosaccharinic acid was synthesized from lactose (Whistler and BeMiller, 1963). Cellobionic acid was produced from cellobiose using CBQase with TBBQ as the electron acceptor. CBQase (0.1 U/ml) was incubated with 4 mM TBBQ and 2 mM cellobiose in 200 ml of 10 mM acetate buffer (pH 5.0). TBBQ and hydroquinone were separated from the cellobionic acid in the reaction mixture with a C18 SepPak^{Tm} cartridge (#43345; Waters div. Millipore Corp.) preconditioned with 10 mM acetate buffer (pH 5.0). The aqueous phase, containing cellobionic acid was recovered and lyophilized, and this material was used without further purification.

Reduction of Mn(IV) was determined using either "activated" MnO_2 (Aldrich; lot no. PP01206MP; purity of ≈86%) or a high purity (99.9+%) preparation (Alfa Products, Danvers, MA.; lot number 120384).

5.2.4. Assays

MnP activity was measured either by following the formation of malonate-Mn(III) complexes by their 270 nm absorbance (*Wariishi et al.*, 1989) or by continuously monitoring phenol red oxidation at 431 nm, using a modification of the original end point assay where the reaction was first stopped using NaOH and the absorbance read (610 nm) at pH 12 (Pick and Keisari, 1980). One unit of MnP activity was defined as the quantity of enzyme mediating a change in absorbance of 1 ODU per min per ml. The malonate MnP assays contained 0.1 mM H_2O_2, 0.2 mM $MnSO_4$, and enzyme in 50 mM malonate

buffer (pH 4.5) in a total volume of 1.5 ml. CBQase activity was measured by monitoring the reduction of TBBQ at 420 nm (Westermark and Eriksson, 1974).

MnP catalyzed Mn-complexing activities were determined by comparing the rates of phenol red (67 μM) oxidation (color loss at 431 nm) supported by the Mn-complexing agent being tested to the rate obtained using a standard MnP assay buffer (50 mM malonate, pH 4.5). An amount of MnP catalyzing a change of 0.05 ODU_{431} min^{-1} standard malonate buffer was used in each assay. Sodium acetate buffer (5 mM; pH 4.5) was added to increase the buffering capacity when low, or unknown concentrations of Mn-complexing agents were assayed, controlling pH to ± 0.1. At 5 mM, sodium acetate supported negligible phenol red oxidation by MnP.

5.2.5. Measurement of manganese dioxide reduction.

Sodium pyrophosphate was deferrated using 3-hydroxyquinoline-chloroform extraction (Waring and Werkman, 1942), Mn(III)-pyrophosphate was prepared by mixing 10 mM $MnSO_4$ and 10 mM MnO_2 in 100 mM pyrophosphate (pH 7) in acid cleaned glassware as described (Kenten and Mann, 1950). Reduction of Mn(IV) was measured in two ways: (1) measurement of the appearance of Mn(III)-pyrophosphate at 478 nm and wavelength scans of the reaction mixture to confirm the characteristic absorbance profile of Mn(III)-pyrophosphate complex (Kenten and Mann, 1950); (2) measuring the appearance of Mn(II) with electron paramagnetic resonance spectroscopy (EPR) (see below).

5.2.6. EPR measurements

EPR spectra were recorded on a Bruker ER 200D-SRC X-band (9.5 GHz) spectrometer using 100 kHz modulation. The free radical standard 2,2-diphenyl-1-picryl-hydrazyl (DPPH) was used to determine the exact microwave frequency. Each of the aqueous samples (5µl) was placed in a standard quartz tube (4 mm i.d.). The 5µl volume was sufficient to obtain the spectra without excessively broadening the sample resonance and reducing the signal to noise ratio.

All the samples containing Mn(II) ions exhibited a 6-line isotropic hyperfine structure at room temperature. Thus the isotropic g_0 factor and the hyperfine splitting parameter A_0 values for Mn(II) could be determined by fitting the line positions corresponding to the transitions M = 1/2, m ↔ M = -1/2, m; where M and m are, respectively, the electronic and nuclear magnetic quantum numbers (m = ± 5/2, ± 3/2, ± 1/2) of the six lines belonging to the isotropic central sextet, to the theoretical expression (Atherton, 1973):

$$B_m = B_0 - A_0 m - \frac{A_0^2}{8B_0}(35 - 4m^2) \qquad (2)$$

In eq. (1) $B_0 = h\nu/g_0\mu_B$, where ν, and h are the frequency of the klystron and Plank's constant, respectively. The fitting yielded the values of A_0 = -95.40 ± 0.05 G and g_0 = 2.0040 ± 0.0008 for Mn(II) for all the samples investigated at 295 K. Solutions with measured amounts of $MnSO_4$ (0.1 to 10 mM) in the various Mn-complexing agents tested were used to calibrate the EPR, and the peak to peak height (amplitude) of the first

derivative trace of the EPR absorption signal was then used to measure the concentration of Mn(II) ions.

5.2.7. Formation of cellobionic acid.

Cellobionic acid formed by CBQase was quantified using 2 mM cellobiose, 15 IU CBQase, 20 mM MnO_2, all in 15 ml of deferrated (Waring and Werkman, 1942) 100 mM sodium pyrophosphate buffer, pH 5.0. A series of duplicate 1 ml samples were taken at 0, 2, 5, 10, 30, and 60 min and analyzed for cellobionic acid by first hydrolyzing the acid and residual cellobiose with 40 IU of β-glucosidase at 37°C for 1 h. The pH of the reaction mixture was raised to ≈9 to convert gluconolactone to gluconic acid, the pH was readjusted to ≈7 with 5 M HCl, and gluconic acid measured using an enzyme-based assay kit (Boehringer Mannheim, 428 191). No detectable gluconic acid was produced in controls containing all assay components less cellobiose or CBQase.

5.3. RESULTS

5.3.1. *Trametes versicolor* extracellular Mn-complexing activity

To measure potential Mn-complexing agents in ligninolytic cultures of *T. versicolor*, a MnP assay following phenol red oxidation was used (Kuan and Tien, 1993; Pick and Keisari, 1980; Kuwahara *et al.*, 1984). Comparing the Mn-complexing efficiencies of crude culture supernatant fractions indicated (Table 5.1) that with constant added MnP activity, phenol red oxidation rates varied between 0 and approximately 300% of the rate seen using the standard 50 mM malonate. This was expected as a number of

Table 5.1. Promotion of MnP activity by *T. versicolor* culture supernatants.

Trial[1]	MnP-mediated phenol red oxidation (Δ ODU·min^{-1})			
	Untreated[2]	Heat treated (100°C, 10 min)	>1K fraction	>12K fraction
Fungus alone	0.38 ± 0.01	0.21 ± 0.01	0.14 ± 0.03	0.16 ± 0.00
Fungus + HWKP	0.23 ± 0.01	0.28 ± 0.01	0.09 ± 0.01	0.11 ± 0.01
HWKP alone	0.001 ± 0.001	0.015 ± 0.002	0.00 ± 0.00	0.00 ± 0.00
Control[3]	0.14 ± 0.02	-	-	-

[1] Supernatants were from 7-day cultures of *T. versicolor* grown in TDM as described in Materials and Methods. Trials were in triplicate and mean values ± standard deviation are shown. Acetate buffer (5 mM) alone supported a rate of 0.0015 ODU min^{-1}.

[2] Supernatants clarified by centrifugation (10,000 x g, 10 min) to remove particulates followed by microfiltration (0.45 μm).

[3] The standard Mn-complexing assay with 50 mM malonate buffer in place of the culture supernatant.

organic acid metabolites produced by *T. versicolor* (Roy and Archibald, 1993) and *P. chrysosporium* (Wariishi *et al.*, 1992; Kuan and Tien, 1993) has been shown to efficiently promote the phenol red oxidizing activity of MnP. Most of this supernatant Mn-complexing activity was resistant to degradation or inactivation by heating and, surprisingly, a significant fraction was nondialyzable, i.e. >12K (Table 5.1).

This HMW fraction of the Mn-complexing activity present in *T. versicolor* culture supernatants was investigated further. The dialyzed HMW material showed two titratable groups with pKa values of about 5 and 8, the former being close to the pH found in actively biobleaching *T. versicolor* cultures (Roy and Archibald, 1993; Addleman and Archibald, 1993; Paice *et al.*, 1989). A determination of the total carbohydrate content of samples of this HMW material gave values of 25.6 ± 0.3 μg /ml for cultures grown in the presence of pulp to 3.4 ± 0.4 μg/ml for the fungus alone, while a sterile pulp control contained 4.7 ± 0.3 μg/ml. Less than 10% of the Mn-complexing activity was lost from the HMW material when it was passed over a C18 Sep Pak column, although approximately 60% of the UV absorbance at 280 nm was removed, indicating that the Mn-complexing material was hydrophilic.

When the HMW material was treated with β-1,4-glucanase (containing both endo- and exocellulases), its ability to support the oxidation of phenol red by MnP was completely lost upon dialysis (12-14 K cutoff). Nucleases and proteinase K had no effect. Approximately 70% of the HMW Mn-complexing activity was recoverable from actively bleaching cultures of *T. versicolor* by ethanol precipitation, like the polysaccharide isolated from ligninolytic cultures of *P. chrysosporium* (Buchala and Leisola, 1987; Bess

et al., 1987). A purified fraction of this HMW material (100 mg ml^{-1}) supported the reductive activity of CBQase when TBBQ was the electron acceptor, but at a 300-fold lower rate, and to a lesser extent (8%) than 1 mM cellobiose (0.342 mg ml^{-1}). Nonetheless, the extracellular polysaccharide secreted by *T. versicolor* can act as a limited source of CBQase reducing equivalents and can complex Mn(III), thus promoting MnP activity.

5.3.2. Sugar acids as Mn-complexing agents

Since CBQase slowly oxidized the carbohydrate fraction of the HMW Mn-complexing material from *T. versicolor* cultures, and this HMW material could function as a Mn-complexing agent for MnP activity, we thought that simple sugar acids might also be effective Mn(III) complexing agents. Gluconic and cellobionic acids produced by glucose oxidase and CBQase both worked very well as Mn complexing (and MnP activity promoting) agents as evidenced by phenol red oxidation (Table 5.2). Glucoisosaccharinic acid, produced in large quantity from cellulose and hemicelluloses during the kraft pulping process, was also effective.

5.3.3. Mn-complexing activity produced in cellulose by CBQase

Do sugar acid residues produced on cellulosic fibers by CBQase-mediated oxidation of exposed reducing ends (Kremer and Wood, 1992; Bao and Renganathan, 1992) allow the fibers to function as effective Mn(III)-complexing (MnP activity-promoting) agents? Cellulose (Solka Floc, a purified cellulose derived from wood fibre)

Table 5.2. Efficacy of gluconic, cellobionic and glucoisosaccharinic acids as Mn-complexing agents in an MnP activity assay.

Mn-complexing agent[1]	Concentration (mM)	Phenol red oxidation (ΔODU·min^{-1})[2]
cellobionic acid[3]	0.1	0.52 ± 0.02
	1.0	0.71 ± 0.01
	10	0.82 ± 0.08
gluconate	0.5	0.16 ± 0.01
	2	0.66 ± 0.02
	8	0.48 ± 0.01
glucoisosaccharinic acid	0.1	0.01± 0.00
	1.0	0.21 ± 0.00
	10	0.22 ± 0.01
acetate + cellobiose	10 and 4	0.01 ± 0.01
acetate	10	0.00 ± 0.00
malonate	50	0.18 ± 0.01

[1] Each assay contained, in addition to the sugar acid, 5 mM acetate buffer (pH 4.5) to control the pH.

[2]What proportion of these sugar acid effects is due to altered substrate (Mn(II)) complexation and how much is due to altered product (Mn(III)) complexation is unknown.

[3] Cellobionic acid was synthesized enzymatically from cellobiose using CBQase as described in Materials and Methods.

was incubated with 0.1 U/ml of CBQase for 4 h in the presence of TBBQ, which acted as an electron acceptor for CBQase. The results in Table 5.3 show that a pretreatment of a 2% w/v cellulose preparation with CBQase increased the rate of MnP-mediated oxidation of phenol red in the absence of any added Mn-complexing agent. The action of CBQase was also seen as more titratable groups on the cellulose fibers (Table 5.3), presumably related to an increase in Mn-complexing sites. Combining endocellulase and CBQase further increased the extent of CBQase-enhanced phenol red oxidation, and number of titratable groups in the cellulose preparation. Furthermore, the rate of MnP-mediated phenol red oxidation supported by the solubilized material also increased substantially (Table 5.3). Since only the soluble fraction (≈4% with CBQase and 15% with CBQase and cellulase) of the incubation mixture was used in the Mn-complexing assay, the total number of potential Mn-complexing sites formed by CBQase and the consequent improvement in MnP activity may have been underestimated.

5.3.4. Reduction of insoluble MnO_2 by CBQase

CBQase reduces, in addition to quinones, both Mn(III) (Bao *et al.*, 1993) and Fe(III) (Kremer and Wood, 1992a). We therefore speculated that CBQase might also be capable of reducing Mn(IV), thus rendering the insoluble MnO_2 deposits found in wood (Blanchette, 1984; 1984a) available as Mn(II) or Mn(III) for MnP-dependent reactions. When the experiment was run, we did in fact observe substantial reduction of Mn(IV) in a reaction mixture that contained insoluble MnO_2, cellobiose, CBQase, and sodium pyrophosphate (Figure 5.1). Mn(IV) reduction was readily detected by the appearance

Table 5.3. Oxidation of cellulose by *T. versicolor* CBQase and the effect of this on the specific activity of MnP.

Mn-complexing agent	Pretreatment[1]	Viscosity[2] (mPa.s)	Titratable groups[2] (meq g^{-1})		Phenol red oxidation[3] ($\Delta ODU \cdot min^{-1}$)
			Total	pH 3-5	
Control[4]	None	-	-	-	0.19
Cellulose[5]	None	10.4	38	27	0.01
	CBQase	9.8	60	45	0.07
	CBQase + Cellulase	8.0	83	70	0.12

[1] Samples were incubated with CBQase (0.1 U/ml), 5 mM acetate buffer (pH 4.5) and 2 mM TBBQ for 4 h at 25°C. TBBQ and reduced TBBQ were removed by passing the reaction mixture over a preconditioned C18 Sep Pak cartridge twice, which removed >80% of the 280nm -absorbing compounds.

[2] The insoluble fraction of the cellulose preparation present after preincubation.

[3]The ability of the cellulose to function as the Mn-complexing agent in the phenol red MnP assay. This was measured using the soluble fraction of the cellulose (15% with cellulase and CBQase, and 4% with CBQase alone). Determined for an aliquot of the supernatant after centrifugation (10,000xg, 10 min).

[4] The standard Mn-complexing assay using 50 mM malonate (pH 4.5)

[5] A 2% w/v suspension of Solka Floc in 5 mM acetate buffer (pH 4.5).

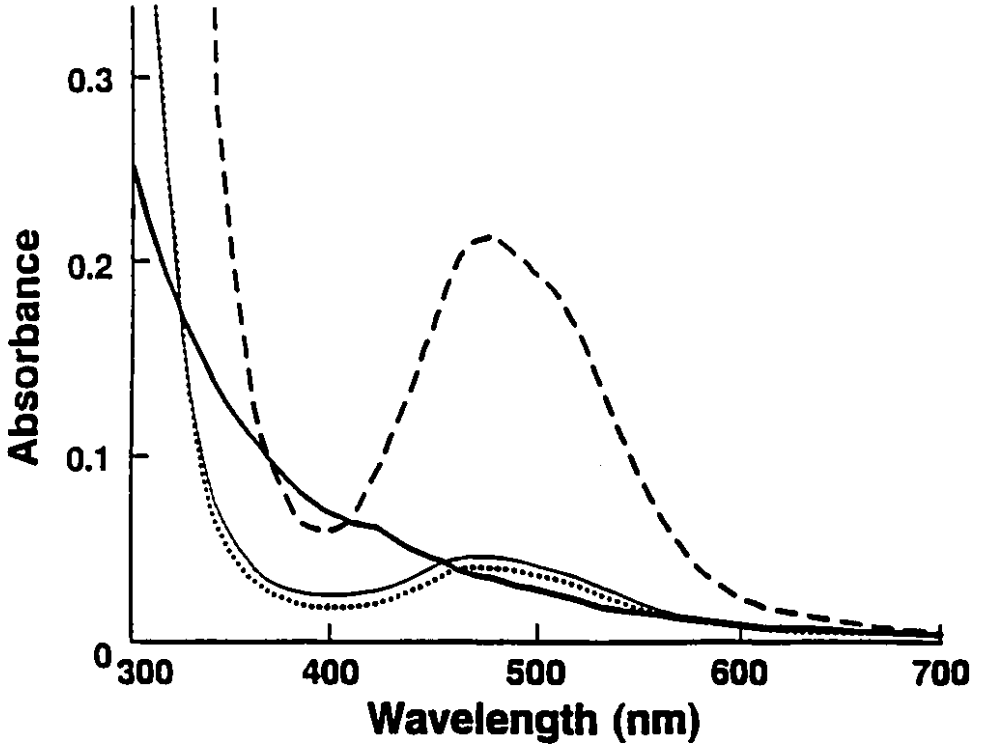


Figure 5.1. Visible light absorbance spectrum of a reaction mixture containing MnO_2 (10mM), cellobiose (2 mM), CBQase (0.1 U per ml), and sodium pyrophosphate (100 mM; pH 5) after 4 h of incubation at 25°C. Peak height corresponds to approximately 2 mM Mn(III)-pyrophosphate based on an extinction coefficient at 478 nm of 0.104 $mM^{-1}cm^{-1}$. Trace (A), complete system; Trace (B) like (A) less CBQase; Trace (C) like (A) less cellobiose; Trace (D) like (A) less MnO_2.

of the characteristic visible absorbance spectrum of the Mn(III)-pyrophosphate complex (Archibald and Roy, 1992; Kenten and Mann, 1950) and by the appearance of free Mn(II) ions as shown by EPR spectra (Figure 5.2). These reductions were CBQase-dependent and observed with all MnO_2 preparations tested. Reduction rates were higher when amorphous "activated" or powdered MnO_2 preparations were used, consistent with the greater reactivity of such preparations. Possible routes of formation for the Mn(III)-pyrophosphate complex in this system are outlined in Equations (2) and (3-4). CBQase is assumed to be acting as the electron donor in the reactions described in Equations (2) and (3). In both cases, the overall stoichiometry of complex formation is the same. This is consistent with the observed consumption of one millimole of cellobiose per millimole of Mn(III)-pyrophosphate formed, based on a molar extinction coefficient (478 nm) of 104 (Archibald and Fridovich, 1982).

Mn(IV) + e- → Mn(III) + pyrophosphate → Mn(III)-pyrophosphate **(2)**

Mn(IV) + 2e- → Mn(II) **(3)**

Mn(II) + Mn(IV) → 2 Mn(III) + 2 pyrophosphate → 2 Mn(III)-pyrophosphate **(4)**

To determine whether CBQase activity formed Mn(III) directly (Equation **2**) or via Mn(II) (Equations **3** and **4**), EPR was used to distinguish between Mn(II) and Mn(III). The amplitude of the 6-line isotropic hyperfine structure diagnostic for Mn(II) was used to measure the rate of Mn(II) formation. Authentic Mn(II) gave signals with g_o values of 2.0039, 2.0046 and 2.0056 in acetate, pyrophosphate, and malonate buffers (50 mM;

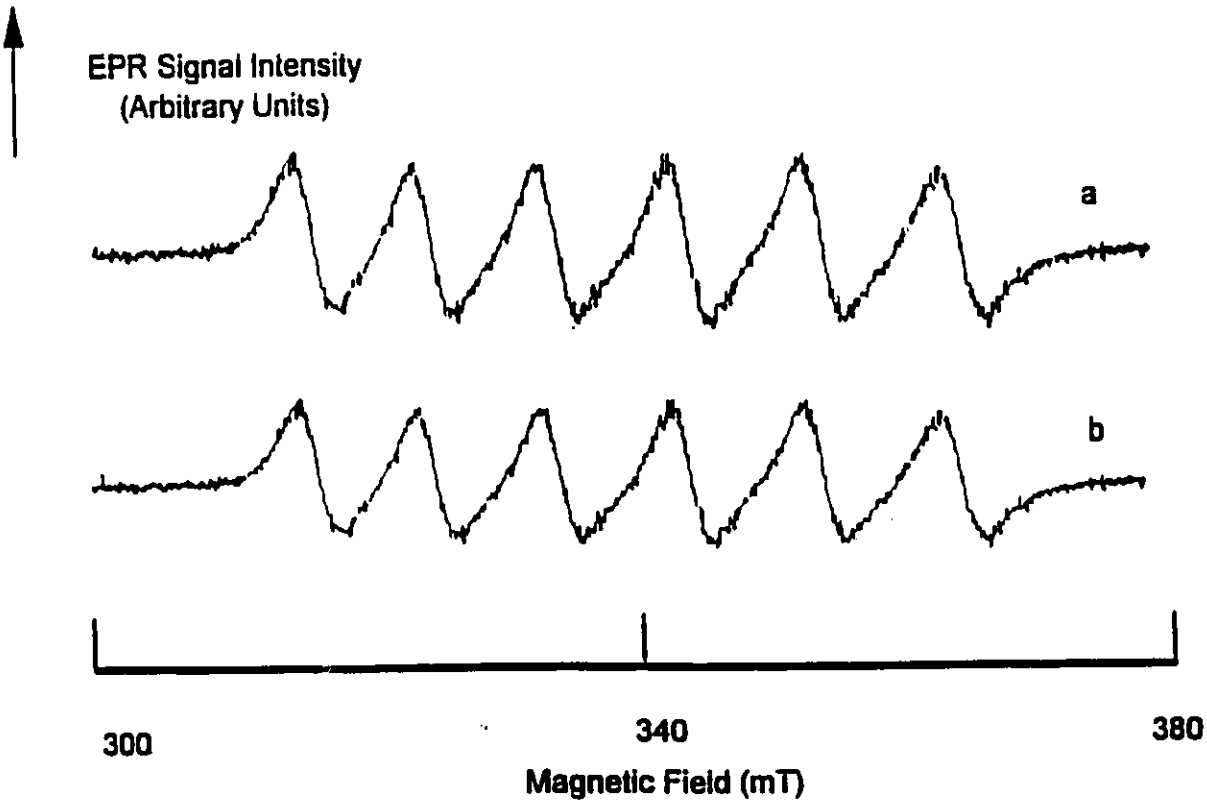


Figure 5.2. First-derivative EPR spectra of different manganese preparations. Trace A is of a solution of $MnSO_4$ (1 mM) in H_2O. Trace B is a scan of a reaction mixture containing insoluble MnO_2 (10 mM), cellobiose (2 mM), CBQase (0.1 U ml^{-1}) in pyrophosphate buffer (pH 5) taken 45 min after the addition of the enzyme. Conditions were as follows: centerfield = 340 mT, ΔH = 80 mT, sweep time = 100s, , microwave power = 2 mW, 20 db attenuation, with readings taken at room temperature (295° K). Absorption signals for A and B had calculated g_o values of 2.0039 and 2.0048, respectively.

pH 4.5) respectively. Broadening of the peak-to-peak widths of each of the six isotropic hyperfine lines of the central sextet was observed and increased in the order acetate > pyrophosphate > malonate buffers. The observed line broadening was likely caused by increased dipolar interactions between the chelators and Mn(II). When MnO_2 was incubated with sodium pyrophosphate, in the presence of 0.1 U/ml of CBQase and cellobiose, a 6-line Mn(II) central sextet (g_o=2.0048) was observed within several minutes, demonstrating that a fraction of the Mn(IV) had been reduced to Mn(II) (Figure 5.2). No EPR signals were observed from genuine Mn(III) complexed with pyrophosphate, nor from MnO_2 in the presence of cellobiose and pyrophosphate, even after 24 h.

CBQase-mediated promotion of Mn(III)-complex formation by the simultaneous reduction of MnO_2 and oxidation of cellobiose to cellobionic acid was demonstrated by incubating 15 units of the enzyme with 2 mM cellobiose and 10 mM MnO_2 in sodium pyrophosphate buffer. Samples of the reaction mixture were taken at intervals and cellobionic acid derived gluconic acid was determined (Table 5.4). The overall stoichiometry suggested that one mole of cellobiose was oxidized per mole of Mn(III)-pyrophosphate formed.

The particular Mn(III)-complexing agent employed had a large effect on the rate at which CBQase reduced MnO_2 and Mn(III) (Table 5.5). When Mn(II) formation was monitored by EPR, a similar dependence of the MnO_2 reduction rate on buffer composition was observed (data not shown). The MnO_2 reduction rate was highest with buffers, such as acetate and succinate that do not form stable Mn(III)-complexes. These findings also suggest that Mn(II) formation is enhanced when poor Mn(III)-complexing

Table 5.4. Production of cellobionic acid during MnO_2 reduction by CBQase.

Time (min)	Absorbance of reaction mixture (478 nm)[1]	Mn(III)-pyrophosphate formed (mM)[1,2]	Cellobionic acid produced (mM)[1,3]
0	0	0	0
5	0.08	0.76	0.54 ± 0.07
10	0.14	1.35	1.16 ± 0.12
30	0.21	2.02	1.60 ± 0.02

[1] At the times indicated, an aliquot of the reaction mixture was removed and the absorbance at 478 nm (due to Mn(III) pyrophosphate complex formation) determined.

[2] The production of Mn(III) was followed using deferrated 100 mM sodium pyrophosphate buffer (pH 5). Mn(III)-pyrophosphate production was calculated using an extinction coefficient of 0.104 $mM^{-1}cm^{-1}$ for the complex (Archibald and Fridovich, 1982). The reaction mixture contained 0.1 U ml^{-1} CBQase, 2 mM cellobiose, and 10 mM MnO_2.

[3] Cellobionic acid was determined by hydrolysis to gluconic acid as described in Materals and Methods. Values are the mean of two determinations ± standard deviations.

Table 5.5. Effect of Mn-complexing agents on CBQase-mediated reduction of MnO_2 and Mn(III)-complexes.

Mn-complexing agent[1]	Rate of Mn(III)-complex formation[2] (μmole $min^{-1}ml^{-1}$)		Rate of Mn(III)-complex reduction[3] (μmole $min^{-1}ml^{-1}$)		Wavelength monitored
	- CBQase	+ CBQase	- CBQase	+ CBQase	(nm)
malonate	0.001 ± 0.0002	0.06 ± 0.01	0.10 ± 0.2	13.2 ± 2.3	458
gluconate	<0.00001[4]	0.09 ± 0.02	0.11 ± 0.3	15.4 ± 4.0	235
pyrophosphate	<0.0002[4]	0.40 ± 0.02	<0.0002[4]	27.9 ± 0.9	478

[1] All buffers were 100 mM, pH 5.0. CBQase was added at 0.1 $U ml^{-1}$ with 20 mM MnO_2 and 2 mM cellobiose. Extinction coefficients were; Mn(III)-malonate ϵ_{458}= 1.93 $mM^{-1}cm^{-1}$ (Wariishi *et al.*, 1992), Mn(III)-gluconate ϵ_{235}= 9.5 $mM^{-1}cm^{-1}$ (Bodini *et al.*, 1976) and Mn(III)-pyrophosphate ϵ_{478}= 0.104 $mM^{-1}cm^{-1}$ (Archibald and Fridovich, 1982).

[2] Rate of formation of Mn(III)-complex using 0.1 $U ml^{-1}$ CBQase, 2 mM cellobiose, 10 mM MnO_2, and 100 mM complexing agent.

[3] Rate of preformed Mn(III)-complex disappearance using 0.1 U/ml CBQase, 2 mM cellobiose, 1.0 mM Mn(III), and 100 mM complexing agent. Mn(III)-malonate and gluconate were formed using MnP followed by ultrafiltration. Mn(III)-pyrophosphate was formed by the dismutation reaction of $MnSO_4$ (10 mM) with solid MnO_2 (10 mM)(Kenten and Mann, 1950).

[4] The reaction was followed for 1 h at 478 nm.

agents are used, and as the efficacy of an Mn(III)-complexing agent increases, the short-term yield of Mn(II) produced by the CBQase-mediated reduction of MnO_2 is decreased.

5.4. DISCUSSION

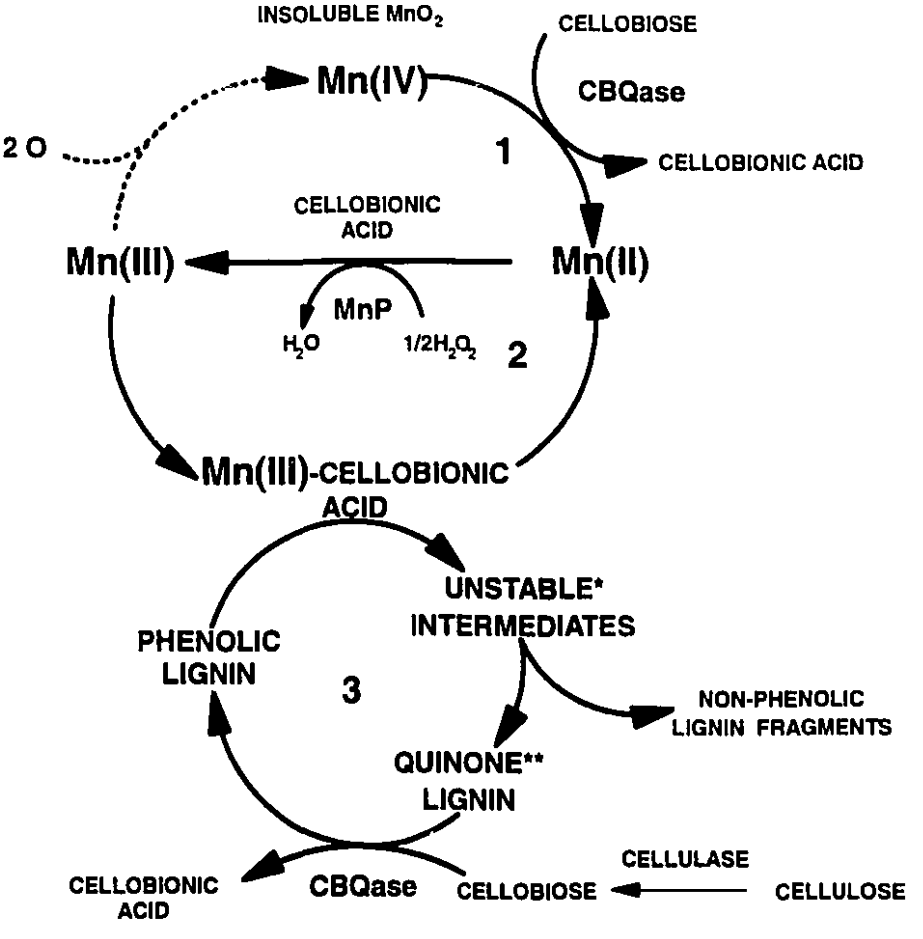
A long-term goal of the work with the *T. versicolor* delignification system has been to develop a cell-free biological delignification system which will bleach and delignify unbleached kraft pulp as effectively as the complete *T. versicolor* bleaching system (Paice *et al.*., 1993; Roy and Archibald, 1993; Paice *et al.*, 1989). The most promising *T. versicolor* secreted enzyme isolated to date is MnP; alone it can demethylate the kraft lignin extensively, but only delignify and biobleach slightly (Paice *et al.*., 1993). Another enzyme commonly secreted during kraft pulp bleaching by *T. versicolor* is CBQase (Paice *et al.*., 1993; Roy and Archibald, 1993) and one theory of biological delignification proposes that this enzyme works in concert with lignin peroxidase, laccase and/or MnP by regenerating peroxidizable substrates and by limiting peroxidase-mediated polymerization (Westermark and Eriksson, 1974; Eriksson *et al.*, 1990).

The work reported here shows that CBQase can promote and complement the action of MnP in several ways in a kraft pulp delignifying and bleaching system. MnP requires three accessory substances in order to oxidize lignin: 1. H_2O_2; 2. Mn(II) ions; and; 3. one or more appropriate Mn(II)/Mn(III) complexing agents. In this work we show that *T. versicolor* CBQase can oxidize the reducing ends of cellulose, creating Mn-complexing organic acids from soluble and insoluble substrates (pulp), thus enhancing MnP activity. If cellulase is present with the CBQase, as is probably always the case *in*

vivo, production of soluble Mn-complexing capacity is greatly enhanced. CBQase can also oxidize cellobiose to form cellobionic acid which acts as an effective Mn(III)-complexing agent promoting increased MnP activity. Finally, CBQase can directly reduce insoluble MnO_2, often the main form of Mn found in wood and pulp, to more accessible (soluble) Mn(II) and Mn(III) ions.

Depletion of cellulose and the terminal reducing sugars in pulp or wood after extensive cellulose attack by the fungus may serve to modulate Mn(III)-dependent lignolytic activity, since at very low Mn-complexing agent concentrations MnP tends to form insoluble MnO_2 (Glenn *et al.*, 1986), thus eliminating the Mn(II) necessary for both the induction and function of MnP. Such deposits of insoluble MnO_2 are often associated with wood delignified by white-rot fungi (Blanchette, 1984; 1984a). Conversely, the enzymatic reduction of insoluble MnO_2 by CBQase is an effective mechanism for inducing *T. versicolor* MnP secretion and for returning manganese to the MnP catalytic cycle (Blanchette, 1984; 1984a). Our results indicate that cooperativity between CBQase and MnP in kraft pulp delignification and bleaching by *T. versicolor* may occur in at least four different ways; CBQase can render Mn available to MnP; CBQase generates the Mn-complexing agents necessary for MnP activity; CBQase elevates available Mn, thereby inducing MnP secretion; and each enzyme provides or regenerates aromatic substrates susceptible to the other (Figure 5.3).

Figure 5.3. Model of proposed *in vivo* cooperativity between MnP and CBQase. CBQase reduces $Mn(IV)O_2$ (**cycle 1**) making it available for the MnP catalytic cycle and furnishes Mn-complexing agents required for the proper function of MnP (**cycle 2**). The number of carbohydrate reducing end groups available for oxidation by CBQase is determined by the level of cellulolytic activity in the culture. CBQase also reduces quinones present in the lignin substrate, making these susceptible to further oxidation by Mn(III)-complexes (**cycle 3**). Cellobionic acid is used as an example of a sugar acid Mn-complexing agent. Mn(III) oxidation to Mn(IV) may be catalytic or occur via dismutation of two Mn(III) molecules to form Mn(II) and insoluble MnO_2. The generation of non-phenolic lignin fragments occurs via non-enzymatic oxidation and fragmentation ($*$). The formation of quinone lignin can occur non-enzymatically ($**$) or phenolic lignin can be formed by direct reduction (not via a quinone) of the unstable intermediates by CBQase. Although the CBQase and MnP-dependent cycle (**3**) of lignin oxidation and reduction is shown as endless, the production of peroxidase-generated small lignin fragments (Umezawa *et al.*, 1986; Umezawa and Higuchi, 1991; Tuor *et al.*, 1992; Forrester *et al.*, 1988 Miki *et al.*, 1987), which cannot be reduced by CBQase allows a progressive net degradation of lignin.


INSOLUBLE MnO_2
CELLOBIOSE
Mn(IV)
CBQase
2 O
1
CELLOBIONIC ACID
CELLOBIONIC ACID
Mn(III)
Mn(II)
MnP
H_2O
$1/2H_2O_2$
2
Mn(III)-CELLOBIONIC ACID
UNSTABLE* INTERMEDIATES
PHENOLIC LIGNIN
3
NON-PHENOLIC LIGNIN FRAGMENTS
QUINONE** LIGNIN
CELLULASE
CELLOBIONIC ACID
CBQase
CELLOBIOSE
CELLULOSE

Preface to CHAPTER 6

The fact that CBQase was secreted into biobleaching cultures of *T. versicolor* (Chapter 2) suggested that it might be an important in lignin biodegradation. Clearly some of the results reported here (Chapter 5) show that its role may be to furnish Mn(II) and Mn(III)-complexing agents, both important components of the MnP delignification system (Paice *et al.*, 1993). CBQase also reduces a wide range of quinones (Tables 3.4, 3.5, Figure 3.9), which could make MnP or laccase-oxidized lignin available for subsequent re-oxidation by laccase or MnP (Figure 5.3, this thesis). In Chapter 4, evidence that CBQase can interact directly with free radicals formed by the one-electron oxidation of CPZ suggested that CBQase might be an important component of the *T. versicolor* delignification system. It suggested that CBQase might establish a redox cycle similar to what had been proposed in the literature (Green, 1977; Westermark and Eriksson, 1974). Data presented in Chapter 6 show that in a number of *in vitro* enzyme systems, purified CBQase can and does interact with LP, laccase and MnP as well as many of the aromatic reaction intermediates formed by these enzymes.

CHAPTER 6. INTERACTIONS OF CELLOBIOSE:QUINONE OXIDOREDUCTASE AND OXIDATIVE ENZYMES IMPLICATED IN LIGNIN DEGRADATION

6.1. INTRODUCTION

A role for cellobiose:quinone oxidoreductase (CBQase) and cellobiose oxidase (CBO) was first proposed by Eriksson and coworkers in the 1970's (Westermark and Eriksson, 1974; 1974a; Ayers *et al.*, 1978). Both CBQase and CBO can catalyze the oxidation of cellobiose and structurally similar disaccharides (Table 3.3, this thesis) and subsequent reduction of quinones (Ayers *et al.*, 1978; Morpeth, 1985; Morpeth and Jones, 1986; Tables 3.4 and 3.5, this thesis). It was proposed that quinones formed during oxidation of lignin substructures by peroxidases and laccases could be reduced by CBQase/CBO (Ander and Eriksson, 1978). Odier *et al.* (1988) reported that the polymerization of certain lignin model compounds by LP was unaffected by CBQase and found that the free radical formed by LP from tetramethoxybenzene was not quenched by CBQase. However Samejima and Eriksson (1991) and Ander *et al.* (1990) clearly demonstrated that a number of phenoxy and cation radicals formed by LP were reduced by CBQase. Ander *et al.* (1990) showed that the polymerization of kraft lignin by a crude preparation of fungal peroxidase enzymes was inhibited by CBQase.

There are other possible roles for CBQase/CBO besides the reduction of oxidized lignin components. Morpeth (1985) showed that CBQase can catalyze the reduction of O_2 to form H_2O_2 via a superoxide intermediate; however this reaction is slow relative to the rate of quinone reduction. Another possible role for CBQase is the reduction of

peroxidase heme compound II, a catalytic intermediate in the oxidation cycles of MnP and LP. Ander *et al.* (1993) found that this reaction occurs at an insignificant rate when the normal substrates of the peroxidases are present. Another potential role for CBQase is to provide a source of soluble Mn(II) required for the proper functioning of MnP's catalytic cycle (Chapter 5, this thesis). Blanchette (1984) has shown that most manganese in wood is present as insoluble MnO_2. The oxidation of cellobiose itself may also be an important, since cellobionic acid is a good complexing agent of Mn(III) (Wariishi *et al.*, 1992; Chapter 5, this thesis). There have been several reports on the metal-reducing activity of CBQase (Kremer and Wood, 1992; Bao *et al.*, 1993) and Roy *et al.* (1994) recently demonstrated that chelators generated by CBQase gave enhanced MnP-mediated phenol red oxidation. Call and Muecke (1994) has proposed that effective delignification by laccase or MnP requires the maintenance of a certain redox potential, and CBQase may be important in maintaining this potential.

The bleaching of kraft pulp involves a combination of removal (solubilization) and modification (decolorization of chromophores) of the residual lignin. There is an interest in supplementing or replacing chemicals currently employed in the bleaching process with enzymes. The white-rot fungus *T. versicolor* extensively bleaches and delignifies kraft pulp, although the process is too slow to be applied industrially (Paice *et al.*, 1989; Reid *et al.*, 1990). Recently it has been found that laccase (Bourbonnais and Paice, 1992) and MnP (Paice *et al.*, 1993), but not LP (Kirkpatrick *et al.*, 1990; Archibald, 1992) were secreted by *T. versicolor* during this fungal pulp bleaching. When applied as purified proteins to kraft pulps, both laccase and MnP were found to slightly delignify,

but not bleach (Bourbonnais and Paice, 1992; Paice *et al.*, 1993). One possible reason for the small effects of isolated enzymes is that a combination of enzymes is required. CBQase is present in culture supernatants during bleaching by *T. versicolor* (Paice *et al.*, 1993; Roy and Archibald, 1993; Chapter 2, this thesis). In the present study, the behavior of purified CBQase in combination with purified MnP and laccase as well as LP and VAO is investigated.

6.2. MATERIALS AND METHODS

6.2.1. Enzyme preparations and assays

6.2.1.1. CBQase

CBQase 4.2 was purified from culture supernatants of *T. versicolor* 52J (Roy and Archibald, 1994). *T. versicolor* flavin and heme-flavin CBQases were purified to homogeneity as described in Chapter 3 of this thesis. *Sporotrichum thermophile* CBQase (containing both heme and flavin cofactors) was produced as previously described (Canevascini, 1987) and partially purified as described in Chapter 4 of this thesis.

CBQase activity was determined by monitoring the cellobiose-dependent reduction of 3,5-di-tert-butylbenzoquinone(1,2) (TBBQ) at 420 nm (Westermark and Eriksson, 1974a). The assay mixture contained sodium acetate (100 mM; pH 4.5), ethanol (20% v/v), cellobiose (2 mM), and TBBQ (0.33 mM) in a total assay volume of 1.5 ml. To inhibit interference in the assay caused by laccase-mediated re-oxidation of the hydroquinone formed from TBBQ by CBQase, the reaction mixture was made anoxic by bubbling N_2 through the reaction mixture for 3 min at a flow rate of 0.5 ml/min. One

unit of enzyme was defined as the amount of enzyme that reduced 1 μmole TBBQ / min/ml.

6.2.1.2. Mn peroxidase

Partially purified *Phanerochaete chrysosporium* MnP was obtained from a commercial source (Tienzyme Inc., State College, Pa.). MnP activity was measured either by following the formation of malonate-Mn(III) complexes by their 270 nm absorbance (Wariishi *et al.*, 1989) or by continuously monitoring phenol red oxidation at 431 nm, using a modification of the original end point assay where the reaction was first stopped using NaOH and the absorbance read (610 nm) at pH 12 (Pick and Kiersi, 1980). One unit of MnP activity was defined as the quantity of enzyme mediating a change in absorbance of 1 ODU per min per ml. The malonate MnP assays contained 0.1 mM H_2O_2, 0.2 mM $MnSO_4$, and enzyme in 50 mM malonate buffer (pH 4.5) in a total volume of 1.5 ml.

6.2.1.3. Veratryl alcohol oxidase

Veratryl alcohol oxidase (VAO) purified from cultures of *P. saju-cajor* was kindly provided by R. Bourbonnais (Pulp and Paper Research Institute of Canada, 570 St Jean Blvd., Pointe Claire, Quebec, Canada, H9R 3J9). VAO activity was measured by following the oxidation of veratryl alcohol to veratraldehyde at 310 nm (Bourbonnais and Paice, 1988). Assays contained 2.5 mM veratryl alcohol and enzyme in 20 mM Na-acetate buffer pH 3.0.

6.2.1.4. Lignin peroxidase

Lignin peroxidase (LP) was measured by the oxidation of veratryl alcohol to veratraldehyde at 310 nm (Tien and Kirk, 1988). Assays contained 2.5 mM veratryl alcohol, 0.1 mM H_2O_2, and sample in 20 mM Na-succinate, pH 3.0.

6.2.1.5. Laccase

Laccase activity was determined by the oxidation of 2,2'-azino-di-(3-ethylbenzthiazoline)-6'-sulfonate (ABTS) measured as the increase in absorbance at 420 nm in 100 mM Na-acetate buffer, pH 4.5 containing 0.5 mM ABTS and one unit (U) of activity corresponds to 1 μmole of ABTS oxidized per minute.

6.2.2. Chemicals

6.2.2.1. Chemicals

Cellobiose, 3,5-di-tert-butylbenzoquinone(1,2)(TBBQ), $MnSO_4$, chlorpromazine (CPZ), were purchased from Sigma Chemical Co. ABTS was from Boeringer Mannheim Chemical Co. and indulin AT was from Westvaco Chemicals. Veratryl alcohol was from Aldrich Chemical Co. All other chemicals were of reagent grade.

6.2.2.2. Pulp samples and enzyme treatments

The pulp used in this study was an unbleached mixed hardwood furnish kraft pulp (HWKP) from an eastern Canadian pulp mill. Alkaline extractability of an enzyme

treated pulp was also evaluated on a mixed furnish softwood kraft pulp (SWKP) from a western Canadian pulp mill.

Pulp (2% w/v) was suspended in 200 ml of distilled water or buffer in 500 ml erlenmeyer flasks. MnP treatments were performed in 50 mM malonate buffer containing glucose (10 mM), glucose oxidase (Sigma Chemical Company, St. Louis, MO., USA) (0.025 U/ml), $MnSO_4$ (0.5 mM), and MnP (1 U/ml based on the Mn(III)-malonate complex assay). The pulp was mixed at 200 rpm on a rotary shaker (r=8 mm) for 24 h at 25°C. Where CBQase (0.05 or 0.5 U/ml) was added in the pulp in the pulp treatments, cellobiose (10 mM) was normally added to the reaction mixture. In some experiments cellobiose was omitted. In sequential incubations, the pulp was washed with distilled water between the enzyme treatments.

6.2.3. Analytical Methods

6.2.3.1. Electron paramagnetic resonance spectroscopy (EPR) measurements

EPR spectra were recorded on a Bruker ER 200D-SRC X-band (9.5 GHz) spectrometer using 100 kHz modulation. The free radical standard 2,2-diphenyl-1-picryl-hydrazyl (DPPH) was used to determine the exact microwave frequency. Each of the aqueous samples (5µl) was placed in a standard quartz tube (4 mm i.d.). The 5µl volume was sufficient to obtain the spectra without excessively broadening the sample resonance and reducing the signal to noise ratio. Spectra were recorded at ambient temperature (≈21°C).

6.2.3.2. Methanol determinations and alkaline extraction

Samples assayed for volatile metabolites were initially clarified with activated charcoal followed by 0.45 μm filtration and resolved by gas chromatography (GC) as described (Bourbonnais and Paice, 1992). An HP 5890A GC with a Chromosorb 102 packed column (1.85 m x 3.2 mm; 80/100 mesh) was used with injector, oven, and FID detector at 130,120, and 150°C, respectively, and (30 ml/min) helium carrier. There was a linear relationship between peak area and concentration for methanol (5-50 mg/l) and ethanol (2-50 mg/l).

6.2.3.3. Gel permeation chromatography

Gel permeation chromatography of reacted guaiacol and indulin samples were performed using an FPLC system employing a Superose 12 10/30 column (Pharmacia Chemical Co.) The column was eluted at a flow rate of 0.25 ml per min with a water: ethanol mixture (1:4) containing 20 mM NaOH, 50 mM NaCl. The column was calibrated using blue dextran, bovine serum albumin, cytochrome c, and vitamin B12. Samples of 0.1-0.2 ml were injected onto the column for fractionation and, when desired, 1 ml fractions of the column eluate were collected. The eluant was monitored at either 280 nm or a rapid scanning diode array detector to characterize the UV-visible spectra of eluting species.

6.2.3.4. Oxygen consumption

Cellobiose-dependent O_2 uptake was measured with a Clark oxygen electrode in a water jacketed cell (Rank Brothers, Cambridge, U.K.) at 25°C in a total volume of 3 ml. The reaction mixtures consisted of 0.03 U of laccase and 2 mM cellobiose in 100 mM sodium acetate buffer and between 0-0.3 U/ml CBQase as required.

6.2.3.5. Pulp Properties

Handsheets were formed by draining a 200 ml samples of 2% w/v pulp suspension through an apparatus with a 150-mesh screen and. After 24 h drying, sheet reflectance (brightness) was measured at 457 nm (CPPA standard method E1) and kappa number, which corresponds to the amount of pulp required to consume 50% of an added amount of potassium permanganate when incubated using a standard conditions using the CPPA standard method G18.

6.2.3.6. UV-visible spectroscopy

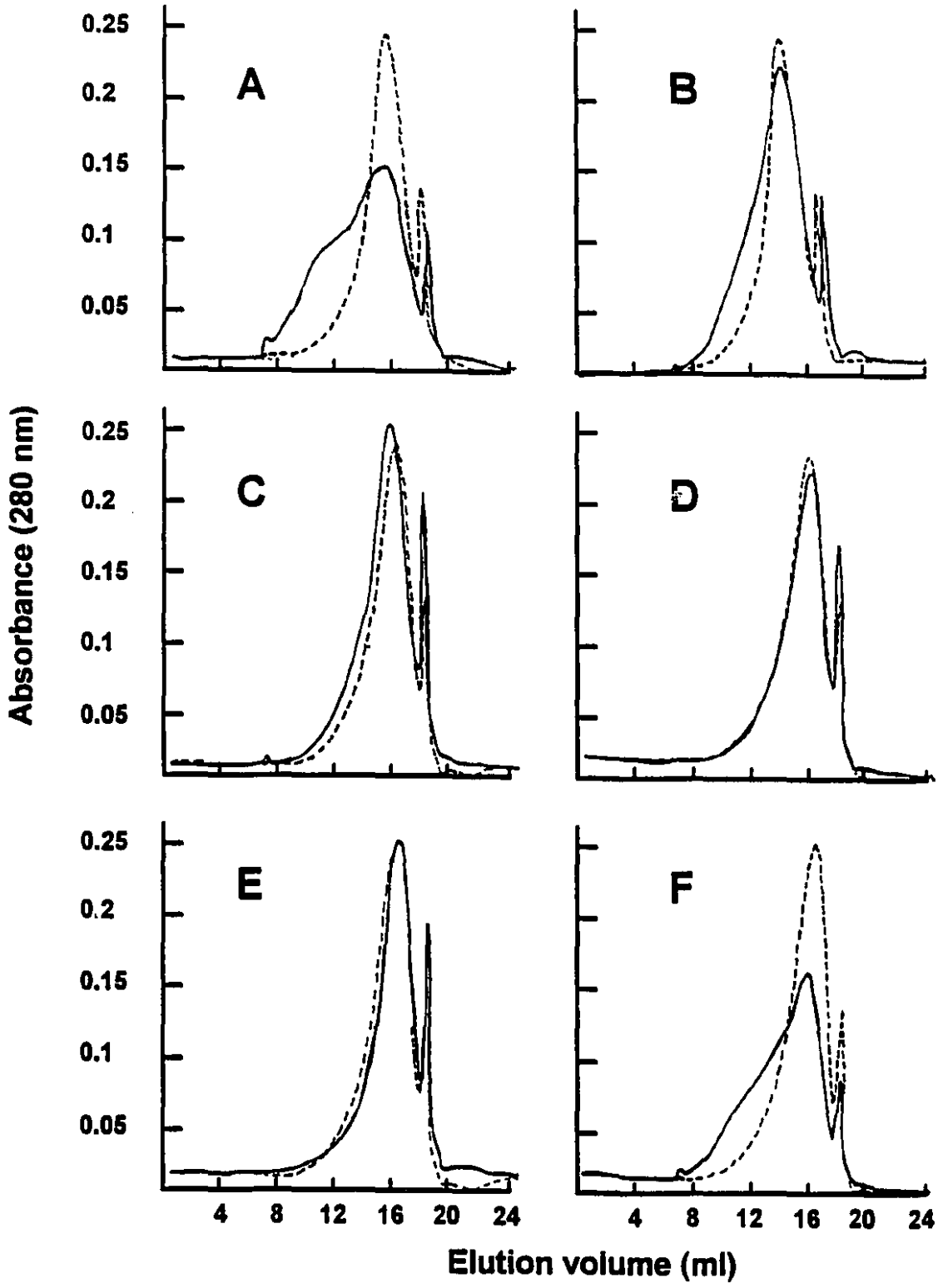
Absorption spectra were obtained using a Perkin-Elmer Lambda 3 spectrophotometer at room temperature in a 1 cm light path cuvette. Spectra of various reaction mixtures in 20 mM sodium acetate buffer (pH 4.5) were recorded.

6.3. RESULTS

6.3.1. Interactions of CBQase with laccase, kraft lignin, and phenolic substrates

Can CBQase influence the darkening and polymerization reactions accompanying the oxidation of kraft lignin by *T. versicolor* laccase? The gel permeation elution profile of dialyzed (MW>1000) Indulin AT (a kraft lignin preparation from black liquor) incubated with various combinations of enzyme is shown in Figure 6.1. Laccase substantially increased the mean molecular weight of the kraft lignin (Figure 6.1, panel A), as shown by the treated lignin eluting earlier from the gel filtration column. When sufficient CBQase was present, this increase in MW of the lignin sample was decreased substantially (Figure 6.1, panel B). A similar, though less extensive polymerization was seen when Indulin was incubated with MnP (Figure 6.1, panel C), and this was also inhibited in the presence of CBQase (Figure 6.1, panel D). CBQase alone produced a slight, though reproducible shift in the elution profile of the reaction mixture towards a lower average molecular weight (Figure 6.1, panel E). It was not clear whether the observed shift in the elution profile was due to a decrease in the molecular weight of the Indulin preparation, or to a change in the properties of the polymer by CBQase-mediated reduction of quinones or other functional groups. Such functional group changes could have altered the behavior of the Indulin and its subsequent elution from the gel filtration column. The absorbance at 465 nm of the reaction mixture was decreased by approximately 17% from 1.2 to 1.0 absorbance units in 24 h (data not shown), demonstrating that the Indulin was reactive with CBQase in the absence of added MnP or laccase. When Indulin was incubated with MnP, laccase and CBQase, substantial polymerization of Indulin was observed (Figure 6.1, panel F). This was due to an excess

Figure 6.1. Gel filtration chromatography profiles of Indulin (kraft lignin) after 24 h treatments with CBQase, laccase, and MnP. Samples of dialyzed (>12,000 MW) Indulin (5 μg/ml) were incubated in 5 mM sodium acetate buffer (pH 4.5) containing 10 mM cellobiose for 24 h at 27°C plus enzyme(s). Reaction mixtures with MnP, also contained 0.2 U/ml GO, 10 mM glucose, and 0.2 mM $MnSO_4$. Shown as solid lines are the elution profiles of Indulin treated as follows: 0.03 U/ml laccase (panel A); 0.03 U/ml of laccase plus 0.3 U/ml CBQase (panel B); 1.0 U/ml MnP (panel C); 1.0 U/ml MnP plus 0.3 U/ml CBQase (panel D); 0.3 U/ml CBQase (panel E); and 0.03 U/ml laccase plus 1.0 U/ml MnP plus 0.3 U/ml CBQase (panel F). In each case, the elution profile of the untreated Indulin control is shown with a dashed line. The control sample was incubated for 24 h under identical conditions without enzyme(s). Chromatography was as described in Materials and Methods (Section 6.2).


A
B
C
D
E
F
0.25
0.2
0.15
0.1
0.05
Absorbance (280 nm)
4
8
12
16
20
24
Elution volume (ml)

of Indulin-oxidizing over Indulin-reducing activity under the reaction conditions used here. If the CBQase to laccase or MnP ratio was raised sufficiently (20:1 for laccase and 1:1 for MnP), then the elution profile of Indulin was identical to that of the control (not shown). It has been suggested that MnP and laccase can act synergistically to promote more extensive lignin degradation then what is seen with either enzyme alone (Galiano *et al.*,1990). However, in another study using a kraft pulp system, no evidence of a synergistic effect between MnP and laccase was found (Paice *et al.*, 1993). No evidence of lignin depolymerization by MnP and/or laccase, in either the presence or absence of CBQase was ever observed during the course of these studies.

The enzymatic oxidation of guaiacol (2-methoxyphenol; MW=124.1) leads to the formation of a colored polymer (MW=5,000-15,000). This polymerization reaction (Westermark and Eriksson, 1974) and its product (Crawford *et al.*, 1981) have been proposed as a useful lignin model. Guaiacol is initially oxidized (and polymerized) by intact cultures of *T. versicolor* and *P. chrysosporium*, then subsequently decolorized (Westermark and Eriksson, 1974). It was shown that CBQase could mediate the decolorization reaction (Westermark and Eriksson, 1974). Addleman and Archibald (1994) used both guaiacol- and guaiacol polymerizate-containing agar plates to screen for *T. versicolor* isolates deficient in their ability to decolorize this material. All of their stable isolates which failed to decolorize were subsequently shown to also have impaired abilities to bleach kraft pulps.

Both the amount and rate of oxygen consumption by laccase are affected by the quantity and type of substrate present. As more substrate molecules are oxidized in the laccase-catalyzed reactions, the overall rate of oxygen consumption decreases. If CBQase

efficiently reduces the reaction intermediates formed by laccase back to their original phenol, then the oxygen consumption rate of laccase should remain constant or only decrease slowly over time. The oxygen consumption rate of laccase with a number of substrates was monitored in the presence and absence of CBQase (Figures 6.2,6.3). Though the specific consumption rate of oxygen by laccase was lower in the presence of Indulin and guaiacol polymerizate (poorer substrates), when CBQase and cellobiose were added to the reaction mixture, the oxygen consumption rate was maintained, suggesting that laccase-oxidizable substrates were being (re)formed by CBQase. Clearly, as the oxidation of guaiacol proceeds, the initial oxidation products formed by laccase stabilize into forms not reducible by CBQase, thus decreasing the total concentration of CBQase-reducible substrate. This is shown by the increasingly lower oxygen consumption rates in the reaction mixtures as the CBQase was added at increasing times after the reaction was initiated with laccase (Figure 6.2). These data also suggest that the interaction between laccase and CBQase occurs primarily via unstable laccase reaction intermediates with half-lives of seconds to minutes. Since the guaiacol polymerizate formed by laccase is, after a period of time, only a very poor substrate for CBQase (data not shown), the most likely candidates for reduction are the free radical reaction intermediates formed by laccase (see below). Guaiacol-containing reaction mixtures were analyzed by gel permeation chromatography after incubation with laccase with and without CBQase added at different times after the reactions were initiated. The results showed that the formation of polymeric products from guaiacol by laccase was completely inhibited when CBQase was added simultaneously (data not shown).

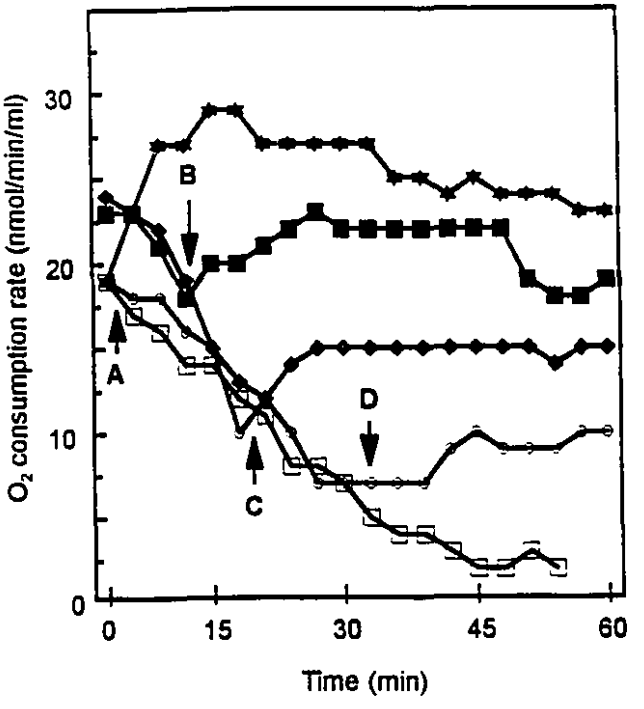


Figure 6.2 Oxygen consumption during the laccase-mediated oxidation of guaiacol in the presence and absence of CBQase. The rate of oxygen consumption was measured at three minute intervals in the presence of laccase alone (□) (0.03 U/ml) and laccase plus CBQase (0.3 U/ml) added 3 min (A) (✱), 12 min (B) (■), 20 min (C) (◆), and 32 min (D) (○) after the reaction was initiated with the addition of laccase. The reaction mixture (3 ml) contained; 2.1 mM guaiacol, 17.9 mM cellobiose, in acetate buffer (100 mM; pH 5.0). After each measurement of oxygen uptake a stream of air was passed through the reaction mixture in the oxygen cell for one minute to attain >90% oxygen saturation.

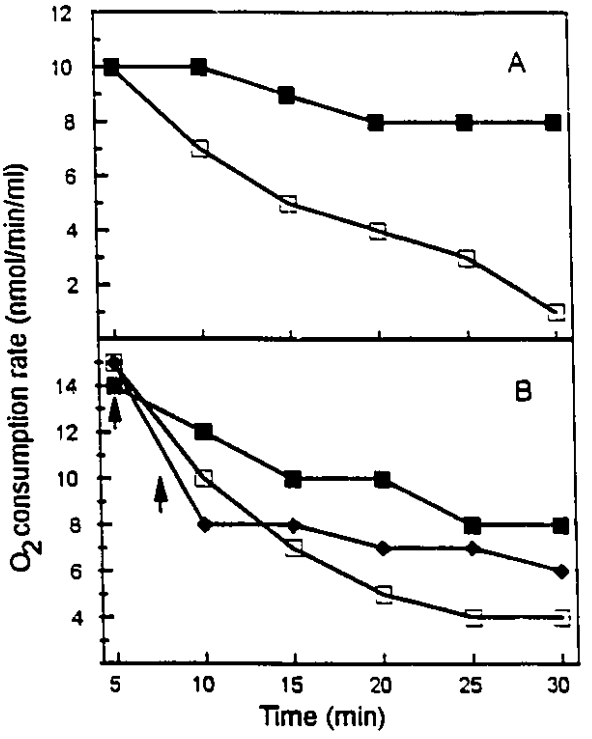


Figure 6.3. Oxygen consumption during the laccase-mediated oxidation of guaiacol polymerizate and Indulin in the presence and absence of CBQase. The reaction conditions were the same as described in Figure 6.2 except that the guaiacol polymerizate concentration was 420 μg/ml (panel A) and Indulin was 5 μg/ml Indulin (panel B). The oxidation of guaiacol polymerizate was initiated with the addition of laccase (0.03 U/ml), either alone (□), or with CBQase (0.3 U/ml) added to the reaction at 0 min (■). The oxidation of Indulin was initiated with the addition of laccase (0.03 U/ml), either alone (◆), or with CBQase (0.3 U/ml) added to the reaction at 0 min (■) or 8 min (•) after the reaction was started.

A further implication is that CBQase is not reducing laccase directly, Had there been direct reduction of laccase by CBQase, then the oxygen consumption in the reaction mixture should have increased to the initial rate irrespective of the time of CBQase addition.

6.3.2. EPR studies

EPR was used to confirm that CBQase was reducing the radical intermediates formed during the oxidation of various laccase substrates. The EPR signals of free radical species were first identified, and were then used to measure the kinetics of their formation and degradation. First laccase was incubated with ABTS and an EPR signal was found centered on a field strength of 341.7 mT having an isotropic g value of 2.0032 (Figure 6.4). The kinetics of formation of the free radical were followed by monitoring the peak to peak height of the EPR signal. The relationship between the EPR signal intensity and the ABTS radical concentration was calculated based on an extinction coefficient for the radical of 36 $mM^{-1}cm^{-1}$ at 420 nm (Wolfenden and Wilson, 1984). A concentration of 1 mM ABTS radical gave an EPR signal with a peak-to-peak height of the first derivative signal of 54 units for a 5µl sample. The EPR signal intensity was a linear function of the ABTS radical concentration in the range of 0.05 to 1 mM. The EPR signal intensity was followed over time in the presence of 0.3 U/ml *T. versicolor* laccase in the presence and absence of CBQase (Figure 6.5). These data illustrate the effect of CBQase on the fate of the ABTS free radical formed by laccase. As the concentration of CBQase was increased in the presence of a constant amount of laccase, the intensity of the EPR signal from the ABTS radical species was decreased. When the CBQase to laccase ratio was

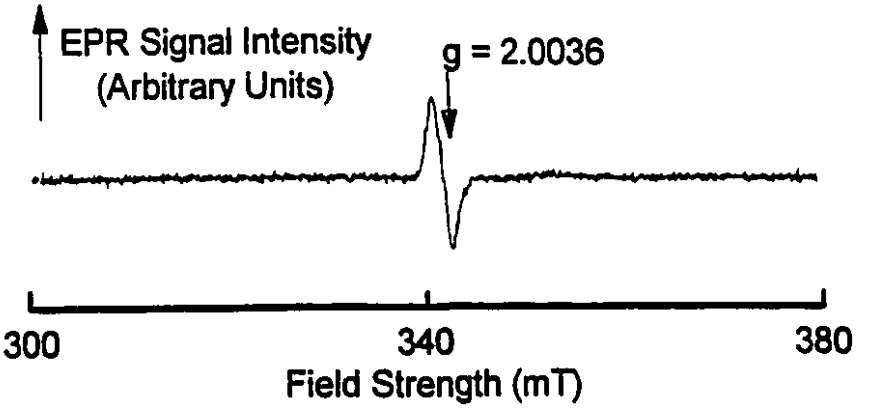


Figure 6.4. EPR signal from ABTS oxidized with laccase. The reaction mixture contained laccase (0.3 U/ml) and 1 mM ABTS in 100 mM acetate buffer (pH 4.5). Trace was recorded after 5 min incubation at ambient temperature (24°C). The sample volume was 5 µl. The instrument conditions were as follows: centerfield 340 mT, $\Delta H = 2.5$ mT, sweep time 100s, microwave power 2 mW, 20 db attenuation, with reading taken at room temperature (295K). The ABTS radical presumably producing this signal is shown in Figure 3.9.

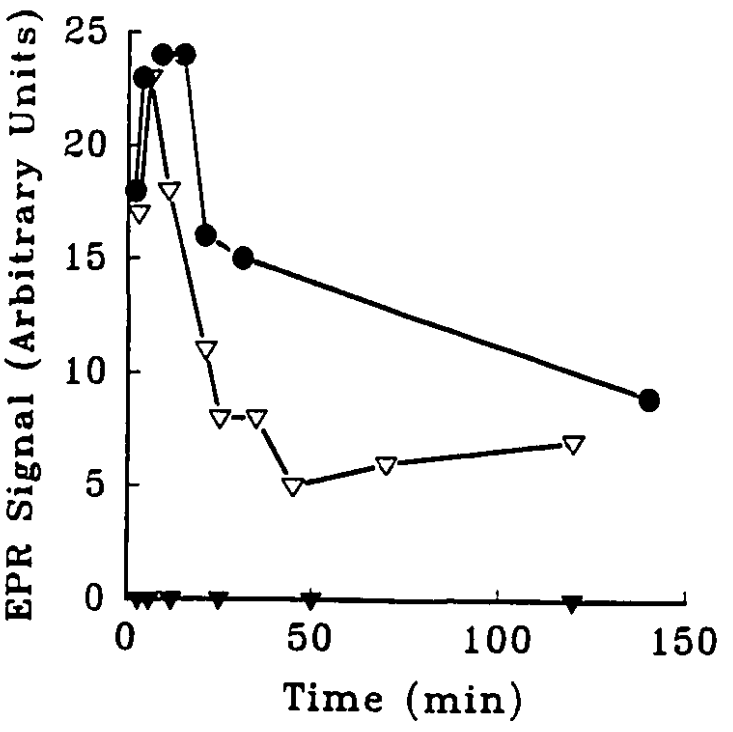


Figure 6.5 Effect of CBQase on the intensity of the EPR signal of laccase-oxidized ABTS (an ABTS radical). Repeated scans at the indicated time intervals were taken of a 5 µl aliquot of a reaction mixture containing 1 mM ABTS and 2mM cellobiose in 20 mM acetate buffer (pH 4.5) and either 0.3 U/ml laccase (•) or 0.3 U/ml laccase plus 0.001 U/ml (□), 0.01 U/ml (▿), or 0.13 U/ml (▼) CBQase. The concentration of the ABTS cation radical was measured as the peak-to-peak height of the first derivative of EPR signal.

low, the rate of increase in intensity of the EPR signal was inhibited in proportion to the amount of CBQase added. However, when the amount of CBQase was increased, the excess of reductive activity resulted in a decrease in the EPR signal intensity. If the CBQase to laccase ratio was large enough (≈3:1), then no EPR signal was seen (Figure 6.5). These data clearly show that CBQase will efficiently reduce the free radical produced from ABTS by laccase-mediated oxidation. Comparable results were also obtained when MnP was used to oxidize ABTS (data not shown).

The oxidation of CPZ by laccase was monitored by EPR, since like ABTS, it is oxidized to form a quasi-stable cation radical (Figure 3.9; Sariaslani *et al.*, 1984). The EPR signal from CPZ• radical was centered on a field strength of 341.8 mT and had an isotropic G value of 2.0047 (Figure 6.6). As seen with ABTS (Figure 6.5) the intensity of the EPR signal from laccase-oxidized CPZ decreased in proportion to the amount of added CBQase (Figure 6.7). These data support the model of CPZ oxidation and CPZ• radical reduction by CBQase proposed in Figure 4.3.

To determine how CBQase and laccase interact with a more lignin-like substrate, samples of dialyzed (MW> 1000) kraft lignin (Indulin AT) were incubated in the presence of laccase and the formation of free radicals in the reaction mixture was followed using the EPR. An EPR signal from the laccase-containing reaction mixture was observed at a field strength of 341.4 mT (g = 2.0028), typical of the signal commonly observed with phenoxy free radicals (Figure 6.8, trace a). Neither the position or the shape of the first derivative trace of the EPR signal, and hence the nature of the free radical formed, were changed in the presence of CBQase (Figure 6.8, trace b). Consistent with the results obtained with ABTS (Figure 6.5) and CPZ (Figure 6.7), the EPR signal observed with

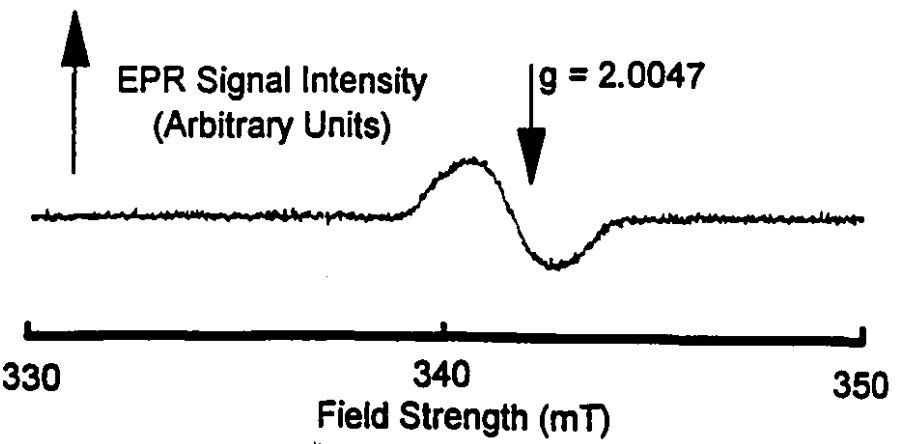


Figure 6.6 EPR signal from laccase-oxidized CPZ. The reaction mixture contained laccase (0.5 U/ml) and 2 mM CPZ in 100 mM acetate buffer (pH 4.5). The EPR signal was recorded after 12 min incubation at ambient temperature (24°C). The sample volume was 5 µl. The instrument conditions were as follows: centerfield 340 mT, ΔH = 2.5 mT, sweep time 100s, microwave power 1 mW, 23 db attenuation, with reading taken at room temperature (295K).

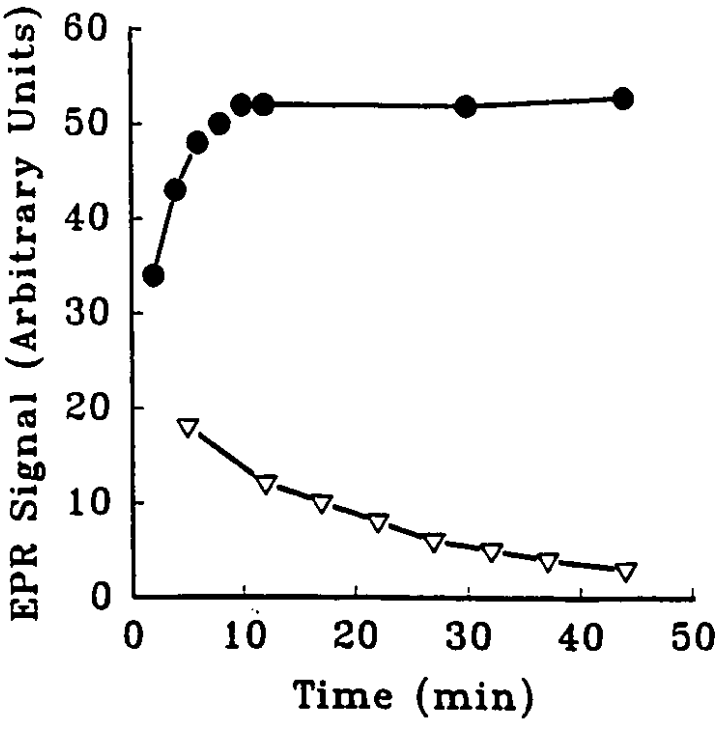


Figure 6.7. Effect of CBQase on the intensity of the EPR signal from CPZ. Repeated scans at the indicated times were taken of a 5 μl aliquot of a reaction mixture containing 2 mM CPZ and 2mM cellobiose in 20 mM acetate buffer (pH 4.5) and either 0.5 U/ml laccase (•) or 0.5 U/ml laccase and 0.3 U/ml CBQase (▵). The concentration of the ABTS cation radical was measured as the peak-to-peak height of the first derivative of the EPR signal.

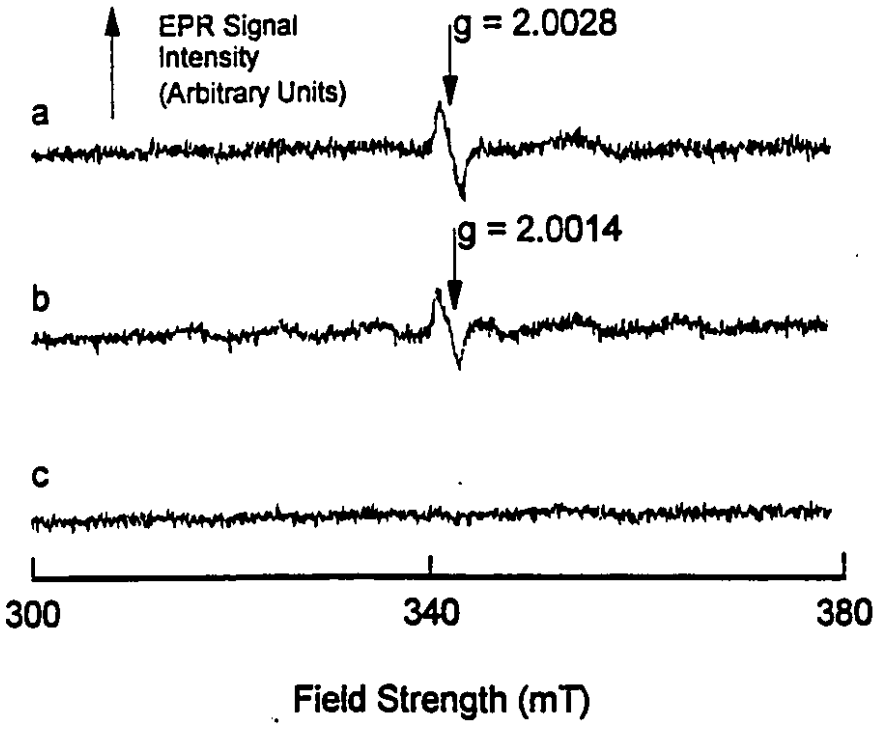


Figure 6.8 EPR signals from Indulin treated with laccase and CBQase. The reaction mixtures contained dialyzed (MW >12,000) Indulin (5 μg/ml) in 20 mM acetate buffer (pH 4.5) and: (a) 0.3 U/ml laccase; (b) 0.3 U/ml laccase plus 2 mM cellobiose and 0.13 U/ml CBQase; and (c) 0.3 U/ml laccase plus 2 mM cellobiose and 0.6 U/ml CBQase. The sample volume was 5 μl. No signal from Indulin was detected in the absence of added laccase The instrument conditions were as follows: centerfield 340 mT, $\Delta H = 2.5$ mT, sweep time 100 s, microwave power 2 mW, 20 db attenuation, with reading taken at room temperature (295K).

Indulin as substrate disappeared when the CBQase to laccase ratio was greater than 2:1 (Figure 6.8, trace c). The intensity of the EPR signal produced during this laccase-mediated oxidation of Indulin was decreased in proportion to the amount of CBQase present (Figure 6.9). Like oxygen consumption by laccase when Indulin was the substrate (Figure 6.3), the EPR signal and therefore the amount of substrate available for laccase-mediated oxidation decreased with time, and the substrate was completely oxidized after 3 h of incubation. In the presence of CBQase, an EPR signal (4.5 mm peak to peak height of the first derivative trace) was still detectable after 16 h. This demonstrates that CBQase can reduce not only relatively stable free radicals such as ABTS and CPZ, but also those having much shorter half life, such as the phenoxy radical species which are formed by laccase during the oxidation of Indulin. These data also show that CBQase can reduce radicals formed on large polymeric substrates. This property is probably essential if CBQase participates in the extracellular attack on residual lignin present in kraft pulps.

6.3.3. Protein-protein Interactions

Oxygen consumption by laccase (as seen in Figures 6.2 and 6.3) could occur in the absence of substrate redox cycling if the CBQase protein were able to directly reduce the laccase protein. In the absence of added CBQase and cellobiose, there was no oxygen consumption by laccase alone (except for the small demand of the polarographic cell electrode itself). When laccase (10 U per ml) was placed in the oxygen cell with CBQase (10 U per ml) without a phenolic substrate, an oxygen consumption rate of 0.8 nmoles per minute was observed. However, this was much lower than the rate at which laccase reacted with most phenolic substrates as well as the rate for the standard assay substrate

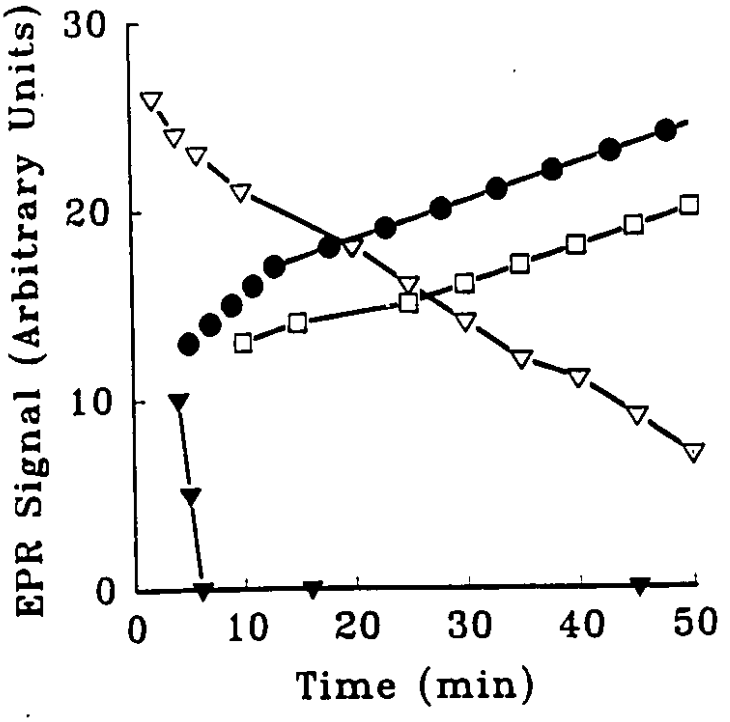


Figure 6.9 Intensity of an Indulin radical EPR signal produced by laccase in the presence of different concentrations of CBQase. EPR signals from the reaction mixtures described in Figure 6.8 were monitored at the times indicated and the peak-to-peak height of the first derivative of the EPR signals were determined. The reaction mixtures contained dialyzed (MW >12,000) Indulin (5 μg/ml) in 20 mM acetate buffer (pH 4.5) and either 0.3 U/ml laccase (•), 0.3 U/ml laccase plus 2 mM cellobiose and 0.13 U/ml CBQase (▽), or 0.3 U/ml laccase plus 2 mM cellobiose and 0.6 U/ml CBQase (▼). The sample volume was 5 μl. In the absence of added laccase, no signal was detected from any indulin reaction mixture.

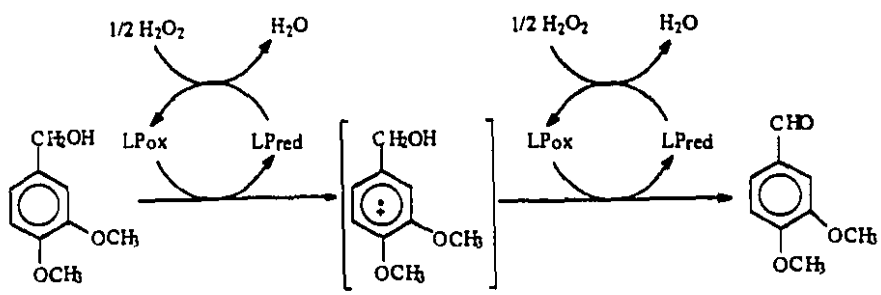
where 10 μmoles of ABTS were being oxidized per minute under the same conditions.

6.3.4. Interaction of CBQase with lignin peroxidase and veratryl alcohol oxidase

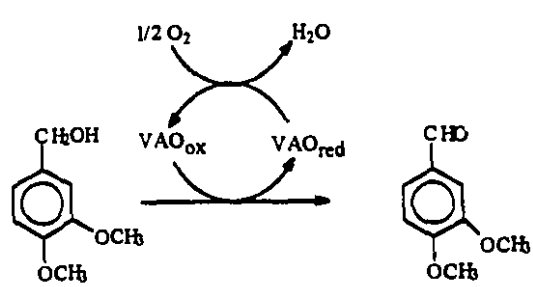
The rates of veratryl alcohol oxidation by lignin peroxidase (LP) and veratryl alcohol oxidase (VAO) were compared in the presence and absence of *T. versicolor* CBQase. VAO is believed to oxidize veratryl alcohol via a two-electron abstraction mechanism with no intermediate leaving the active site of the enzyme, as shown in Scheme I (p. 194). In contrast, LP is believed to catalyze two one-electron oxidations with the formation of a free transient (soluble) veratric cation radical intermediate as shown in Scheme II, although the existance of the radical intermediate could not be conclusively demonstrated (Tien *et al.*, 1986). If these hypotheses are correct then CBQase in the presence of cellobiose should inhibit the formation of veratrylaldehyde by LP but not by VAO. When such an experiment was performed, this was the case, providing further evidence that CBQase directly reduces the organic free radicals formed by LP and not the stable product (veratraldehyde) (Table 6.1). In controls, CBQase was unable to reduce the LP protein or veratrylaldehyde directly. When equimolar amounts of LP and CBQase were incubated in the presence of cellobiose and H_2O_2, no H_2O_2 was consumed over 45 min as measured by $\Delta OD_{240\ nm}$.

6.3.5. Interaction of CBQase with MnP

As with LP, oxidized MnP (compound II) was not directly reduced at a detectable rate by CBQase in the absence of phenolic substrates. A reaction mixture (1.5 ml) containing malonate buffer (50 mM, pH 4.5), MnP (0.2 U), CBQase (0.2 U), cellobiose



Scheme I



Scheme II

Table 6.1. Effect of *T. versicolor* CBQase on veratryl alcohol oxidation by LP and VAO, and phenol red oxidation by MnP.

Oxidative enzyme	Quantity of CBQase added[1] (U/ml)	Rate of veratraldehyde production/ phenol red oxidation[2] (nmoles min^{-1})	Inhibition (%)
LP	0	32	0
	0.012	16	50
	0.06	0.5	84.3
	0.12	0	100
VAO	0	32	0
	0.012	32	0
	0.06	32	0
	0.12	32	0
MnP	0	26	0
	0.012	18	31
	0.06	11	58
	0.12	0	100

[1] Reaction was started with addition of H_2O_2 (MnP and LP) or enzyme (VAO) and allowed to continue until a steady rate of oxidation was obtained (15-30 s), then CBQase was added (2 µl aliquots). The reaction was allowed to proceed until the new equilibrium rate was established, then a second aliquot of CBQase was added and so on.

[2] Phenol red oxidation was determined using an experimentally determined extinction coefficient of 10.8 mM^{-1} cm^{-1} (420 nm) in 50 mM sodium malonate buffer (pH 4.5). VAO and LP activities were determined in 50 mM sodium tartrate buffer (pH 4.0).

(2 mM) and H_2O_2 (0.1 mM) was monitored (240 nm) to measure the disappearance of H_2O_2. However, when MnP activity was measured using phenol red as the oxidizable substrate, a decrease in the rate of oxidation was observed with increasing additions of CBQase until the reaction was completely inhibited (Table 6.1). The reduction in the rate of phenol red oxidation observed here was undoubtably due to the CBQase-mediated reduction of the Mn(III)-malonate formed by MnP, since this Mn(III)-complex is an excellent substrate for CBQase (Figure 3.9, Group IV). Clearly, many if not all free radicals formed by one-electron oxidations of aromatic substrates by laccase, LP, or MnP will be efficiently reduced if CBQase and an appropriate electron donor (cellobiose or cellulose) are present (see Chapter 5). Thus, the relative amounts of reductive and oxidative activities produced by the white-rot fungi are likely to be coordinately regulated, since, as shown in Table 6.1, the presence of an excess of CBQase may defeat or decrease the net oxidative activities of MnP, laccase and LP.

6.3.6. Combined MnP and CBQase treatments of kraft pulps

As the results given in sections 6.3.1.-6.3.5., and reports in the literature (Ander *et al.*, 1990; Samejima and Eriksson, 1992) clearly show, CBQase can and does readily interact with and quench the reaction intermediates (free radicals) commonly formed by laccase, LP and MnP. These three oxidative enzymes being the most commonly proposed as being central to fungal lignin biodegradation.

Though it was shown in Chapter 2 that *T. versicolor* 52J secreted a large number of both low and high molecular weight metabolites, many of which were induced by the presence of pulp, during the course of lignin biodegradation, I speculated that it might be

possible to obtain enhanced lignin degradation using a simpler cell-free system. The limited kraft pulp residual lignin degradation which was obtained using MnP (Paice *et al.*, 1993) and laccase in the presence of ABTS (Bourbonnais and Paice, 1992) suggested this was a reasonable approach.

The most promising oxidative enzyme to combine with CBQase for kraft pulp trials was MnP, since it has been shown that manganese was essential for *T. versicolor*-mediated bleaching (F. Archibald, pers. commun.). Laccase was not chosen because a mediator functioning like ABTS has not been identified in *T. versicolor* cultures, and without a mediator, the extent of laccase-mediated delignification is much less than that obtained using MnP and Mn (Paice *et al.*, 1993).

Since the quinones produced by the MnP-mediated oxidation of phenols are likely substrates for CBQase, it seemed logical to try both simultaneous and sequential treatments of pulp with MnP and CBQase. Furthermore, since during the growth of *T. versicolor* on kraft pulp, CBQase activity is secreted before MnP (Chapter 2), the reverse sequence was also tried. The lignin content (kappa number) and brightness of the enzyme-treated pulp were determined, together with the release of methanol which has previously been associated with fungal and enzymatic delignification (Paice *et al.*, 1993; Bourbonnais and Paice, 1992). The results (Table 6.2) indicate that CBQase (0.5 U/ml) added simultaneously with or after MnP gives slightly enhanced delignification as measured by a decrease in kappa number. However, at least in this system, the use of CBQase alone, or when added to kraft pulp prior to MnP addition, did not bleach and delignify significantly.

6.3.6. Formation of Mn-complexing agents promoting MnP activity on residual kraft lignin

Previous studies demonstrated that a 50 mM malonate buffer at pH 4.5 gave better MnP-mediated oxidation of lignin in kraft pulp than oxalate at the same concentration (Paice *et al.*, 1993). It was also found that cellobionic acid from the CBQase-mediated oxidation of cellobiose or cellulose and gluconate from glucose oxidase oxidation of glucose supported MnP-mediated oxidation of phenol red by acting as Mn(III) complexing agents (Paice *et al.*, 1993; Roy *et al.*, 1994; this thesis Chapter 5). Could CBQase form effective Mn(III)-complexes in a cell-free enzyme-catalyzed delignification system? A hardwood kraft pulp was treated with both MnP and CBQase, where the only source of Mn(III)-complexing agents were the sugar acids formed by CBQase-mediated oxidation of either cellulose or both cellobiose and cellulose. The presence of CBQase in the cell-free delignification system increased both extent of delignification and demethylation of the hardwood kraft pulp (Table 6.3). CBQase provided the Mn(III)-complexing agents essential for MnP activity in this system. The increase in the extent of delignification and demethylation of kraft pulp mediated by cellobionic acid and/or the longer-chain cellulose-derived carboxylic acids formed by CBQase is consistent with the increase in phenol red oxidation by MnP seen when purified sugar acids were used in place of a malonate buffer (Chapter 5, this thesis; Roy *et al.*, 1994). As before (Chapter 5, this thesis), MnP activity, as estimated by the extent of delignification and demethylation occurring, was increased when CBQase-generated cellobionic acid replaced malonate. The formation of Mn(III)-complexing sugar acids thus represents a second

Table 6.2. Simultaneous and sequential 24-h treatments of hardwood kraft pulp with MnP and CBQase.[1]

Enzyme treatment	Methanol (mg/l)	Kappa number
MnP (1 U/ml)[2]	16.4 ± 0.3[3]	12.5 ± 0.1
CBQase (0.5 U/ml)[4]	3.3 ± 1.3	13.4 ± 0.2
Control (no enzyme)	2.0 ± 2.0	13.2 ± 0.2
Simultaneo		
MnP + CBQase (0.05 U/ml)	15.3 ± 0.2	12.8 ± 0.1
MnP + CBQase (0.5 U/ml)	20.3 ± 0.7	12.1 ± 0.1
Sequential[5]		
MnP then CBQase (0.05 U/ml)	16.4 ± 0.3	12.6 ± 0.1
MnP then CBQase (0.5 U/ml)	16.4 ± 0.3	12.1 ± 0.1
CBQase (0.05 U/ml) then MnP	2.8 ± 2.8	12.6 ± 0.1
CBQase (0.5 U/ml) then MnP	4.3 ± 1.3	12.9 ± 0.0

[1] Reaction mixtures (200 ml) contained 2% w/v hardwood kraft pulp (initial kappa number 14.1) at 2% consistency in 500 ml Erlenmeyer flasks, in malonate buffer (50 mM; pH 4.5), and were incubated at 27°C on a gyratory shaker (r = 8 mm) at 200 rpm for 24 h.

[2] MnP (1 U/ml) was added with $MnSO_4$ (0.5 mM), glucose (10 mM), and glucose oxidase (0.025 U/ml).

[3] Values shown are the means of duplicate determinations ± standard deviations.

[4] CBQase at the concentrations shown was added with cellobiose (10 mM).

[5] Sequential additions were for 24 h treatments with CBQase and MnP in the order shown with their appropriate accessory components.

Table 6.3. Ability of CBQase to provide Mn-complexing agents permitting MnP to function in kraft pulp.

Sample treatment[1]	Methanol production (mg/l)				Kappa number
	Day 1	Day 2	Day 3	Day 4	Day 4
MnP + CBQase + cellobiose[2]	8.0	12.0	11.2	11.0	12.3
	11.7	16.2	16.5	18.5	11.2
MnP + CBQase[3]	15.8	18.5	18.5	17.8	10.9
	15.2	16.6	18.0	17.9	11.1

[1] Treatments were on 2% w/v hardwood kraft pulp (kappa number 14.1) (4 g) at 2% consistency in 500 ml Erlenmeyer flasks agitated at 200 rpm (r= 8 mm) kept at 27°C for 4 days, with samples (5 ml) taken daily for methanol analysis. Duplicate samples are shown for each treatment.

[2] Reaction mixture contained pulp plus 1.0 U/ml MnP, 0.5 U/ml CBQase, 0.5 mM $MnSO_4$, and 10 mM cellobiose incubated for 24 h at 27°C. H_2O_2 additions were made using a peristaltic pump to maintain a concentration of 1 mM in the reaction flask.

[3] Reaction mixture as is 2, less cellobiose

possible role for CBQase in the extracellular kraft pulp delignification and biobleaching system of *T. versicolor*.

6.3.7. Effect of CBQase on the alkaline extractability of residual lignin

The solubility of lignin in neutral and alkaline aqueous solutions is a function of both its size and charge. Since phenols formed by CBQase will become more soluble due to their ionization at high pH, the effect of CBQase on the kappa number of extracted pulp was investigated. As shown in Table 6.4, there is clearly an increased solubility of lignin (a very desirable change when viewed from the perspective of an industrial process) when CBQase is the last enzyme in the sequence. The combined demethylation and reduction mediated by MnP and CBQase, respectively, probably results in a net increase in phenolic content as shown in the Scheme III. The proposed mechanism of demethylation (Scheme III) by MnP leads to the formation of *ortho*-quinones, many of which are good substrates for CBQase. MnP treatments demethylate hardwood kraft pulp leading to a significant decrease in phenolic methoxyl content of residual lignin (Paice *et al.*, 1993). Demethylation is therefore likely to lead to *ortho*-quinone formation. Such *ortho*-quinones are more difficult to ionize at alkaline pH than are phenols, likely making the residual lignin less soluble. Furthermore, the oxidation of the residual lignin by MnP likely decreases the total phenolic content of the hardwood kraft pulp, further decreasing its alkaline extractability.

Thus, the sequential action of MnP and CBQase may also account for the increased alkali extractability of lignin observed when softwood kraft pulp was bleached with *T. versicolor* (Reid *et al.*, 1990).

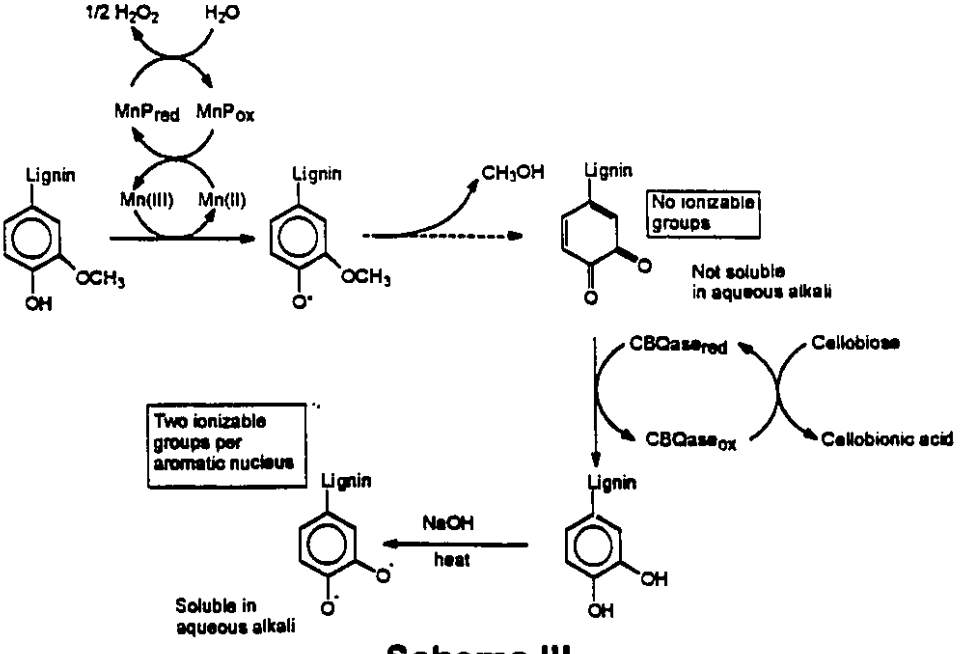
Table 6.4. Effect of MnP and CBQase treatments of kraft pulps on alkaline extractability of lignin.

Enzyme Treatment[1]	Hardwood pulp		Softwood pulp	
	Kappa before extraction	Kappa extracted[2]	Kappa before extraction	Kappa extracted
MnP	11.6 ± 0.4[3]	1.0 ± 0.0	23.0 ± 0.1	3.9 ± 0.2
CBQase	12.4 ± 0.1	2.3 ± 0.0	24.7 ± 0.0	3.9 ± 0.2
MnP then CBQase	11.8 ± 0.2	2.1 ± 0.1	23.6 ± 0.1	5.3 ± 0.7
Control, no enzyme	13.2 ± 0.3	2.1 ± 0.2	24.2 ± 0.1	1.8 ± 0.2

[1] Enzyme treatment was on 2% w/v pulp containing 1 U/ml MnP with 0.5 mM $MnSO_4$, 50 mM malonate (pH 4.5), 10 mM glucose, and 0.025 U/ml glucose oxidase incubated at 27°C for 24 h. The CBQase treatments followed MnP treatments and contained 0.5 U/ml CBQase, 10 mM cellobiose in 50 mM malonate buffer (pH 4.5), and were incubated for 24 h at 27°C . Initial kappa numbers were 14.1 and 29.5 for hardwood and softwood pulps, respectively.

[2] Pulps were extracted NaOH (0.11 times the pulp kappa number in grams of NaOH per gram of oven dry pulp) at 70°C for 90 min. They were then washed with deionized water until the pH was <8.

[3] Values shown are the means of duplicate determinations ± standard deviations.


1/2 H2O2
H2O
MnPred
MnPox
Lignin
Mn(III)
Mn(II)
OCH3
OH
Lignin
OCH3
O•
CH3OH
Lignin
No ionizable groups
O
O
Not soluble in aqueous alkali
CBQasered
Cellobiose
CBQaseox
Cellobionic acid
Two ionizable groups per aromatic nucleus
Lignin
NaOH
heat
O•
O•
Soluble in aqueous alkali
Lignin
OH
OH

Scheme III

6.4. DISCUSSION

The observation that white-rot fungi could decolorize quinones and quinonoid phenol oxidation products was first reported by Law (1959). When grown on phenol-containing agar plates, *Polyporus sanguineus* would initially oxidize the phenols to form colored products, then slowly decolourize them (Law, 1959). Observation of a similar phenomenon around growing colonies of *Polyporus* (*Trametes*) *versicolor* and *Phanerochaete chrysosporium* led to the discovery of the enzyme CBQase (Westermark and Eriksson, 1974). CBQase catalyzed the carbohydrate-dependent decolorization of the phenol oxidation products and the reduction of many different quinones (Westermark and Eriksson, 1974a). In a related study, it was found that the polymerization of a lignosulphonate by cultures of *Fomes annosus* was inhibited by cellobiose (Hütterman *et al.*, 1977), and CBQase was subsequently identifed in their culture system (Hütterman and Noëlle, 1980). It was shown that *P. chrysosporium* CBQase decreased the consumption of guaiacol by laccase (Westermark and Eriksson, 1974) presumably by inhibiting or reversing the formation of coloured polymerization products similar to those described here (Figure 6.2) and elsewhere (Bao *et al.*, 1993). That the high initial oxygen consumption of laccase was unaffected by CBQase (Figure 6.3 a,b,c) suggests that the reduction mediated by CBQase was of the organic radical intermediates formed by laccase from the substrate and not a direct effect on the laccase protein. This was confirmed by protein only controls.

The CBQase-dependent decrease in the EPR signals associated with the cation free radicals formed from CPZ and ABTS by *T. versicolor* laccase (Figures 6.5, 6.7) is consistent with a published report showing that the EPR signal associated with the cation

radical formed from tetramethoxybenzene when oxidized with LP was not detectable in the presence of CBQase and cellobiose oxidase (Eriksson and Samejima, 1992). Though Odier *et al.* (1988) had suggested that CBQase has no effect on the tetramethoxybenzene radical, evidence published since suggests that CBQase does indeed reduce this cation radical (Samejima and Eriksson, 1991). In addition, other cation and phenoxy radical intermediates are also susceptible to CBQase-mediated reduction (Figures 6.6, 6.7, 6.8) (Ander *et al.*, 1990; Bao *et al.*, 1993; Samejima and Eriksson, 1992). The EPR scan shown in Figure 6.8 clearly shows that CBQase reduces the phenoxy radical intermediates formed from kraft lignin (Indulin AT). This is the first direct evidence that CBQase reduces free radical intermediates formed in a high molecular weight lignin-derived substrate.

Recently Ander *et al.*(1993) demonstrated that the H_2O_2-oxidized form of LP (compound II) could be reduced directly by CBQase and cellobiose with the concommitant formation of H_2O_2. That CBQase-mediated reduction was indicated by a shift in the Soret absorption peak associated with the oxidized heme of both MnP and LP (Ander *et al.*, 1993). However, these results were obtained using 100-fold higher concentrations of CBQase and LP proteins than were used in this study. In the system used here, a very low rate of peroxidase reduction could have been masked by a low level of H_2O_2 production via various mechanisms (Chapter 3, this thesis; Kremer and Wood, 1992). However, Ander *et al.* (1993) have also shown that the reactions of oxidized LP with its "normal" range of substrates (e.g. aromatic alcohols) are much faster than with CBQase, and therefore direct reduction of LP by CBQase was not a significant reaction in the presence of aromatic (phenolic and nonphenolic) substrates.

CBQase can also interact with LP, MnP, and laccase enzymes by reducing the free radical species which are their initial oxidation products. Ander *et al.* (1990) suggest that inclusion of CBQase in a reaction mixture with kraft lignin, and a crude enzyme preparation containing laccase and LP leads to the depolymerization of the lignin in the reaction mixture. However, in the systems described here containing *T. versicolor* MnP or laccase and CBQase, no evidence was found for a net depolymerization of lignin (Figure 6.1), though in the presence of CBQase, further polymerization of the Indulin AT kraft lignin was strikingly inhibited. This evidence (Figure 6.1) coupled with the direct observation of CBQase quenching phenoxy radicals (Figure 6.8) formed from this polymeric lignin-derived substrate, makes it clear that CBQase, if present with cellobiose, will be an active participant in any such system.

Can the interaction of CBQase with MnP lead to increased rates and extents of delignification of the residual lignin in kraft pulp? Data in Tables 6.2, 6.3, and 6.4 indicate that there are small but probably significant benefits when combinations of MnP and CBQase are present compared to using either enzyme alone. Clearly, CBQase has a significant impact on the reactions which occur on the residual lignin. When CBQase is added together with MnP, it complimented the activity of the latter enzyme leading to a slightly greater delignification (Tables 6.3, 6.4). The ability of CBQase to furnish MnP with a useful complexing agent (cellobionic acid) for Mn(III) (Table 6.4), may be a significant role of CBQase as was proposed in Chapter 5 (Figure 5.3). That CBQase rendered the residual kraft lignin more alkali soluble presumably by reducing quinones to hydroquinones (Table 6.4) may contribute to the overall delignification obtained with whole cultures of *T. versicolor*, especially with softwood pulps (Reid *et al.*, 1990; Reid

and Paice, 1994). It is also a very useful result in the industrial context, since alkaline extraction stages are nearly always used between pulp bleaching stages.

Possible reasons for the relatively small effects of CBQase on the delignification of the kraft pulps tested include: (1) the inaccessibility of Mn(III)-oxidized residual lignin species to CBQase; (2) the use of inappropriate concentrations or ratios of CBQase and MnP; (3) the use of inappropriate Mn-complexing agents or incorrect Mn(III): complexing agent ratios or concentrations; (4) the incorrect temporal addition of each enzyme; (5) the absence of one or more important delignifying cofactors. Some of these possibilities, for example the inaccessibility of the residual lignin to protein-size molecules and the large effects that Mn(III):complexing agent ratios and types have on Mn(III) reactivity have been demonstrated (Strebotnik and Messner, 1991; Archibald and Valeanu, Unpublished data). Since the reactions of CBQase and MnP are competitive/complimentary, their addition in the correct proportions and at appropriate times (as illustrated in Figure 5.3) are probably crucial to the proper and extended functioning of the delignification system. Unlike us, *T. versicolor* knows how to control both the amount and timing of enzyme addition in a fashion which favours delignification.

CHAPTER 7. OVERALL CONCLUSIONS

Cultures of *T. versicolor* 52J can actively bleach and delignify hardwood kraft pulp concurrently with mycelial growth (primary metabolism), by inducing the required complement of ligninolytic activities. An extensive and detailed examination of the extracellular components appearing and present during the course of *T. versicolor*-mediated bleaching of kraft pulps revealed a complex mixture of metabolites, enzymes and polymeric oligosaccharides. A carbon balance of *T. versicolor* in the presence and the absence of hardwood kraft pulp clearly showed that overall, the intermediary metabolism of the fungus was little affected by kraft pulp under the biobleaching conditions used. Therefore pulp biobleaching and delignification do not require or induce any major intermediary metabolic shifts by *T. versicolor*. However there were quantitatively small, but potentially significant shifts in the types and quantities of acidic metabolites secreted by *T. versicolor*. A cocktail of those metabolites most induced by the presence of pulp proved to be an effective Mn(III)-complexing mixture, supporting the oxidation of phenol red, a polyaromatic phenol, by MnP. That the fungus responds to the presence of pulp by secreting Mn-complexing species, suggests that Mn-complexes are important in pulp delignification.

Furthermore, the secretion of Mn(III)-complexing high molecular weight polysaccharides by *T. versicolor* cultures suggests that a range of complexing agents may be involved in kraft pulp delignification. Kraft pulp also induced the secretion of both MnP and CBQase isozymes in *T. versicolor* cultures, providing further evidence that manganese likely plays a central role in fungal delignification.

Though *T. versicolor* MnP, in the presence of H_2O_2, probably produces the greatest flux of Mn(III) ions, it is not the sole enzyme capable of oxidizing Mn(II) to Mn(III). Indeed it was found that laccase, in the presence of appropriate phenolic substrates, produces a significant flux of Mn(III) ions. This may be an important source

of phenolic lignin oxidizing Mn(III) ions when there is no H_2O_2 available for the proper functioning of MnP.

T. versicolor secreted two CBQase proteins that reduce a broad range of substrates that included quinones, manganese (Mn(III)-complexes and insoluble MnO_2), and many free radical species while oxidizing cellobiose and a few other carbohydrates. Though the *T. versicolor* CBQases reduced most quinones, these enzymes reduced organic free radicals and Mn(IV) even more avidly, suggesting that reduction of species other than quinones may be an important function for this enzyme *in vivo*. The direct demonstration of free radical quenching and Mn(II) formation from Mn(IV) by CBQase by EPR supports this hypothesis.

CBQase(s) appear to be an important component of the kraft pulp bleaching and delignification system of *T. versicolor*. Three catalytic activities of CBQase illustrate how I believe CBQase is involved in the delignification process. First, CBQase catalyzes the extracellular reduction of insoluble MnO_2 to Mn(II) or Mn(III), which can then return to the MnP catalytic cycle. Second, CBQase oxidizes oligosaccharides and its preferred substrate cellobiose to sugar acids, which are effective Mn(III)-complexing agents and provide both soluble and imobilized Mn(III)-complexing sites within lignocellulosic materials. Finally, the ability of CBQase to directly reduce organic free radical intermediates provides the fungus with a means of inhibiting the polymerization reactions initiated by the one- electron oxidations catalyzed by laccase, LP and MnP. This free radical-reducing activity of CBQase was shown to substantially inhibit the polymerization of kraft lignin (Indulin) mediated by laccase and MnP. Unlike all other biological polymers, whose synthesis and degradation are mediated by very different enzymes and reactions, lignin synthesis and degradation both occur via organic free radical reactions. Therefore, whether quenching free radicals promotes synthesis or degradation will depend on more subtle factors such as: (1) the concentration of free radicals in the system; (2) the availability of radical coupling sites on both the substrate (lignin) and the lignin fragments formed during degradation; and (3) the rate of removal of lignin fragments from the

reaction system; (4) the number of potential sites (e.g. free phenolic groups) for the formation of free radicals present in the lignin; (5) the availability and concentration of oxygen or hydrogen peroxide for laccase and MnP, respectively; and (6) the availability of reducing equivalents for CBQase.

A simple cell-free delignification system using CBQase and MnP with a source of non-enzymatic substrates was able to mediate small, but significant delignification of kraft pulps. Both the sequence of addition and the relative amounts of CBQase and MnP are likely critical for obtaining a significant amount of kraft pulp delignification, yet a simple sequential addition of MnP followed by a CBQase treatment gave a small increase in the extent of delignification of kraft pulp. This provides direct evidence that CBQase is an important, though perhaps not essential, component of the *T. versicolor* delignification system. In these enzyme trials, only a small range of enzyme addition ratios, rates and conditions were tried, nonetheless small effects on kraft pulp lignins were observed. These findings suggest that cooperativity between these two enzymes exists.

To date, the most effective enzymatic delignification effects observed are indirectly mediated by small molecules such as the Mn(III)-complexes and the ABTS free radical, which presumably can diffuse into regions of the pulp fibres inaccessible to protein-sized molecules like CBQase. A possible limitation of this *in vitro* system is that no reductive mediators (e.g. glutathione) were identified or used in these studies. Furthermore, the availability of numerous reducing ends on cellulose chains may be required for proper functioning of CBQase in a wood pulp system; however, no cellulases were present in our *in vitro* delignification systems to produce them.

Preface to Appendix A.

Early work by Kenten and Mann (1950) demonstrated that horseradish peroxidase could, in the presence of certain simple phenolic substrates, mediate the oxidation of Mn(II) to Mn(III). Laccase(s) carry out one-electron oxidations of phenolic substrate similar to those of the peroxidases and are always present in biobleaching cultures of *T. versicolor* 52J (Chapter 2). Since Mn(II) oxidation was proposed to occur via the production of phenoxy radicals by HRP from its phenolic substrates, many of which are also substrates for laccase, it seemed reasonable that laccase might also be able to produce Mn(III). This study was aimed at determining whether such a laccase-based system could produce a significant flux of Mn(III) ions.

APPENDIX A. Production of manganic chelates by laccase from the lignin-degrading fungus *Trametes (Coriolus) versicolor*

A.1. INTRODUCTION

Many, but not all, white rot (ligninolytic) fungi have been shown to secrete two classes of heme peroxidases which are thought to be central to the lignin degradation (Kirk and Farrell, 1987). Lignin peroxidases can catalyze the oxidation of phenolic and nonphenolic aromatic lignin fragments and precursors by mechanisms involving the formation of substrate cation radicals (Gold *et al.*, 1984,Kersten *et al.*, 1990,Tien and Kirk, 1984). The second enzyme class, the manganese peroxidases, specifically oxidize Mn(II) ions to Mn(III) ions in the presence of H_2O_2 and appropriate Mn(III) chelating

agents. The resulting Mn(III) complexes can substantially oxidize a broad spectrum of phenolic and related compounds, including a variety of synthetic lignins (DHP) (Johannson and Nyman, 1987; Wariishi *et al.*, 1989; Wariishi *et al.*, 1991) *in situ* wood lignins (Datta *et al.*, 1991; Galliano *et al.*, 1991) and pulp mill effluent chlorolignins (Lockner *et al.*, 1991; Roy-Archand and Archibald, 1991). The small size of Mn(III) chelates with anionic species such as malonate, oxalate, pyrophosphate and short-chain polyphosphates may allow these oxidants to penetrate regions of wood and lignin structure inaccessible to proteins (Strebotnik and Messner, 1990).

The electron withdrawing vigour of Mn(III) chelates can be greatly modulated. Free (hexaquo) Mn(III) ions are 1.52 eV electropositive in respect to Mn(II) ions, sufficient to split water directly, while aqueous Mn(III) complexed in a large excess of iron-free pyrophosphate anions is stable for years. Thus, the nature and concentration of the chelator will determine: (a) the readiness with which a Mn(III) chelate is formed; (b) the chelate's half-life, and; (c) its substrate range and reaction rate.

Recently the white rot fungus *Trametes (Coriolus) versicolor* was shown to extensively bleach and delignify unbleached hardwood kraft pulp over 3-5 d (Paice *et al.*, 1989; Reid *et al.*, 1990). Although our strain of *T. versicolor* (52) can produce and secrete several isozymes of both lignin and Mn peroxidases, it does not appear to produce lignin peroxidase under pulp biobleaching conditions (Archibald, 1992). However under these conditions, substantial levels of laccase (phenoloxidase) and cellobiose quinone oxidoreductase proteins are always secreted. Recently, it was shown that a lignin peroxidase of *Phanerochaete chrysosporium* also had the ability to oxidize Mn(II) to

Mn(III), but only in the presence of an aromatic compound capable of being directly oxidized by the enzyme (Popp *et al.*, 1990). The mechanism was thought to involve the aryloxy radical formed from the aromatic substrate by lignin peroxidase acting as the proximal agent, oxidizing Mn(II) to Mn(III). Neither this report (Popp *et al.*, 1990) nor the earlier ones on the white-rot fungal Mn-peroxidases (Glenn and Gold, 1985; Karhunen *et al*, 1990; Kuwahara *et al.*, 1984; Paszczynski *et al.*, 1985) noted the work of Kenten and Mann 40 years ago in which horseradish and turnip peroxidases were shown capable of efficiently generating Mn(III) chelates in the presence of any of several monophenolics (Kenten and Mann, 1950). If phenoxy and other aryloxy radicals, as well as $O_2^{\cdot}$ (Archibald, 1986; Archibald and Tyree, 1987) can readily generate Mn(III) from Mn(II) chelates, laccase proteins, which apparently carry out the same 1-electron abstractions from phenolics as do peroxidases (Kersten *et al.*, 1990; 1985) should also be able to oxidize Mn(II) to Mn(III). The following data shows that this is so.

A.2. MATERIALS AND METHODS

A.2.1 Enzymes and Chemicals

Laccase was purified from *T. versicolor* 52 (ATCC 20869) to a single peak on a Mono Q column and a single band on an SDS polyacrylamide gel as previously described (Roy-Arcand and Archibald, 1991). Horseradish peroxidase (type II), *Arthromyces ramosus* peroxidase, microperoxidase (MP-11), monoamine oxidase, potato phenolase, *Agaricus bisporus* tyrosinase and *Pyricularia oryzae* laccase were from Sigma. *Coprinus macrorhizus* peroxidase was from Chemical Dynamics Corp., So. Plainfield, N.J.

Common chemicals and phenolic substrates were from Fisher, Aldrich, and Sigma. Sodium pyrophosphate buffer was deferrated by the 8-hydroxyquinoline-chloroform extraction method (Waring and Werkman, 1942) before use. Mn(III)-pyrophosphate was prepared by mixing 10 mM $MnSO_4$ and 10 mM MnO_2 in 100 mM Na-pyrophosphate, pH 7, in an acid-clean flask for 24 h (Kenten and Mann, 1950). Mn(III)-pyrophosphate was quantified (15.5 mM) in the resulting deep wine red solution using its extinction coefficient [$\sum$M(478 nm) = 104] (Archibald and Tyree, 1987).

A.2.2 Assays

The effect of Mn on the reaction of phenolics with each of the various enzymes was followed in polycarbonate tubes for at least 15 h at 25°C in 1.5 ml of 100 mM Fe-scrubbed Na-pyrophosphate. The buffer was pH 7.0 for all enzymes except *T. versicolor* laccase, for which it was set to pH 5.0. Phenolic substrate (2mM) and $MnSO_4$ (2mM) were added and the reaction initiated with 0.38 U of laccase or approximately 0.4 U of the other enzymes. Hydrogen peroxide (2mM) was also added to the peroxidase enzyme reactions. Reaction rates and spectra were obtained using a Perkin-Elmer λ3 spectrophotometer calibrated against holmium oxide wavelength and neutral density O.D. standards.

Electron paramagnetic resonance spectra of the reaction mixtures were obtained using a Bruker ER 200D-DRC X-band (9.5 GHz) spectrometer using 100 kHz modulation. The free radical standard diphenyl-picryl-hydrazyl (DPPH) was used to determine the precise microwave frequency. Aqueous samples (5μl) were placed in a standard quartz

tube (4 mm i.d.). The 5µl sample size was sufficient to obtain the desired spectra without excessively broadening the sample resonance and reducing the signal to noise ratio. Mn(II) ions produced a diagnostic 6-line hyperfine structure with a g_0 value of 2.0040 at room temperature (see CHAPTER 4 for further details). Mn(II) oxidation by the laccase-phenol system was followed by monitoring the decrease in the peak to peak height of the first derivative signal associated with the first peak of the spectrum.

A.3. RESULTS AND DISCUSSION

Manganic (Mn(III)) ions are very similar to ferric ions in their hydrated and crystalline radii and their chelation behaviour including stability constants (Archibald, 1986). Consequently, Mn(III), like Fe(III), forms a much more stable complex with 1,2, or 3 pyrophosphate ions than do Mn(II) or Fe(II). Therefore, a mixture of Mn(II) ($MnSO_4$) and Mn(IV) (MnO_2) efficiently dismutes to give primarily Mn(III) complexed with 1,2, or 3 pyrophosphate anions (Figure A.1). These forms may account for the 3 peaks at 478, 486 and 500 nm (Figure A.1). Figure A.1 also shows that both horseradish peroxidase and *T. versicolor* laccase in the presence of phenol likewise efficiently produce Mn(III), confirming Kenton and Mann's 1949 plant peroxidase results and demonstrating that, like Mn-peroxidase, *T. versicolor* laccase is fully capable of generating Mn(III) chelates. All components (pyrophosphate, phenol, and the enzyme) were shown to be necessary for detectable Mn(III) generation. Phenol is a relatively poor substrate for both laccase and horseradish peroxidase, but when laccase and phenol were allowed to react for 48 h in the standard assay, about 70% of the original Mn(II) was oxidized to Mn(III).

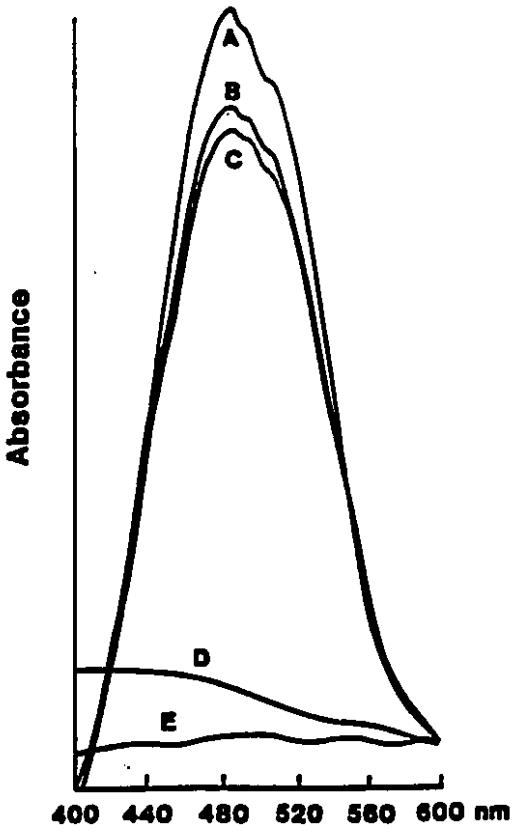


Figure A.1. Visible absorbance spectra of various reaction mixtures incubated overnight in 1.5 ml of 100 mM Na-pyrophosphate buffer. The traces represent; A, 0.38 U laccase, 2 mM $MnS0_4$, 2 mM phenol, pH 5; B, Mn(III)-pyrophosphate produced as described in Materials and Methods; C, 0.4 U horseradish peroxidase, 2 mM H_2O_2 2mM $MnSO_4$, 2 mM phenol, pH 7. D, as in C, but less $MnSO_4$; and E, as in C, but less peroxidase. Traces D and E are slightly raised vertically from their true positions. In peaks A,B, and C λmax = 478 nm and the shoulders are at 486 and 500 nm. By manipulating the spectrophotometer a slight shoulder can also be observed at ~ 445 nm.

This high efficiency is probably in part due to the low reactivity of phenol with Mn(III)-pyrophosphate under these conditions. Since Mn(III)-pyrophosphate does react rapidly with many simple phenolics, particularly those with ortho hydroxyl (catechol) structures or adjacent hydroxyl and methoxyl groups (Archibald, 1986) the absence of visible Mn(III)-pyrophosphate in other laccase-phenolic reaction mixtures may be due to either no Mn(III) formation or Mn(III) complex reduction by a phenolic substrate or some product of phenol oxidation. Since Mn(III)-pyrophosphate (1:20) per se was shown to be stable for months, the disappearance over time of Mn(III) from some of the systems must be via Mn(III) reduction by the phenolic substrate or its oxidation products. Also, changes in the relative rates of laccase-mediated Mn and phenolic oxidation over time were commonly observed and varied with the phenolic employed (Figure A.2).

Mn(II) ions produce a diagnostic 6 line first derivative signal in the EPR, with an intensity proportional to the amount of Mn(II) present in the sample cavity. Other oxidation states, most importantly Mn(III) and Mn(IV), do not produce signals under these conditions. When an aliquot of the standard reaction mixture (deferated pyrophosphate buffer (pH 5.0), $MnSO_4$, phenol, and laccase), was placed in the EPR (Figure A.3, line A) a typical signal from Mn(II) ions was seen . The oxidation of Mn(II) to Mn(III) in the reaction mixture over time was accompanied by the dissappearance of the Mn(II) 6-line signal (Figure A.3, lines B and C). At a field strength of 340 mT, a sharp signal typical of phenoxy radicals was also observed decreasing with time through the course of the reaction (Figures A.3 and A.4). This provides further evidence that the mechanism

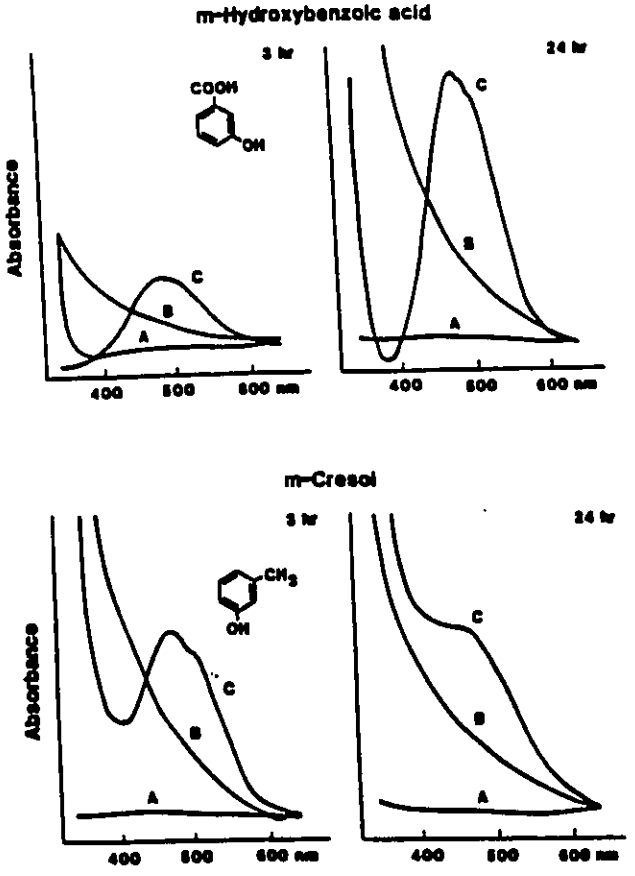


Figure A.2. Effects of the presence of $MnSO_4$ on the reaction of *T. versicolor* laccase with 2 phenolics. Assays employed 0.38 U of laccase in 100 mM Na-pyrophosphate, pH 5. Legend: A, 2 mM $MnSO_4$, 2 mM phenolic, no laccase; B, laccase, 2 mM phenolic, no $MnSO_4$, and C, laccase, 2 mM phenolic, 2 mM $MnSO_4$. Traces A are slightly offset vertically from their true positions.

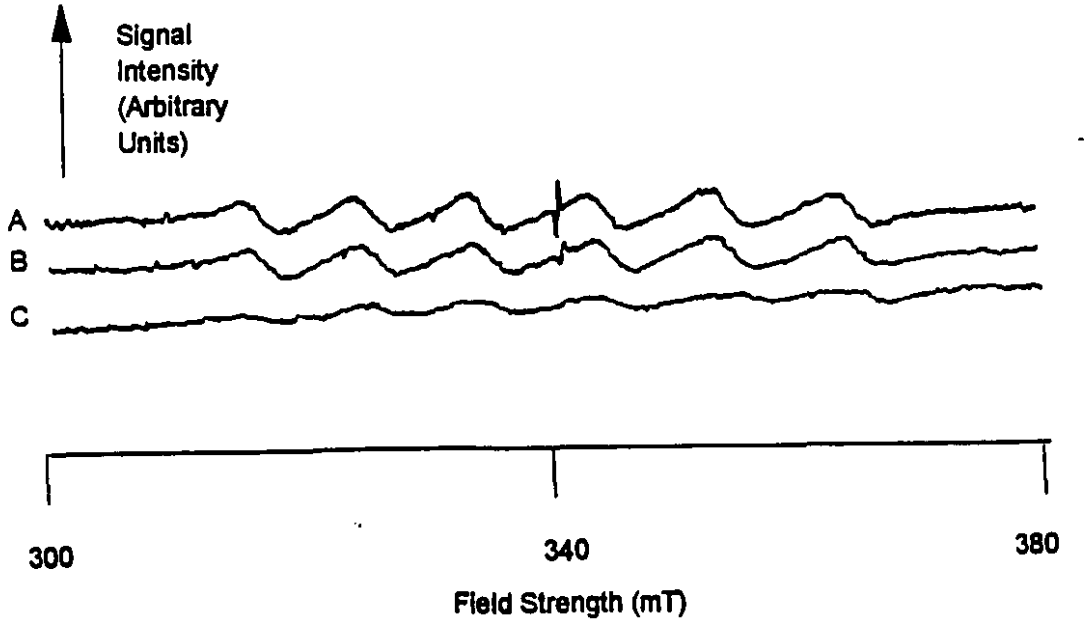


Figure A.3. EPR signal from a reaction mixture containing laccase (0.3 U/ml), 2 mM $MnSO_4$, and 1 mM phenol in 100 mM iron-free sodium pyrophosphate (pH 5.0). Spectra after 5 min (A), 120 min (B), and 72 h (C) are shown. Instrument conditions were as follows: centerfield 340 mT, ΔH = 80 mT, sweep time = 100 s, microwave power 2 mW, 20 db attenuation, with readings taken at room temperature (295 K). Absorption signals for A and B were 2,0039 and 2,0018, respectively.

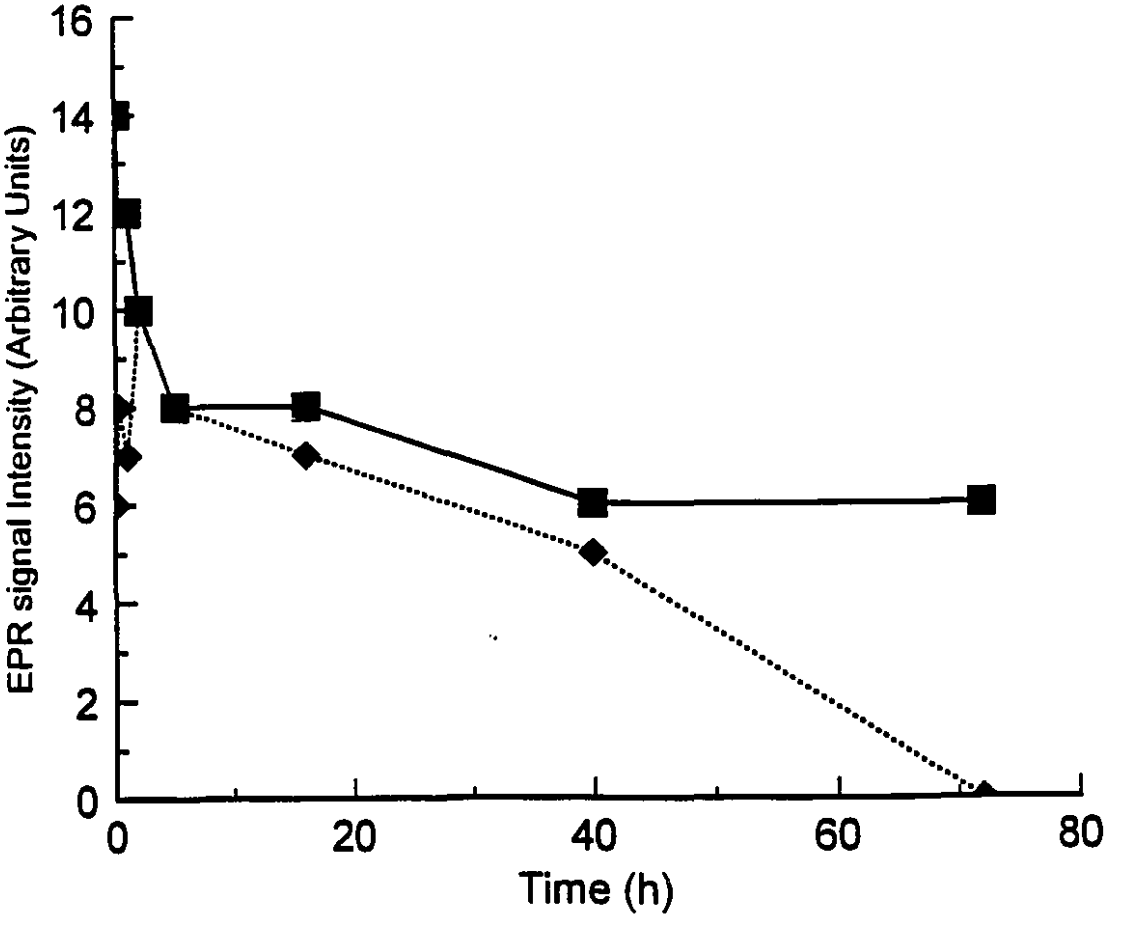


Figure A.4. Time course of Mn(II) oxidation by laccase in the presence of phenol. The reaction mixture was as described in the legend to Figure 3. The peak-to-peak height of the first derivative signal intensity for the first peak of the 6 line hyperfine signal for Mn(II) was used to quantify the amount of Mn(II) present (■). Also shown is the peak-to-peak height of the free radical signal produced by the laccase-mediated oxidation of phenol by laccase (◆). EPR instrument conditions were as described in Figure 3.

originally proposed by Kenten and Mann (1950), that Mn(II) oxidation is mediated via a phenoxy radical intermediate is correct.

Recently, it has been reported that an H_2O_2-free aqueous mixture of glutathione, oxygen, Mn(II)-lactate, and horseradish peroxidase can oxidize veratryl alcohol to veratraldehyde (McEldoon and Dordick, 1991). However, the catalysis of this atypical peroxidase reaction, in which the proximal oxidant of veratryl alcohol is postulated to be a glutathione-Mn complex, would appear to have little in common with the present system in which H_2O_2 is essential (and glutathione unnecessary) for the oxidation of Mn(II) by horseradish peroxidase. Table A.1 shows that not only can laccase and horseradish peroxidase form Mn(III) via the agency of a variety of simple phenolics, but other laccases and non ligninase-type peroxidases can as well. More than half of the enzyme-phenolic reactions (Table A.1) showed no clear Mn(III) peaks but Mn substantially altered the UV-visible spectrum of the reaction products. This suggests, but does not prove, that Mn(III) was generated during those reactions, as would be expected if these enzymes catalyzed similar 1-electron abstractions (Kersten *et al.*, 1985).

Increasing Mn(II) and Na-pyrophosphate concentrations will promote both the formation and stability of Mn(III)-pyrophosphate but decrease its reactivity (Archibald, 1986; Archibald and Fridovich, 1982; Archibald and Tyree, 1987). Decreasing the pyrophosphate to 30 mM had little effect, but at 10 mM there was significantly reduced Mn(III)-complex formation in the presence of laccase and phenol. Thus, whether Mn(III) formation and oxidative activity is important in the *in vitro* functioning of laccase in particular delignifying systems will depend on many factors including; concentrations of

Table A.1. Mn(III) complex generation by phenolics combined with peroxidase and phenoloxidase enzymes[1]

Enzyme	Phenolic	Mn(III)-pyrophosphate observed[2]	Reaction products altered by presence of $MnSO_4$[3]
T. versicolor laccase I	phenol	+	+
" "	m-cresol	+	+
" "	resorcinol	+	+
" "	guaiacol	+	+
" "	m-hydroxybenzoate	+	+
" "	p-coumarate	+	+
" "	o-hydroxybenzylalcohol	+	+
P. oryzae laccase	phenol	+	+
" "	guaiacol	-	+
A. bisporus tyrosinase	phenol	-	+
" "	tyrosine	-	+
" "	guaiacol	-	+
Potato phenolase	phenol	-	+
" "	tyrosine	-	+
" "	chlorogenic acid	-	+
Horseradish peroxidase	phenol	+	+
" "	resorcinol	+	+
" "	guaiacol	-	+
A. ramosus peroxidase	phenol	+	+
" "	resorcinol	-	+
" "	guaiacol	-	+
C. macrorhizus peroxidase	phenol	-	+
" "	resorcinol	-	+
" "	guaiacol	-	+
Cytochrome C microperoxidase	phenol	-	+
" "	resorcinol	-	+
Bovine monoamine oxidase	benzylamine	-	-
" "	phenylalanine	-	-

[1] Reaction mixtures and assays as described in Materials and Methods.

[2] Positive indicates that the characteristic Mn(III)-pyrophosphate peak at λ=478 (see Figure 1) was observed in one or more of the spectral scans performed over a 15-48 h incubation. In most cases, the Mn(III) spectrum was absent or reduced in the later scans (see Figure 2). The amount of Mn(III) produced was greatly affected by enzyme, phenolic, and pyrophosphate levels. Maximum Mn(III) production did not always coincide with maximum enzyme addition.

[3] Positive indicates that addition of Mn(II) caused qualitative changes in the visible spectrum of the enzyme-phenolic-pyrophosphate reaction mixture.

Mn(II), phenolics, laccase, and Mn chelators, pH, pO_2, nature of the phenolics, nature of Mn(III) chelators, and presence of Fe. Other than the phenolic dependence, the same caveats apply to the apparently dedicated fungal Mn-peroxidases. Thus, apparently the only novel feature of Mn(III) generation by the Mn-peroxidases is their ability to oxidize Mn(II) to Mn(III) in the absence of a phenolic intermediate. Conversely, the ubiquity of phenoloxidative and peroxidative enzymes presumably capable of indirectly oxidizing Mn, especially in plants, which often contain substantial Mn levels (Archibald, 1986; Kenten and Mann, 1955; Kono *et al.*, 1976) may mean that Mn(III) is important in more than just fungal systems.

To investigate the possible role of laccase-mediated Mn(III) generation in delignification, hardwood kraft black liquor was diluted, dialyzed (1 kD exclusion) and incubated with 0.01, 0.1 and 1 U/ml *T. versicolor* laccase, 30 mM pyrophosphate pH 4.5, and 2 mM $MnSO_4$. Increasing OD_{465} indicated that the laccase could react directly with the lignin, but no Mn(III) peak was seen at 478 nm. However, the strong visible-uv absorbance of the lignin, the tendency of $MnSO_4$ to precipitate the lignin and the direct oxidation of the lignin molecules by laccase may well have masked Mn(III)-forming activity. Some evidence in support of this was obtained from observations obtained in the EPR, where the Mn(II) signal decreased slightly when indulin oxidation was high. The Mn(II) signal increased to its initial level after 20 min incubation under these conditions.

Appendix B Characterization of the extracellular polysaccharide produced by *T. versicolor* during kraft pulp biobleaching.

B.1. INTRODUCTION

The structure of the high molecular weight Mn- complexing agent or agents (Chapter 5) in biobleaching culture supernatants of *T. versicolor* (Chapter 2) has not been previously characterized. In light of the relative importance being attached to MnP and Mn- complexing agents in the degradation of lignin (Kuan and Tien, 1993a; Wariishi *et al.*, 1992; this thesis) the composition of this material was determined. During growth and under biobleaching conditions (Chapter 2) *T. versicolor* frequently produces a considerable amount of extracellular slime. The comparable extracellular gel-like material of *Phanerochaete chrysosporium* has been extensively characterized and found to be composed primarily of a β-1→3, 1→6-type glucan (Buchala and Leisola, 1987). This extracellular glucan was produced in nitrogen-limited cultures, conditions which are necessary for the induction of the ligninolytic system of *P. chrysosporium* (Buchala and Leisola, 1987). In contrast, the high molecular weight material secreted by *T. versicolor* appears under nitrogen-sufficient growth conditions (Chapter 2). When purified, the extracellular material secreted by *T. versicolor* supported the MnP-mediated oxidation of Mn(II) to Mn(III)(Chapter 5). Thus, the nature of this material may be relevant to understanding *T. versicolor*-mediated biological pulp bleaching and delignification.

B.2. MATERIALS AND METHODS

After seven days incubation the extracellular polysaccharide was isolated from a standard hardwood biobleaching culture of *T. versicolor* 52J (2% w/v pulp in 15% mycological broth). The pulp and fungal biomass were removed from the culture supernatant (4 liters) by filtration through cheesecloth and Whatman #4 filter paper. The subsequent purification of the extracellular polysaccharide was essentially as described by Buchala and Leisola (1987). The clarified supernatant was subjected to a cycle of freeze-thaw to precipitate the polysaccharide which was recovered by centrifugation (2 h at 10,000 x g) or alternatively, recovered by precipitation from fresh supernatant with 2 volumes of cold ethanol (Buchala and Leisola, 1987). The precipitated material was washed 3 times with water:ethanol (1:1), then lyophilized. The lyophilized material was sequentially extracted (121°C; 60 min) first with 100 ml H_2O (discarded) per gram. The washed polysaccharide (containing 80% of the original lyophilized material) was then dissolved in methyl sulfoxide (100 ml per gram of insoluble material) and subsequently dialyzed (1000 kDa molecular weight cutoff), and the retentate lyophilized.

B.3. RESULTS AND DISCUSSION

The yield of polysaccharide in a particular culture was highly variable, however, at least a low level of material was nearly always present under biobleaching conditions. Longer incubations of *T. versicolor* in the presence of pulp produced increasing yields of this extracellular material. The amount of polysaccharide produced by *T. veriscolor*

52J cultures was greatest in cultures which contained high levels of glucose in the growth medium.

The net sugar yield after acid hydrolysis for the two samples analyzed was approximately 30% of the material hydrolyzed. The sugars were measured following the separation of their sialylated derivatives by gas chromatography. When hydrolyzed and analyzed for carbohydrate composition the polysaccharide consisted mainly of glucose with a very low mannose content (Table B.1). This was true for two different preparations of the high moleular weight material. The chromatograms of the derivitized polysaccharide hydrolysates did not contain any other unidentified peaks, and therefore it is reasonable to conclude that the material secreted by *T. versicolor* is a glucan.

The mannose present likely comes from secreted proteins, since most of these are glycosylated, and mannose is a common glycosylation sugar in secreted fungal proteins. Such a composition is very similar to that secreted by most fungi (Buchala and Leisola, 1987). As was suggested in Chapter 5, this HMW material may function as an electron donor for CBQase, albeit at a very low rate relative to that obtained when the electron donor is cellobiose.

Table B.1. Composition of glucan secreted by *T. versicolor* during kraft pulp biobleaching.

Sample	Sugar[1]	Composition (mg/liter)	Fraction
T. versicolor 52J [2]	glucose	961	1
HWKP (2% w/v)	mannose	99.6	0.1
	total carbohydrate	1061	-
T. versicolor 52J [3]	glucose	1009	1
SWKP (2% w/v)	mannose	60	0.06
	total carbohydrate	1069	-

[1] No other sugars were detected in the hydrolysates. No unidentified peaks were seen in the derivatized lysates. Standards were run for glucose, mannose, xylose, arabinose, and galactose.

[2] Glucan was isolated from a 5-day HWKP *T. versicolor* 52J biobleaching culture supernatant.

[3] Glucan was isolated from a 7-day oxygen delignified SWKP-*T. versicolor* 52J biobleaching culture supernatant.

Appendix C. Synthesis of *ortho*-quinones from the corresponding phenols using Fremy's salt oxidation.

C. 1. INTRODUCTION

In the first publication describing the identification and isolation of the enzyme CBQase, it was proposed that the enzyme could reduce a wide range of both *ortho*- and *para*-quinones (Westermark and Eriksson, 1974; 1974a). Most subsequent investigations of CBQase have used a limited number of quinones, most commonly 3,5-di-*tert*-butyl-1,2-benzoquinone (TBBQ) (e.g. Bao *et al.*, 1993) or the artificial electron acceptor dichlorophenolindophenol (DCIP). No systematic investigation of the reductive activity of CBQase with more than a few quinone substrates has been published.

Teuber and Staiger (1955) have published a technique for the synthesis of orthoquinones based on the oxidation of suitably substituted phenolic substrates using Fremy's salt (potassium nitrosodisulphonate). The regioselectivity of these reactions has been confirmed by a number of other investigators (Deya *et al.*, 1987; Zimmer *et al.*, 1971). For the present work, a series of phenolic substrates were oxidized using Fremy's salt to synthesize *ortho*-quinones which were then used in the characterization of the substrate range and specificity of *T. versicolor* CBQase 4.2 (Chapter 3). The precise method used in the syntheses, the yields and some properties of the resulting products are reported. In addition, spectra of the oxidized and reduced visible forms of these compounds in an aqeous solvent system are shown, since this information has not been published previously.

C.2. MATERIALS AND METHODS

In general, the phenolic substrates selected and the oxidation method used were as described by Teuber and Staiger (1955). Fremy's salt was obtained from Aldrich Chemical Co. and prepared in an aqueous solution immediately before use (<5 min). After opening and use, the Fremy's salt bottle was flushed for 5 min with anhydrous nitrogen gas, tightly sealed, and stored in a dessicator at 4°C. The phenol substrates, Fremy's salt, buffers, and solvents used in the synthesis were of the highest purity available. Melting points were obtained using a standard melting point apparatus with an integral heating platen. The visible spectra of these orthoquinones were recorded using a Perkin-Elmer λ3 spectrophotometer with an automated chart recorder. The absorbnace spectrum of each orthoquinone compound was obtained from a solution containing: 2 mM of each quinone in an acetate buffer (50 mM, pH 4.5) containing 20%v/v ethanol. The individual orthoquinone compounds were synthesized as follows.

C.2.1. Synthesis of 4-*tert*-octyl-1,2-benzoquinone.

Initially, 0.5 g of 4-tert-octyl-phenol was dissolved in 105 ml of acetone and 1.6 g of Fremy's salt was dissolved in 80 ml of water containing 50 mM KH_2PO_4. The Fremy's salt solution was slowly added to the acetone-phenol mixture (3 min), stirred, and within 5-10 min an intense red colour was formed. The reaction mixture was stirred for an additional 2 h, after which clear inorganic crystals had formed. The mother liquor was decanted and cooled on ice (0°C). After 5-10 min some of the quinone product had precipitated. Two 50 ml aliquots of water were added to the mother liquor after 30 and 60 min incubation and the mixture was left standing for 6 h at 0°C. After 6 h, 0.35 g of

precipitated. Two 50 ml aliquots of water were added to the mother liquor after 30 and 60 min incubation and the mixture was left standing for 6 h at 0°C. After 6 h, 0.35 g of red needle shaped crystals were obtained for a net yield of 70% of the theoretical. After resolution and recrystallization from diethylether:petroleum ether (1:1) at -80°C, red needles were obtained.

C.2.2. Synthesis of 4-*tert*-butyl-1,2-benzoquinone.

Initially, 0.75 g of 4-*tert*-butyl phenol was dissolved in 75 ml of acetone and added to 300 ml of an aqueous solution containing 30 mM KH_2PO_4, 75 mM sodium acetate and 3 g of Fremy's salt. The solution was thoroughly mixed and left to stand for 3 h at room temperature until a deep red color developed (≈22°C). The mother liquor was extracted three times with chloroform. The chloroform was removed by evaporation under reduced pressure and the resulting residue dissolved in ether (50 ml), then concentrated to ≈10 ml. The solvent extract was diluted with 10 ml of petroleum ether, cooled to -80°C and the quinone allowed to crystallize at this temperature overnight. Red crystals which precipitated from the solution were recovered by filtration on a pre-chilled sintered glass funnel.

C.2.3. Synthesis of 3,4-*di*-methyl-1,2-benzoquinone.

Initially, 0.5 g of 3,4-dimethyl phenol were dissolved in 10 ml of ether. Fremy's salt (3 g) was dissolved in 195 ml of a 20 mM solution of KH_2PO_4, added to the phenol and ether mixture and shaken vigourously for 10 min. After incubating the solution with

periodic shaking for 30 min, during which the reaction mixture turned a deep red colour. After 30 min, the solution was extracted with chloroform and the extract dried over 10 g of anhydrous Na_2SO_4. The Na_2SO_4 was removed from the mother liquor by filtration (Whatman No. 4). The anhydrous chloroform was removed from the reaction mixture under reduced pressure, yielding about 0.4 g of bright red crystals. Teuber and Staiger (1955) reported that 3,4-di-methyl-1,2-benzoquinone was not stable, degrading and the melting point of the product gradually increasing after 10 days of storage at room temperature in a dessicator. However, it was found that when stored at -80°C that the material was relatively stable with no change in melting point observed in 3 months.

C.2.4. Synthesis of 3,4,5-*tri*-methyl-1,2-benzoquinone.

Initially, 1 g of 3,4,5-tri-methyl phenol was dissolved in 30 ml of acetone to which was added 4.5 g of Fremy's salt was dissolved in 260 ml of water containing 16 mM KH_2PO_4 and 50 mM sodium acetate. The solution was mixed, and kept on ice at 0°C, and after a short period (2-3 min) red crystals appeared in the solution. The reaction mixture was extracted into chloroform (3 x 50 ml) and the extracts combined and washed with 30 ml of water. Residual water in the washed chloroform extract was removed by adding 10 g of Na_2SO_4 which was then removed by filtration (Whatman No. 4). The chloroform was removed by evaporation under reduced pressure to yield a solid black residue. This residue was redissolved in ether (20 ml) and the quinone was recrystallized twice from the ether solution at -80°C. The method yielded 0.8 g of red crystals (80%). 3,4,5-tri-methyl-1,2-benzoquinone was stable for at least 3 months at -80°C.

C.2.5. Synthesis of 3-methoxy-5-methyl-1,2-benzoquinone.

Initially, 0.5 g of 4-methyl-guaiacol was dissolved in 10 ml of ether, to which was added 2.2 g of Fremy's salt dissolved in 150 ml of water containing 33 mM KH_2PO_4. The stirred mixture was kept at 0°C for 30 min, until a deep red colour had developed. The quinone in formed in the reaction mixture was extracted into 40 ml of chloroform. The reaction mixture was extracted five times, the extracts pooled and water removed by adding 10 g of Na_2SO_4, which was then removed by filtration (Whatman No. 4). The chloroform was removed from the reaction mixture by evaporation under reduced pressure to yield a solid residue. The residue was dissolved in 20 ml of diethyl ether and recrystallized twice at -80°C. The synthesis yielded 0.45 g of red crystals.

C.2.6. Synthesis of 4-methyl-1,2-benzoquinone.

Initiallly, 0.4 g of *p*-cresol was dissolved in 6 ml of ether to which 2.8 g of Fremy's salt was dissolved in 170 ml of water containing 60 mM sodium acetate was added. The reaction mixture was kept at 0°C with constant stirring for 60 min, during which an intense red colour developed in the reaction mixture. The aqueous solution was extracted with diethyl ether (5 x 100 ml) and the extracts pooled. Solid Na_2SO_4 was added to the pooled extracts to remove moisture overnight (4°C) after which the Na_2SO_4 was removed by filtration (Whatman No. 4). The dried diethyl ether extract was concentrated under vacuum, and the 4-methyl-1,2-benzoquinone was recovered by low temperature crystallization (-80°C). The quinone was crystallized twice from a solution of diethyl ether solution yield red needle-like crystals.

C.3. RESULTS AND DISCUSSION

The starting materials, melting points, properties, and yield of each of the synthesized quinones are summarized in Table C.1 and at the end of the description of the method used for the synthesis of each compound (see **MATERIALS AND METHODS**). None of the synthesized quinones were sufficiently soluble in aqueous solution to obtain absorbance spectra, therefore all of the spectra had to be recorded in the presence of 20% ethanol by volume. Reducing the volume of ethanol led to precipitation of the quinones.

The visible spectra for each of the quinones are shown in Figures C.1 through C.6 for the quinones synthesized. In addition, Figures C.7 and C.8 are included to show the visible spectra of two quinones, 3,5-di-tert-butyl-1,2-benzoquinone and 3,5-di-methoxy-1,4-benzoquinone which were used in the characterization of CBQase, but which were available commerically.

The spectra presented here are substantially different than those shown by Teuber and Staiger (1955). However the solvent systems used in this work were completely different. Whereas Teuber and Staiger (1955) recorded the spectra of these quinones dissolved in chloroform, an aqueous buffer system containing 20% ethanol was used here. Similar differences in the absorbance profiles of quinones dissolved in aqueous buffers compared to the same quinones dissolved in organic solvents have been reported (Adler *et al.*, 1960). In particular, it was found that methoxy substituted orthoquinones tended to have rather absorbance profiles which tail towards longer wavelengths with absorbance spectra very similar to that of 3-methoxy-5-methyl-1,2-benzoquinone (Figure C.1). It

Table C.1. Properties of *ortho*-quinones synthesized from phenolic compounds using Fremy's salt oxidation

Quinone[1]	Yield[2] (%)	Melting point (°C)		λ_{max}[5] (nm)	Extinction coefficient[6] ($mM^{-1}cm^{-1}$)
		A[3]	B[4]		
4-tert-butyl	75	68-69	66-67	400	0.35
4-tert-octyl	70	122-124	122-123	425	1.1
4-methyl	75	67	68	380	0.57
3,4-di-methyl	50	103	97-102	416	0.42
3,4,5-tri-methyl	80	91-92	94-96	430	0.15
3-methoxy-5-methyl	90	105	89-90	460	0.50

[1] Quinone substituent only is given, all are orthoquinones.

[2] Calculated as yield of quinone (g) divided by amount of phenol (theoretical yield) used times 100.

[3] Experimentally determined melting points for quinones synthesized for this work.

[4] Values given in Teuber and Staiger (1955) for the same quinones.

[5] The wavelength in the visible region (360 to 700 nm) with the highest absorbance.

[6] The calculated extinction coefficient based on the difference in absorbance observed between the oxidized and sodium hydrosulphite reduced spectra.

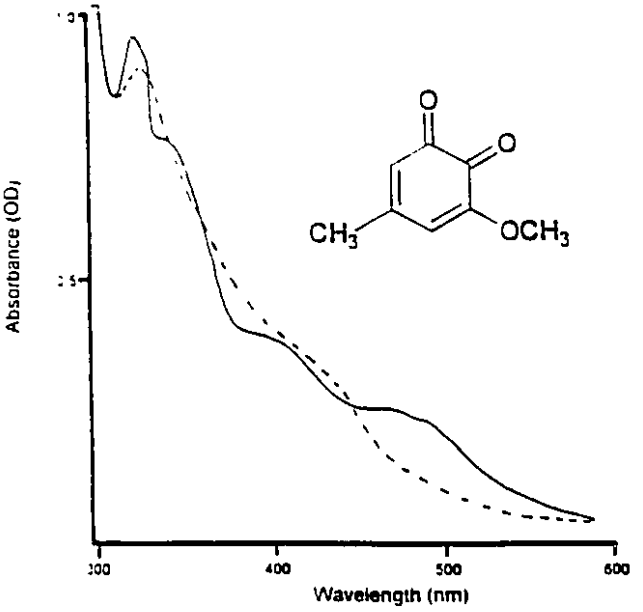


Figure C.1. Visible spectrum of 3-methoxy-5-methyl-1,2-benzoquinone. Both oxidized (solid line) and reduced (dashed line) forms are shown.

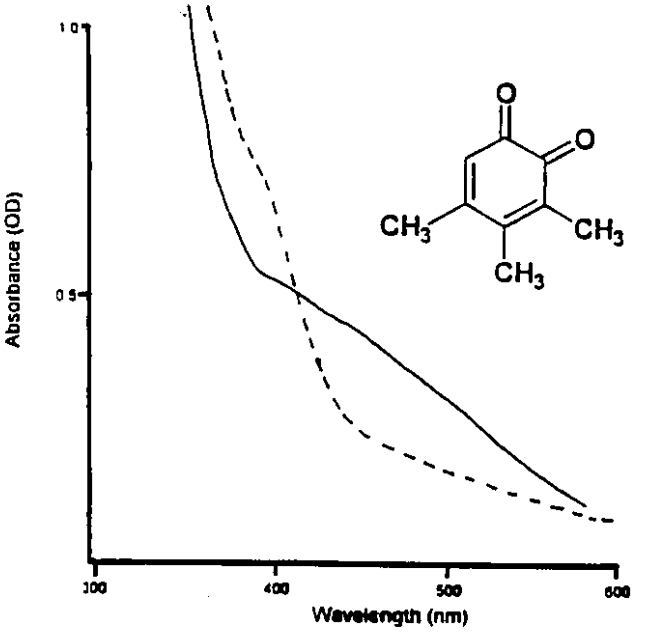


Figure C.2. Visible spectrum of 3,4,5-tri-methyl-1,2-benzoquinone. Both oxidized (solid line) and reduced (dashed line) forms are shown.

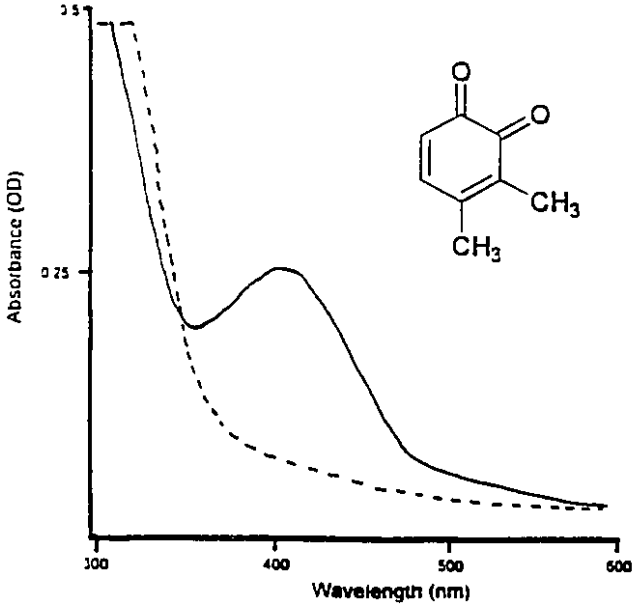


Figure C.3. Visible spectrum of 3,4-di-methyl-1,2-benzoquinone. Both oxidized (solid line) and reduced (dashed line) forms are shown.

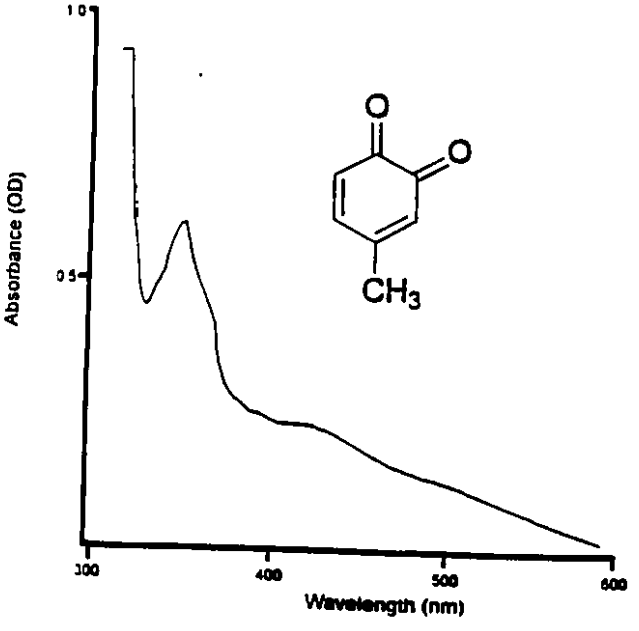


Figure C.4. Visible spectrum of 4-methyl-1,2-benzoquinone. The spectrum is of the oxidized form.

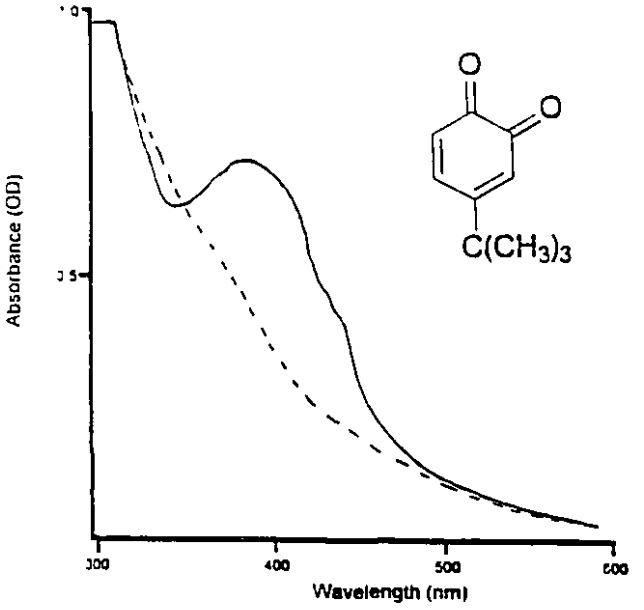


Figure C.5. Visible spectrum of 4-*tert*-butyl-1,2-benzoquinone. Both oxidized (solid line) and reduced (dashed line) forms are shown.

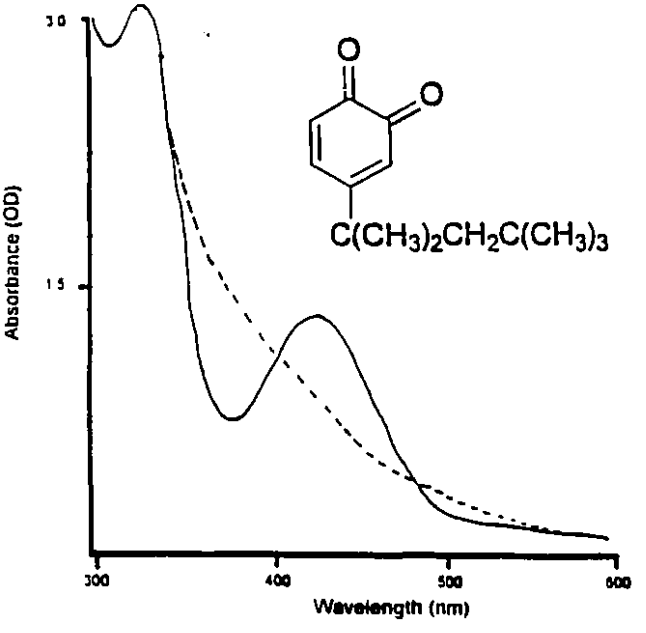


Figure C.6. Visible spectrum of 4-*tert*-octyl-1,2-benzoquinone. Both oxidized (solid line) and reduced (dashed line) forms are shown.

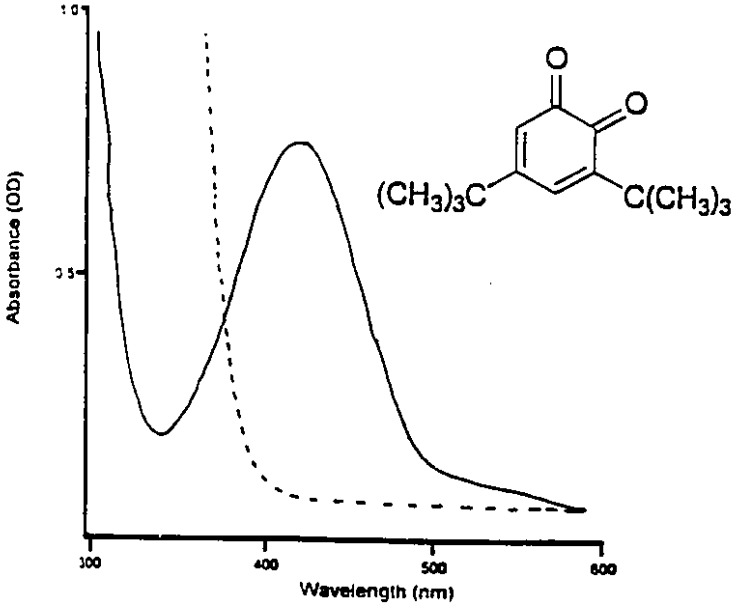


Figure C.7. Visible spectrum of 3,5-di-*tert*-butyl-1,2-benzoquinone (2 mM). Both oxidized (solid line) and reduced (dashed line) forms are shown.

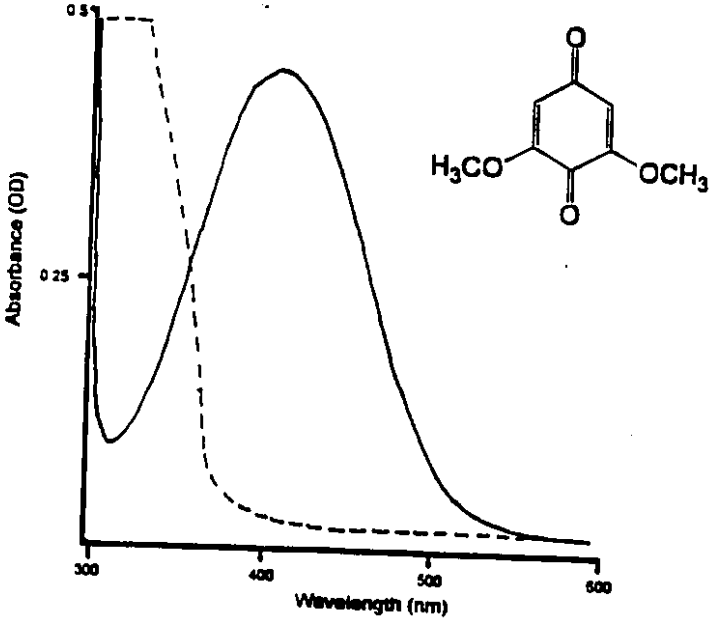


Figure C.8. Visible spectrum of 3,5-dimethoxy-1,4-benzoquinone (2 mM). Both oxidized (solid line) and reduced (dashed line) forms are shown.

was proposed that quinone hydrates are formed in aqueous solutions, as shown in Figure C.9 (Adler *et al.*, 1960).

Among the methyl substituted quinones (Figures C.2-C.4), only the dimethyl quinone shows a distinct absorption peak at around 400 nm, with the mono- and tri-substituted quinones having rather broader absorbance profiles. The 4-methyl-1,2-benzoquinone had the smallest change in absorbance profile upon reduction (Figure C.4). Increasing the size of the substituent at the 4 position on the aromatic ring did lead to an increasingly well defined peak around 400 nm (compare Figures C.4, C.5, and C.6), though the decrease in absorbance upon reduction with sodium hydrosulphite was smaller than for some of the other quinones. Both 3,5-di-tert-butyl-1,2-benzoquinone and 3,5-di-methoxy-1,4-benzoquinone had stronger absorbances at 400 nm, as well as more defined absorbance peaks at these wavelengths (Figures C.7, C.8). All of the quinones were reduced by the addition of stoichiometric amounts of sodium hydrosulphite (dithionite), though the reaction rate of some quinones was much slower than others.

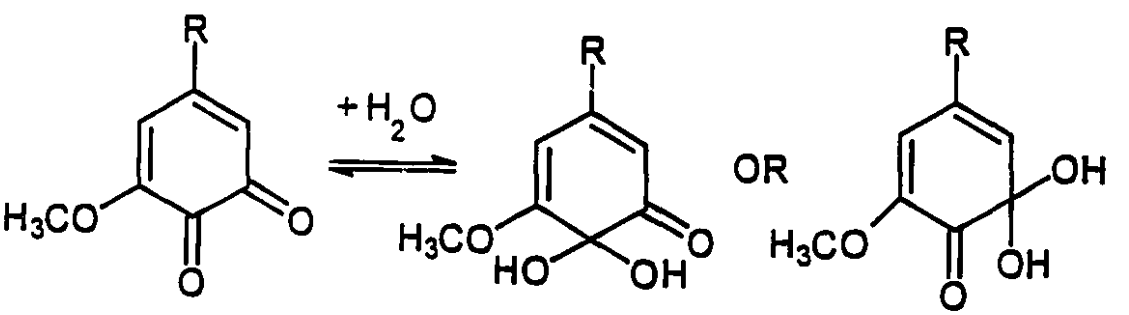


Figure C.9. Proposed formation of *o*-quinol from *o*-quinones in the presence of aqueous solvents. Figure is adapted from Adler *et al.* (1960).

Appendix D Attempts at identification of a chromatographic peak in HPLC separations of organic acids present in the supernatant of cultures of *Trametes versicolor* 52J

D.1. INTRODUCTION

Studies of manganese peroxidase have demonstrated that malonic acid is an effective Mn(III) chelator (Wariishi *et al.*, 1992; Paice *et al.*, 1993). Malonic acid is an organic metabolite which has been found in culture supernatants of *Phanerochaete chrysosporium* (Wariishi *et al.*, 1992) but was not one of the metabolites which was detected in culture supernatants of *Trametes versicolor* 52J during the course of the study presented in Chapter 2.

However, since the time when the results presented in CHAPTER 2 were obtained, a new high pressure liquid chromatograph (HPLC) producing better peak resolution has been aquired. HPLC analysis of numerous *T. versicolor* 52J culture filtrates detected the presence of an absorbance peak at 210 nm from organic acid separations which had a retention time close to that of malonic acid (F. Archibald, pers. comm.). The objective of this suplimentary study was to determine whether malonic acid had been produced by *T. versicolor* 52J under the physiological conditions used in the biobleaching study presented in Chapter 2.

D.2. MATERIALS AND METHODS

D.2.1. Culture supernatants

Samples of *T. versicolor* supernatants from pulp-containing and pulp-free cultures frozen (-20C) since the work described in Chapter 2 was completed, were thawed at 4°C and a 10 ml aliquot taken. These culture supernatants were prepared for analysis by filtration (0.45 μm membrane filter) only.

D.2.2. Analysis of supernatants

Culture filtrates were analyzed using a Waters 600E HPLC with a PAD 996 photodiode array detector. The filtered supernatants were placed in sample vials and 20 μl of the sample was injected onto an Aminex HPX-87H column (7.8 x 300 mm) and eluted with 0.008 M H_2SO_4 at a flow rate of 0.6 ml min^{-1} and a column temperature of 35°C. The only significant differences in the chromatographic method used in this study and that used for the results presented in Chapter 2 were the volume of sample analyzed and the instrument itself.

D.3. RESULTS AND DISCUSSION

Analytical determinations using the old and new instruments of malonic acid standards gave retention times of 10.2 min and 11.5 min, respectively. Most of the difference in retention times can be accounted for by the slightly smaller void volume in the new installation, reflected in the shorter retention time for the material eluting in the first peak (Figures D.1, D.3). The elution of dimethylsuccinate (the non-metabolized

Peak	Retention Time (min)	Peak Area (uv.s)
A	7.06	706
B	7.57	174.5
C	11.0	56.8
D	15.4	1382.3
E	16.9	55.4
F	24.3	63.7

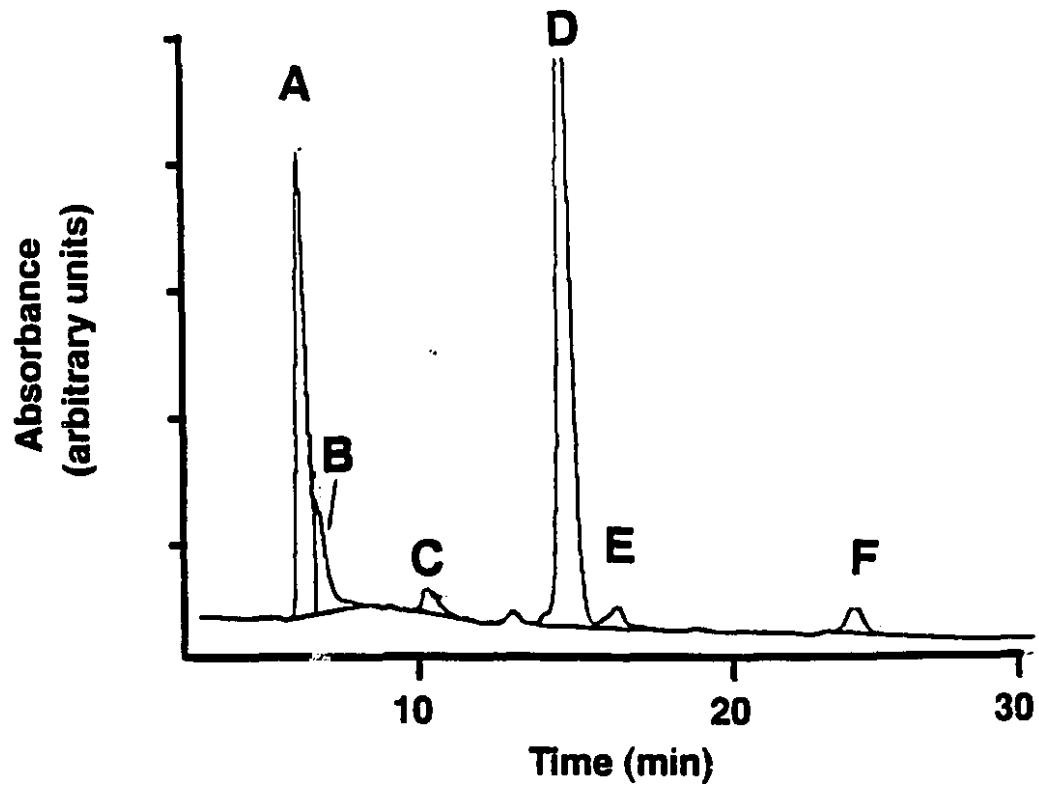


Figure D.1. Elution profile of a *T. versicolor*-TDM culture supernatant applied to an organic acid separating column with the older HPLC and photodiode array detector. Peaks were detected at 210 nm. The y-axis scale was in milliabsorbance units and the x-axis scale in minutes. Injection volume was 25 µl.

Peak	Retention Time (min)	Peak Area (uv.s)
A	7.0	3376
B	9.3	129.8
C	9.7	152.3
D	10.8	272.0
E	13.6	110.4
F	15.2	1365
G	21.3	136.7

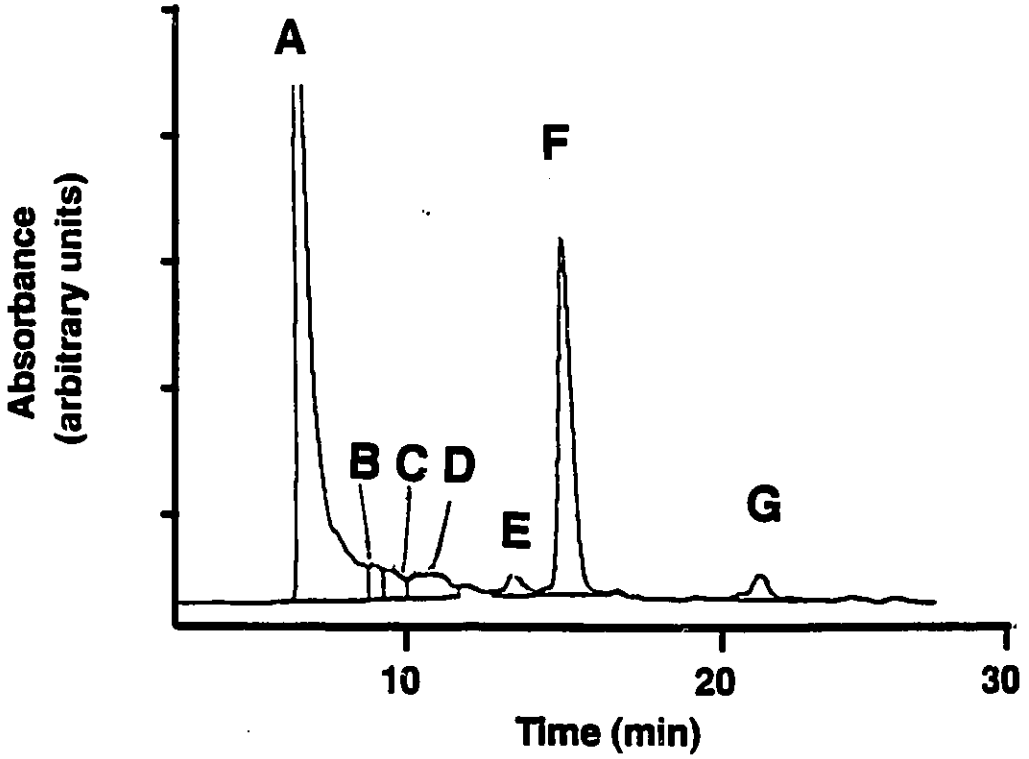


Figure D.2. Elution profile of a *T. versicolor*-TDM plus HWKP culture supernatant applied to an organic acid separating column with the with the older HPLC and photodiode array detector. Peaks were detected at 210 nm. The y-axis scale was in milliabsorbance units and the x-axis scale in minutes. Injection volume was 25 µl.

retention time) later in the old system, perhaps reflecting some inefficiency in the pump system of the older HPLC.

A comparison of the elution profile of components from the organic acid column monitored at 210 nm reveals the significant differences in the peak profile and distribution obtained with the two different instruments when similar sample volumes were injected (compare Figures D.1, D.2 with Figures D.3, D.4). When the sample volume was increased on the older instrument, fewer peaks were detected and integrated than when using a 20 µl sample volume with the new instrument (compare Figures D.3, D.4 with Figures D.5, D.6). Clearly, the two instruments give different component elution profiles at 210 nm.

When an absorbance profile of the peak from the culture supernatant with the same retention time as malonic acid was analyzed, it was very different from that obtained for a malonate standard resolved under the identical conditions. Since the malonic acid peak was not perfectly resolved under these conditions, it is possible that other unidentified components of the culture supernatant may be contributing to the UV absorbance at this retention time. This possibility has not been resolved. In addition to the HPLC analysis performed during the course of the analysis presented in Chapter 2, the culture supernatants were analyzed for acidic metabolites by GC-MS. Malonic acid is one of the organic acids which is readily detected using this method (O. Mamer, pers. comm.) and if present should have been detected but it was not. This coupled with the observed differences in the UV-visible absorbance porfile of malonic acid and the peak which was detected at a retention time corresponding to malonic acid raises some questions as to the

Peak	Retention Time (min)	Peak Area (uv.s)
A	6.3	796172
B	7.1	24532
C	8.9	423084
D	9.9	200888
E	10.2	188271
F	12.3	47081
G	13.9	2212780
H	17.6	9208
I	17.7	2560

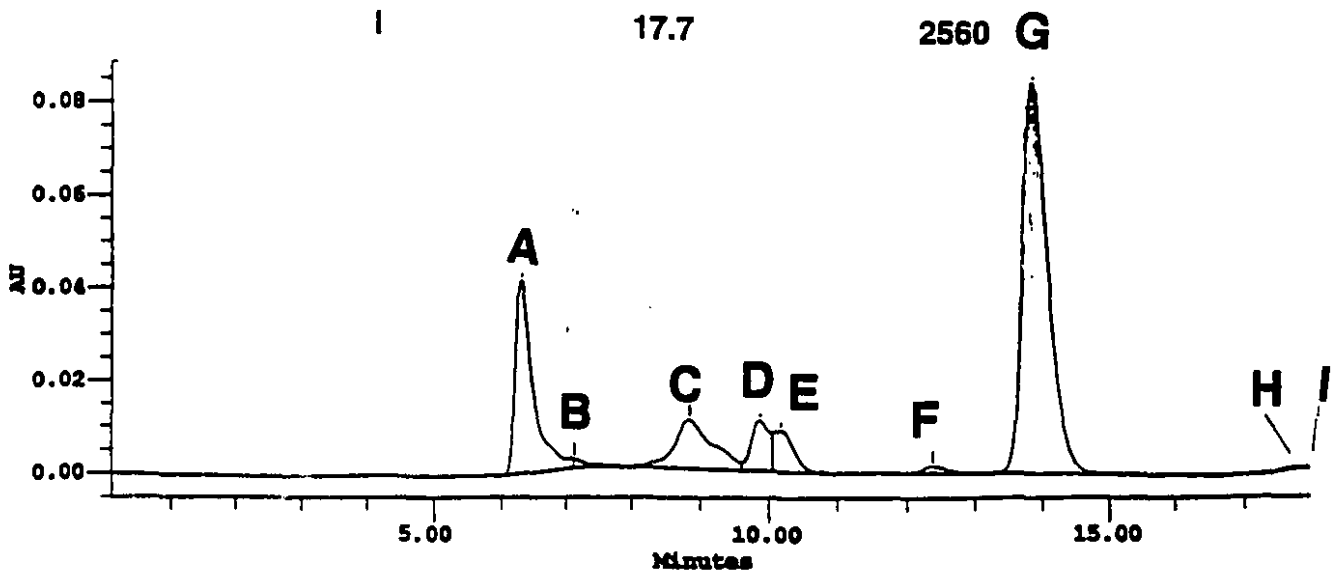


Figure D.3. Elution profile of a *T. versicolor*-TDM culture supernatant applied to an organic acid separating column with the newer HPLC and photodiode array detector. Peaks were detected at 210 nm. The y-axis scale was in absorbance units and the x-axis scale in minutes. Injection volume was 20 µl.

Peak	Retention Time (min)	Peak Area (uv.s)
A	6.3	2538083
B	7.6	92119
C	8.1	103281
D	8.3	59257
E	8.8	241570
F	9.5	173725
G	9.9	428210
H	10.1	363674
I	11.1	42695
J	12.4	95110
K	14.0	2353717
L	14.9	36215

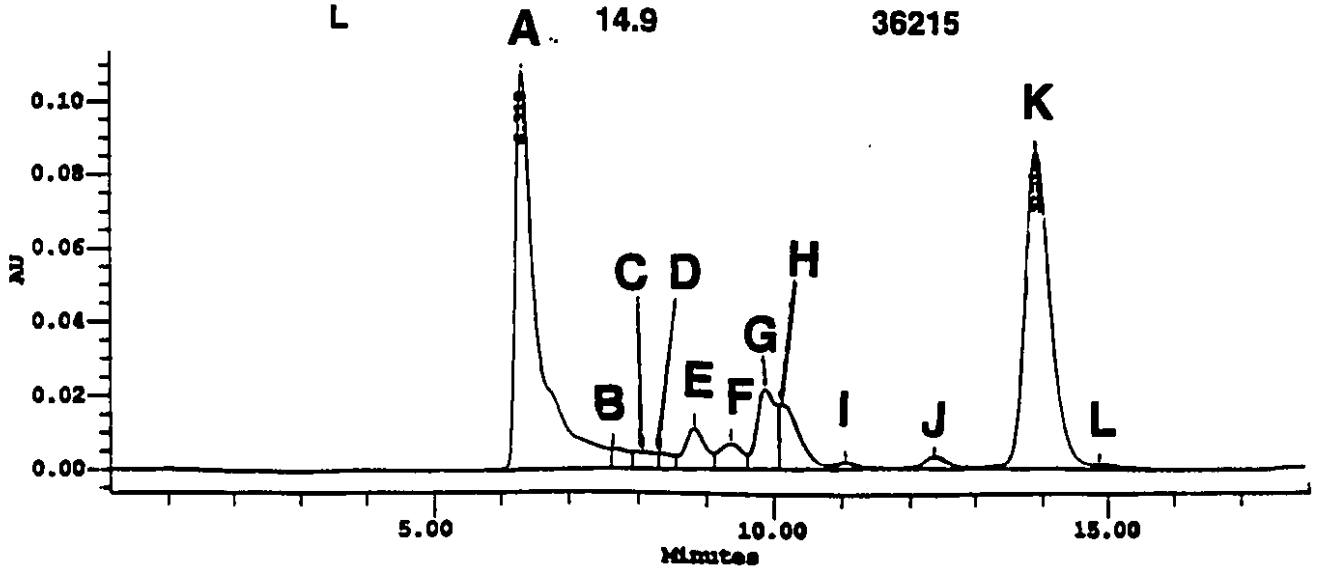


Figure D.4. Elution profile of a *T. versicolor*-TDM plus HWKP culture supernatant applied to an organic acid separating column with the newer HPLC and photodiode array detector. Peaks were detected at 210 nm. The y-axis scale was in absorbance units and the x-axis scale in minutes. Injection volume was 20 µl.

Peak	Retention Time (min)	Peak Area (uv.s)
A	7.3	8811
B	9.5	750.5
C	11.2	1318
D	13.7	453
E	15.4	12611
F	17.0	638
G	19.5	45.5

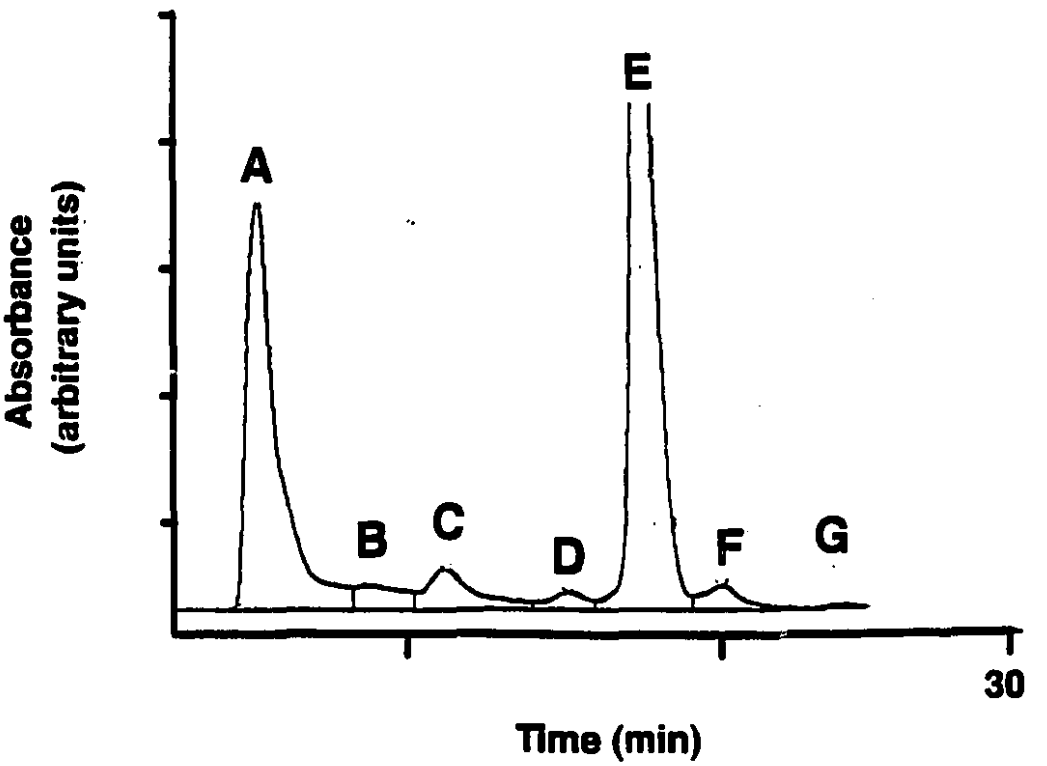


Figure D.5. Elution profile of a *T. versicolor*-TDM plus HWKP culture supernatant applied to an organic acid separating column with the older HPLC and photodiode array detector. Peaks were detected at 210 nm. The y-axis scale was in milliabsorbance units and the x-axis scale in minutes. Injection volume was 200 µl.

Peak	Retention Time (min)	Peak Area (uv.s)
A	4.2	142.3
B	7.2	30745
C	9.6	2976
D	10.9	3078
E	12.3	855
F	13.7	1589
G	15.4	13341
H	16.9	645
I	19.4	74.6

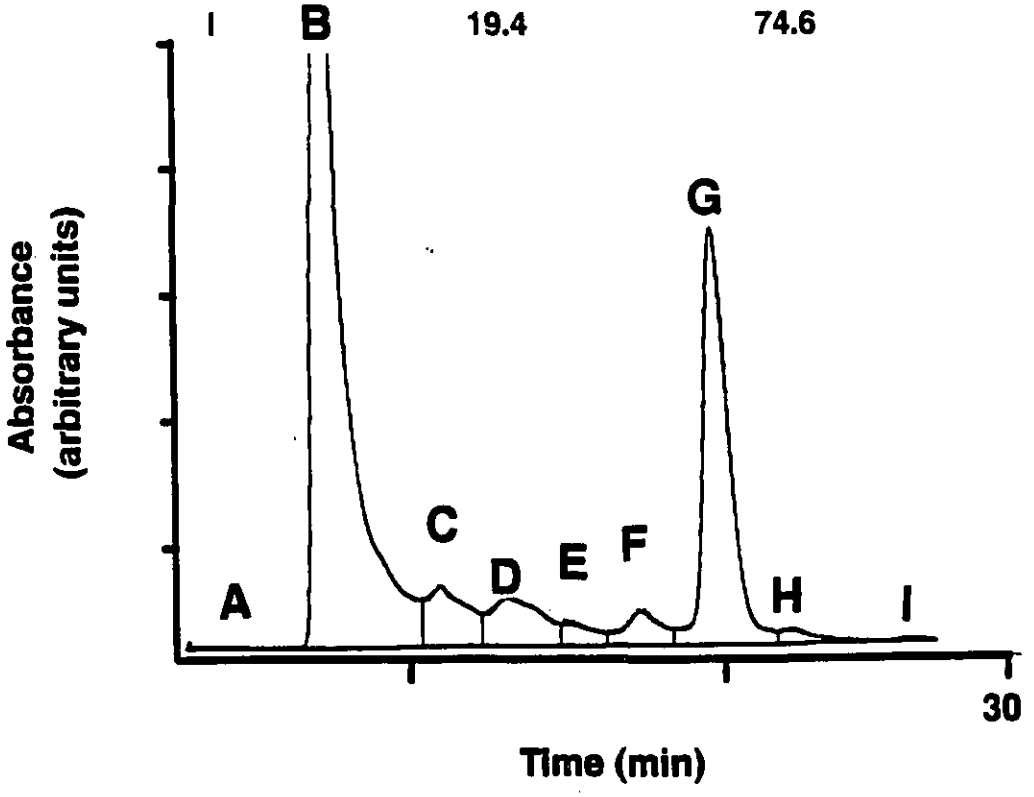


Figure D.6. Elution profile of a *T. versicolor*-TDM plus HWKP culture supernatant applied to an organic acid separating column with the older HPLC and photodiode array detector. Peaks were detected at 210 nm. The y-axis scale was in milliabsorbance units and the x-axis scale in minutes. Injection volume was 200 µl.

identity of the peak.

BIBLIOGRAPHY

Addleman, K. and F.S. Archibald. 1993. Pulp bleaching and delignification by dikaryons and monokaryons of *Trametes versicolor*. Appl. Environ. Microbiol. **59**: 266-273.

Adler, E., R. Magnusson, B. Berggren, and H. Thomelius. 1960. Periodate oxidation of phenols: II. Oxidation of 2,6-dimethoxyphenol and 3-methoxycatechol. Formation of a 1,2-napthoquinone. Acta. Chem. Scand. **14**: 515-528.

Adler, E. 1977. Lignin chemistry-past, present and future. Wood Sci. Technol. **11**: 169-218.

Agosin, E. , J.-J. Daudin, and E. Odier. 1985. Screening of white-rot fungi on (^{14}C)lignin-labelled and (^{14}C)whole-labelled wheat straw. Appl. Microbiol. Biotechnol. **22**: 132-138.

Ander, P. and K.-E. Eriksson. 1978. Lignin degradation and utilization by micro-organisms. In Progress in Industrial Microbiology, vol 14 (ed. M.J. Bull). Elsevier. New York. 1-58.

Ander, P., C. Mishra, R.L. Farrell, and K. -E. Eriksson, 1990. Redox reactions in lignin degradation: interactions between laccase different peroxidases and cellobiose: quinone oxidoreductase. J. Biotechnol. **13**: 189-198.

Ander, P. 1993. The importance of cellobiose oxidizing enzymes (CBQ and CbO). In lignin and cellulose degradation. In Proceedings Lignin Biodegradation and Transformation (J.C. Duarte, M.C. Ferreira, and P. Ander, eds.). Forbitec. Lisbon, Portugal. 115-118.

Ander, P. and K.-E. Eriksson. 1975. Influence of carbohydrates on lignin degradation by the white-rot fungus *Sporotrichum pulverulentum*. Svensk Papperstidning **18**: 643-652.

Ander, P. and K.-E. Eriksson. 1977. Selective degradation of wood components by white-rot fungi. Physiol. Plant. **41**: 239-248.

Ander, P., G. Sena-Martins, and J.C. Duarte. 1993. Influence of cellobiose oxidase on peroxidases from *Phanerochaete chrysosporium*. Biochem. J. **293**: 431-435.

Anon. 1972. Worthington Enzyme Manual, Freehold, New Jersey. p19.

Anonymous. 1993. 1992 reference tables. Canadian Pulp and Paper Association. Montreal. 54 pp.

Archibald, F.S. 1986. Manganese: its acquisition by and function in the lactic acid bacteria. CRC Critical Rev. in Microbiol. **13:** 63-10

Archibald, F.S. 1992. The role of fungus-fiber contact in the biobleaching of kraft brownstock by *Trametes (Coriolus) versicolor*. Holzforschung **46**: 305-310.

Archibald, F.S. 1992. Lignin peroxidase activity is not important in biological bleaching and delignification of unbleached kraft pulp by *Trametes versicolor*. Appl. Environ. Microbiol. **58**: 3101-3109.

Archibald, F.S. and I. Fridovich. 1982. The scavenging of superoxide radical by manganous complexes: *in vitro*. Arch. Biochem. Biophys. **214:** 452-463.

Archibald, F.S. and I. Fridovich. 1982. Investigations of the state of the manganese in *Lactobacillus plantarum*. Arch. Biochem. Biophys. **215:** 589-596.

Archibald, F.S. and B.P. Roy. 1992. Production of manganic chelates by laccase from the lignin-degrading fungus *Trametes (Coriolus) versicolor*. Appl. Environ. Microbiol. **58**: 1496-1499.

Archibald, F.S. and C. Tyree. 1987. Manganese poisoning and the attack of trivalent manganese upon catecholamines. Arch. Biochem. Biophys. **256:** 638-650.

Atherton, N.M. 1973. Electron Spin Resonance. Wiley Press. New York.

Ayer W.A., L.M. Browne, M.-C. Feng, H. Orszanska, and H. Saeedi-Ghomi. 1986. The chemistry of the blue stain fungi. Part 1. Some metabolites of *Ceratocystis* species associated with the mountain pine beetle infected lodgepole pine. Can. J. Chem. **64**: 904-909.

Ayers, A.R. S.B. Ayers, and K.-E. Eriksson. 1978. Cellobiose oxidase, purification and partial characterization of a hemoprotein from *Sporotrichum pulverulentum*. Eur. J. Biochem. **90**: 171-181.

Aziz, S. and K. Sarkanen. 1989. Organosolv pulping-a review. Tappi J. **72(3)**: 169-175.

Banci, L., I. Bertini, P. Turano, M. Tien, and T.K. Kirk. 1991. Proton NMR investigation into the basis for the relatively high redox potential of lignin peroxidase. Proc. Natl. Acad. Sci. U.S.A. **88**: 6956-6960.

Bao, W., and V. Renganathan. 1992. Cellobiose oxidase of *Phanerochaete chrysosporium* enhances crystalline cellulose degradation by cellulases. FEBS Lett. **302**: 77-80.

Bao, W., S.N. Usha, and V. Renganathan. 1993. Purification and characterization of cellobiose dehydrogenase, a novel extracellular hemoflavoenzyme from the white-rot fungus *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **300**: 705-713.

Bauminger, B.B. 1975. Micromethod for manual analysis of true glucose in plasma without deproteinization. J. Clin. Pathol. **27**: 1015-1017.

Bess, B., B. Pettersson, H. Lennholm, T. Iversen, and K.E. Eriksson. 1987. Synthesis, structure and enzyme degradation of an extracellular glucan produced in nitrogen-starved cultures of the white rot fungus *Phanerochaete chrysosporium*. Appl. Biochem. Biotechnol. **9**: 310-318.

Blanchette, R.A. 1984. Manganese accumulation in wood decayed by white rot fungi. Phytopathology. **74**: 725-730.

Blanchette, R.A. 1984. Selective delignification of eastern hemlock by *Ganoderma tsugae*. Phytopathology. **74**: 153-160.

Blanchette, R.A. 1984. Screening wood decayed by white-rot fungi for preferential lignin degradation. Appl. Environ. Microbiol. **48**: 647-653.

Blanchette, R.A. and C.G. Shaw. 1978. Associations among bacteria, yeasts and basidiomycetes during wood decay. Phytopathol. **68**: 631-637.

Bodini, M.E., L.A. Willis, T.L. Riechel, and D.T. Sawyer. 1976. Electrochemical and spectroscopic studies of manganese(II), -(III), -(IV) gluconate complexes. 1. Formulas and oxidation-reduction stoichiometry. J. Inorgan. Chem. **15**: 1538-1543.

Borchardt, L.G. and C.V. Piper. 1970. A gas chromatographic method for carbohydrates as alditol-acetates. Tappi. **53**: 257-260.

Bourbonnais, R. and M.G. Paice. 1988. Veratryl alcohol oxidases from the lignin-degrading basidiomycete *Pleurotus sajor-caju*. Biochem. J. **255**: 445-450.

Bourbonnais, R. and M.G. Paice. 1990. Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. FEBS Lett. **267**: 99-102.

Bourbonnais, R. and M.G. Paice. 1992. Demethylation and delignification of kraft pulp by *Trametes versicolor* laccase in the presence of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulphonate). Appl. Microbiol. Biotechnol. **36**: 823-827.

Bradford, M.M 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem. **72**: 248-254.

Brown. J.A., J.K. Glenn, and M.H. Gold. 1990. Manganese regulates expression of manganese peroxidase by *Phanerochaete chrysosporium*. J. Bacteriol. **172**: 3125-3130.

Buchala, A.J. and M. Leisola. 1987. Structure of the β-D-glucan secreted by *Phanerochaete chrysosporium* in continuous culture. Carbohydr. Res. **165**: 146-149.

Buswell, J.A. and K.-E. Eriksson. 1986. Methanol metabolism in *Phancrochaete chrysosporium*. Proc. 3rd International Conf. on Biotechnol. in the Pulp and Paper Ind., Stockholm, pp 20-23.

Call, H.P. and I. Muecke. 1994. State of the art of enzyme bleaching and disclosure of a breakthrough process. Proc. 1994 Non-Chlorine Bleaching Conf., Amelia Island.

Canevascini, G. 1988. Cellobiose Dehydrogenasc from *Sporotrichum thermophile*. Methods Enzymol. **160**: 443-448.

Canevascini, G. P. Borer, and J.-C. Dreyer. 1991. Cellobiose dehydrogenases of *Sporotrichum* (*Chrysosporium*) *thermophile*. Eur. J. Biochem. **198**: 43-52.

Cassclton, P.J. 1975. Anaplerotic Pathways. In: The filamentous fungi. Biosynthesis and metabolism (J.E. Smith and D.R. Berry, eds.). Edward Arnold Publishers. London. 121-136.

Chen, C.-L. and H.-M. Chang. 1985. Chemistry of lignin biodegradation. In Biosynthesis and biodegradation of wood components T. Higuchi (ed.). Academic Press. Orlando. 535-556.

Clayton, D.W. and J.E. Stone. 1963. The redeposition of hemicelluloses during pulping. Part 1: The use of tritium labelled xylan. Pulp Pap. Can. 64: 459-468.

Clayton, D., D. Easty, D. Einspahr, W. Lonsky, E. Malcolm, T. McDonough, L. Schroeder, and N. Thompson. 1989. Delignification process variables. In Pulp and Paper manufacture, vol 5 alkaline pulping. T.M. Grace and E.W. Malcolm (eds). The Joint Textbook Committee of the Paper Industry. Montreal.

Costa-Ferreira, M., P. Ander, and J.C. Duarte. 1992. Formation of peroxidases and cellobiose:quinone oxidoreductase in relation to degradation of synthetic lignin by *Phanerochaete chrysosporium*. In Biotechnology in Pulp and Paper Industry (M.S. Kuwahara and M. Shimada, eds.). UNI Publishers Co., Ltd., Tokyo, Japan. 351-356.

Coudray, M.R., G. Canevascini, and H. Meier. 1982. Characterization of a cellobiose dehydrogenase in the cellulolytic fungus *Sporotrichum* (*Chrysosporium*) *thermophile*. Biochem J. **203**: 277-284.

Cox, M.C., M.S. Rogers, M. Cheesman, G.D. Jones, A.J. Thomson, M.T. Wilson, and G.R. Moore. 1992. Spectroscopic identification of the haem ligands of cellobiose oxidase. FEBS Lett. **307**: 233-236.

Datta, A., A. Betterman, and T.K. Kirk. 1991. Identification of a specific manganese peroxidase among ligninolytic enzymes secreted by *Phanerochaete chrysosporium* during wood decay. Appl. Environ. Microbiol. **57**: 1453-1460.

Dekker, R.F.H. 1988. Cellobiose dehydrogenase produced by *Monilia* sp. Methods in Enzymol. **160**: 454-463.

Dekker, R.F.H. 1980. Induction and characterization of a cellobiose dehydrogenase produced by a species of *Monilia*. J. Gen. Microbiol. **120**: 309-316.

Dence, C.W. and R.C. Francis. 1991. Delignification and brightness development with non-conventional peroxide systems. In Workshop on emerging pulping and chlorine-free bleaching technologies. Raleigh NC.

Dodson, P.J., C.S. Evans, P.J. Harvey, and J.M. Palmer. 1987. Production and properties of an extracellular peroxidase from *Coriolus versicolor* which catalyses C_α-C_β cleavage in a lignin model compound. FEMS Microbiol. Lett. **42**: 17-22.

Dutton, M.V., C.S. Evans, P.T. Atkey, D.A. Wood. 1993. Oxalate production by basidiomycetes, including the white-rot species *Coriolus versicolor* and *Phanerochaete chrysosporium*. Appl. Microbiol. Biotechnol. **39**: 5-10.

Edwards, S.L., R. Raag, H. Wariishi, M.H. Gold, and T.L. Poulos. 1993. Crystal structure of lignin peroxidase. Proc. Natl. Acad. Sci. USA. **90**: 750-754,

Eriksson, K.-E., B. Pettersson, and U. Westermark. 1974. Oxidation: An important enzyme reaction in fungal degradation of cellulose. FEBS Lett. **49**: 282-285.

Eriksson, K.-E.L., R.A. Blanchette, and P. Ander. 1990. Microbial and enzymatic degradation of wood and wood components. Springer-Verlag. New York. 407 pp.

Eriksson, K.-E., B. Pettersson, J. Volc, and V. Musilek. 1986. Formation and partial characterization of glucose-2-oxidase, a H_2O_2 producing enzyme in *Phanerochaete chrysosporium*. Appl. Microbiol. Biotechnol. **23**: 257-262.

Eriksson, K.E. and B. Pettersson. 1982. Purification and partial characterization of two acidic proteases from the white-rot fungus *Sporotrichum pulverulentum*. Eur. J. Biochem. **124**: 635-642.

Escribano, J. Garcia-Cánofvas, F. Garcia-Carmona, and J.A. Lozano. 1985. Kinetic study of the transient phase of a second-order chemical reaction coupled to an enzymic step: application to the oxidation of chlorpromazine by peroxidase-hydrogen peroxide. Biochem. Biophys. Acta. **831**: 313-320.

Evans, C.S., J.Y. Farmer, J.M. Palmer. 1984. An extracellular haem-protein from *Coriolus versicolor*. Phytochem. **23**: 1247-1250.

Fähnrich, P. and K. Irrgang. 1982. Conversion of cellulose to sugars and cellobionic acid by the extracellular enzyme system of *Chaetomium cellulolyticum*. Biotech. Lett. **4**: 775-780.

Fåhraeus, G. and B. Reinhammmar. 1967. Large scale production and purification of laccase from cultures of the fungus *Polyporus versicolor* and some properties of laccase A. Acta Chem Scand. **21**: 2367-2378.

Falkehag, S.I., J. Marton, and E. Adler. 1966. Chromophores in kraft lignin. In Lignin structure and reactions. Adv. in Chem. Ser. 59: 75-89. Amer. Chem. Soc., Washington.

Fazekas, A.G. and K. Kokai. 1971. Extraction, purification, and separation of tissue flavins for spectrophotometric determination. Methods Enzymol. **18B**: 385-398.

Fenn, P. and T.K. Kirk. 1981. Relationship of nitrogen to the onset and suppression of ligninolytic activity and secondary metabolism in *Phanerochaete chrysosporium*. Arch. Microbiol. **130**: 59-65.

Forrester, I.T., A.C. Grabski, R.R. Burgess, and G.F. Leatham. 1988. Manganese, Mn-dependent peroxidase, and the biodegradation of lignin. Biochem. Biophys. Res. Commun. **157**: 992-999.

Fujita, K., R. Kondo, K. Sakai, Y. Kashino, T. Nishida, and Y. Takahara. 1993. Biobleaching of softwood kraft pulp with white-rot fungus IZU-154. Tappi J. **76(1)**: 91-94.

Fujita, K., R. Kondo, K. Sakai, Y. Kashino, T. Nishida, and Y. Takahara. 1991. Biobleaching of kraft pulp using white-rot fungus IZU-154. Tappi J. **74(11)**: 123-127.

Furman, G.S. Jr. 1986. The contribution of chrarge-transfer complexes to the color of kraft lignin. The institute of Paper Chemistry, Appleton, WI. PhD Thesis. 170pp.

Galliano, H., G. Gas, J.L. Seris, and A.M. Boudet. 1991. Lignin degradation by *Rigidoporus lignosus* involves synergistic action of two oxidizing enzymes: Mn peroxidase and laccase. Enzyme Microb. Technol. **13**: 478-482.

Gilbertson, R.L. 1980. Wood-rotting fungi of North America. Mycologia **72**: 1-49.

Glenn, J.K., and M.H. Gold. 1985. Purification and characterization of an extracellular Mn(II)-dependent peroxidase from the lignin-degrading basidiomycete, *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **242**: 329-341.

Glenn, J.K., L. Akileswaran and M.H. Gold. 1986. Mn(II) oxidation is the principal function of the extracellular Mn-peroxidase from *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **251**: 688-696.

Glenn, J.K., M.A. Morgan, M.B. Mayfield, M. Kuwahara, and M.H. Gold. 1983. An extracellular H_2O_2-requiring enzyme preparation involved in lignin biodegradation by the white rot basidiomycete *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **114**: 1077-1083.

Glossman, H. and D.M. Neville Jr. 1971. Glycoproteins of cell surfaces. A comparative study of three different cell surfaces of the rat. J. Biol. Chem. **246**: 6339-6346.

Gold, M.H., J. Kuwahara, A.A. Chiu, and J.K. Glenn. 1984. Purification and characterization of an extracellular H_2O_2-requiring diarylpropane oxygenase from the white-rot basidiomycete *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **234:** 353-362.

Goring, D.A.I. 1983. Some recent topics in wood and pulping chemistry. Intl. Symp. Wood Pulping Chem. **1**: 3-13.

Green T.R., 1977. Significance of glucose oxidase in lignin degradation. Science **268**: 78-80.

Greene, R.V. and J.M. Gould. 1983. Substrate-induced H_2O_2 production in mycelia from the lignin-degrading fungus *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **117**: 275-281.

Habu, N., M. Samejima, J.F.D. Dean, and K.-E.L. Eriksson. 1993. Release of the FAD domain from cellobiose oxidase by proteases from cellulolytic cultures of *Phanerochaete chrysosporium*. FEBS Lett. **327**: 161-164.

Haemmerli, S.D., M.S.A. Leisola, and A. Fiechter. 1986. Polymerisation of lignin by ligninases from *Phanerochaete chrysoporium*. FEMS Microbiol. Lett. **35**: 33-36.

Harkin, J.M. and J.R. Obst. 1973. Lignification in trees: indication of exclusive peroxidase participation. Science **180**: 296-297.

Harris, P.J., A.B. Blakeney, R.J. Henry, and B.A. Stone. 1988. Gas chromatographic determination of the monosaccharide composition of plant cell wall preparations. J. Assoc. Off. Anal. Chem. **71**: 272-275.

Henriksson, G., G. Pettersson, G. Johansson, A. Ruiz, and E. Uzcategui. 1991. Cellobiose oxidase from *Phanerochaete chrysosporium* can be cleaved by papain into two domains. Eur. J. Biochem. **196**: 101-106.

Highley, T.L., and L.L. Murmanis. 1985. Determination of hydrogen peroxide production in *Coriolus versicolor* and *Poria Placenta* during wood degradation. Mater Org. **20**: 241-252.

Higuchi, T. and Y. Ito. 1958. Dehydrogenation products of coniferyl alcohol formed by the action of mushroom phenol oxidase, *Rhus* laccase, and radish peroxidase. J. Biochem. **45**: 575-579.

Hodge, J.E., and B.T. Hofreiter. 1962. Determination of reducing sugars and carbohydrates. In Carbohydrate Chemistry (R.L. Whistler and M.L. Wolfrom, eds.) Academic Press. New York. 380-394.

Hütterman, A. and A. Noelle. 1982. Characterization and regulation of cellobiose dehydrogenase in *Fomes annosus*. Holzforschung **36**: 283-286.

Hütterman, A., M. Gebauer, C. Volger, and C. Rösger. 1977. Polymerisation und abbau von natrium-ligninsulfonat durch *Fomes annosus* (Fr.) Cooke. Holzforschung **31**:83-89.

Iiyama, K. and J. Nakano. 1973. Studies on the color of lignin. Part X effects of chromophoric groups on the color of various lignins. Japan Tappi: 27(11): 530-542.

Jamieson, S. 1991. Alcell pulping: World class research right here in Canada. Pulp Pap. Can. **92(3)**: 16-18.

Johansson, T., K.G. Welinder, and P.O. Nyman. 1993. Isozymes of lignin peroxidase and manganese(II) peroxidase from the white-rot basidiomycete *Trametes versicolor*. Partial sequences, peptide maps, and amino acid and carbohydrate compositions. Arch. Biochem. Biophys. **300**: 57-62.

Johansson, T. and P.O. Nyman. 1987. A manganese(II)-dependent extracellular peroxidase from the white-rot fungus *Trametes versicolor*. Acta Chem Scand. **B41**: 762-765.

Johansson, T. and P.O. Nyman. 1993. Isozymes of lignin peroxidase and manganese(II) peroxidase from the white-rot basidiomycete *Trametes versicolor*. Isolation of enzyme forms and characterization of physical and catalytic properties. Arch. Biochem. Biophys. **300**: 49-56.

Jönsson, L., T. Johansson, K. Sjöström, P.O. Nyman. 1987. Purification of ligninase isozymes from the white-rot fungus *Trametes versicolor*. Acta Chem. Scand. **B41**: 766-769.

Kaplan, D.L. 1979. Reactivity of different oxidases with lignin and lignin model compounds. Phytochem. **18**: 1917-1919.

Karhunen, E., A. Kantelinen, and M-L Niku-Paavola. 1990. Mn-dependent peroxidase from lignin-degrading white rot fungus *Phlebia radiata*. Arch. Biochem. Biophys. **279**: 25-31.

Kelleher, T.J., B.S. Montenecourt, and D.E. Eveleigh. 1987. Cellobiose-quinone oxidoreductase-application in monitoring cellobiohydrolase purification. Appl. Microbiol. Biotechnol. **27**: 299-305.

Kelley, R.L. and C.A. Reddy. 1986. Purification and characterization of glucose oxidase from ligninolytic cultures of *Phanerochaete chrysosporium*. J. Bacteriol. **166**: 269-274.

Kenten, R.H., and P.J.G. Mann. 1955. The oxidation of manganese by illuminated chloroplast preparations. Biochem. J. **61**: 279-286.

Kenten, R.H., and P.J.G. Mann. 1950. The oxidation of manganese by peroxidase systems. Biochem. J. **46**: 67-73.

Kenten, R.H. and P.J.G. Mann. 1953. The oxidation of certain dicarboxylic acids by peroxidase systems in the presence of manganese. Biochem. J. **53**: 498-505.

Kern, H.W. 1989. Production and stability of lignin peroxidases of *Phanerochaete chrysosporium* cultivated on glycerol in the presence of solid manganese(IV) oxide. Appl. Microbiol. Biotechnol. **33**: 582-588.

Kern, H.W. 1990. Improvement in the production of extracellular lignin peroxidases by *Phanerochaete chrysosporium*: effect of solid manganese(IV) oxide. Appl. Microbiol. Biotechnol. **32**: 223-234.

Kersten, P.J., Kalyanaraman, K.E. Hammel, and T.K. Kirk. 1987. Horseradish peroxidases oxidizes 1,2,4,5-tetramethoxybenzene by a cation radical mechanism. In Lignin enzymic and microbial degradation (Odier, ed.). Symp. Intern. INRA. Paris. 75-79.

Kersten, P.J. and D. Cullen. 1993. Cloning and characterization of a c-DNA encoding glyoxal oxidase: A H_2O_2 producing enzyme from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Proc. Natl. Acad. Sci. U.S.A. **90**: 7411-7424.

Kersten, P.J. and T.K. Kirk. 1987. Involvement of a new enzyme, glyoxal oxidase, in extracellular H_2O_2 production by *Phanerochaete chrysosporium*. J. Bacteriol. **169**: 2195-2201.

Kersten, P. J., S.K. Stephens, and T.K. Kirk. 1988. Glyoxal oxidase and the extracellular peroxidases. In: Biotechnology in Pulp and Paper Manufacture: Applications and Fundamental Investigations. Proceedings of the Fourth International Conference on Biotechnology in the Pulp and Paper Industry. (T.K. Kirk and H.-M. Chang, eds.).Butterworth-Heinemann, Toronto. pp 457-463.

Kersten, P.J., M. Tien, B. Kalyanaraman, and T.K. Kirk. 1985. The ligninase of *Phanerochaete chrysosporium* generates cation radicals from methoxybenzenes. J. Biol. Chem. **260:** 2609-2612.

Kersten, P.J., B. Kalyanaraman, K.E. Hammel, B. Reinhammar, and T.K. Kirk. 1990. Comparison of lignin peroxidase, horseradish peroxidase, and laccase in the oxidation of methoxybenzenes. Biochem. J. **268:** 475-480.

Kersten. P.J. and T.K. Kirk. 1987. Involvement of a new enzyme glyoxal oxidase, in extracellular H_2O_2 production by *Phanerochaete chrysosporium*. J. Bacteriol. **169**: 2195-2202.

Keyser, P., T.K. Kirk, and J.G. Zeikus. 1978. Ligninolytic enzyme system of *Phanerochaete chrysosporium*: synthesized in the absence of lignin in response to nitrogen starvation. J. Bacteriol. **135**: 790-797.

Kirk, T.K. 1984. Degradation of lignin. In Microbial degradation of organic compounds. D.T. Gibson (ed.). Marcel Dekker. New York. 399-437.

Kirk, T.K. 1973. The chemistry and biochemistry of decay. In Wood deterioration and its prevention by preservative treatment. Vol. 1. DD. Nicholas (ed.). Syracuse University Press. Syracuse. 149-181.

Kirk, T.K. 1971. Effects of microorganisms on lignin. Annu. Rev. Phytopathol. **9**: 185-210.

Kirk, T.K. and R.L. Farrell. 1987. Enzymatic "combustion": The microbial degradation of lignin. Annu. Rev. Microbiol. **41**: 465-505.

Kirk, T.K., W.J. Connors, R.D. Bleam, W.F. Hackett, and J.G. Zeikus. 1975. Preparation and microbial decomposition of synthetic (^{14}C)-lignins. Proc. Nat. Acad. Sci. U.S.A. **72**: 2515-2519

Kirk, T.K. and M. Shimada. 1985. Lignin biodegradation: the microorganisms involved and the physiology and biochemistry of degradation by white-rot fungi. In Biosynthesis and biodegradation of wood components, T. Higuchi (ed.). Academic Press. Orlando. 579-605.

Kirk, T.K., M. Tien, P.J. Kersten, M.D. Mozuch, and B. Kalyanaraman. 1986. ligninase of *Phanerochaete chrysosporium*: mechanism of its degradation of the non-phenolic arylglycerol β-aryl ether substructure of lignin. Biochem. J. **236**:279-287.

Kirk, T.K., M. Tien, and B.D. Faison. 1984. Biochemistry of the oxidation of lignin by *Phanerochaete chrysosporium*. Biotech. Adv. **2**: 183-199.

Kirk, T.K., E. Schultz, W.J. Connors, L.F. Lorenz, and J.G. Zeikus. 1978. Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium*. Arch. Microbiol. **117**: 277-285.

Kirk, T.K. and H.H. Yang. 1979. Partial delignification of unbleached kraft pulp with ligninolytic fungi. Biotechnol. Lett. **1**: 347-352.

Kirk, T.K., and R.L. Farrell. 1987. Enzymatic "combustion": The microbial degradation of lignin. Annu. Rev. Microbiol. **41**: 465-505.

Kirkpatrick, N., I.D. Reid, E. Ziomek, C. Ho, and M.G. Paice. 1989. Relationship between fungal biomass production and the brightening of hardwood kraft pulp by *Coriolus versicolor.* Appl. Environ. Microbiol. **55**: 1147-1152.

Kirkpatrick, N., I.D. Reid, E. Ziomek, and M.G. Paice. 1990. Biological bleaching of hardwood kraft pulp using *Trametes* (*Coriolus*) *versicolor* immobilized in polyurethane foam. Appl. Microbiol. Biotechnol. **33**: 105-108.

Koenigs, J.W. 1974. Hydrogen peroxide and iron: a proposed system for decomposition of wood by brown-rot basidiomycetes. Wood. Fiber **6**: 66-80.

Koenigs, J.W. 1972. Production of extracellular hydrogen peroxide and peroxidase by wood-rotting fungi. Phytopathol. **62**: 100-110.

Kondo, R., T. Iimori, and H. Imamura. 1990. Bioconversion of a lignin model compound into its glucoside during the hydrosysis of cellobiose by mucelia of wood-rotting fungi. Mokuzai Gakkaishi **36**: 501-505.

Kondo, R. and H. Imamura. 1989. Formation of lignin model xyloside in polysaccharades media by wood-rotting fungi. Mokuzai Gakkaishi **35**: 501-505.

Kondo, R., K. Kurashiki, and K. Sakai. 1993. Biobleaching of kraft pulp by extracellular enzymes excreted from white-rot fungi in a cultivation system using a membrane filter. Proc. FEMS Symposium on Lignin Biodegradation and Transformation. Lisbon. 245-246.

Kondo, R., H. Yamagami, and K. Sakai. 1993. Xylosylation of phenolic hydroxyl groups of the monomeric lignin model compounds 4-methylguaiacol and vanillyl alcohol by *Coriolus versicolor*. Appl. Environ. Microbiol. **59**: 438-441.

Kondo, R. and H. Imamura. 1988. Formation of lignin model glucosides by wood-rotting fungi. Mokuzai Gakkaishi **34**: 609-617.

Kono, Y., Takahashi, M-A., and K. Asada. 1976. Oxidation of manganese pyrophosphate by superoxide radicals and illuminated spinach chloroplasts. Arch. Biochem. Biophys. **174:** 454-462.

Kremer, S.M. and P.M. Wood. 1992. Evidence that cellobiose oxidase from *Phanerochaete chrysosporium* is primarily an Fe(III) reductase. Kinetic comparison with neutrophil NADPH oxidase and yeast flavocytochrome b_2. Eur. J. Biochem. **205**; 133-138.

Kremer, S.M. and P.M. Wood. 1992b. Production of Fenton's reagent by cellobiose oxidase from cellulolytic cultures of *Phanerochaete chrysosporium*. Eur. J. Biochem. **208**: 807-814.

Kremer, S.M. and P.M. Wood. 1992c. Continuous monitoring of cellulose oxidation by cellobiose oxidase from *Phanerochaete chrysosporium*. FEMS Microbiol. Lett. **92**: 187-192.

Kritzman, G., I. Chet, and Y. Henis. 1977. The role of oxalic acid in the pathogenic behavior of *Sclerotium rolfsii* Sacc. Exper. Mycology. **1**: 280-285.

Kritzman, G., I. Chet, and Y. Henis. 1977. Effect of carbon dioxide on growth and carbohydrate metabolism in *Sclerotium rolfsii*. J. Gen. Microbiol. **100**: 167-175.

Kuan, I-C., M. Tien. 1993. Stimulation of Mn peroxidase activity: A possible role for oxalate in lignin biodegradation. Proc. Natl. Acad. Sci. U.S.A. **90**: 1242-1246.

Kuan, I-C., M. Tien. 1993a. Glyoxylate-supported reactions catalyzed by Mn peroxidase of *Phanerochaete chrysosporium*: Activity in the absence of added hydrogen peroxide Arch. Biochem. Biophys. **302**: 447-454.

Kuan, I-C., K.A. Johnson, and M. Tien. 1993. Kinetic analysis of manganese peroxidase. The reaction with manganese chelators. J. Biol. Chem. **268**: in press.

Kuwahara, M., Y. Asada, Y. Kimura, M. Aokage. 1987. Isolation of mutants of a lignin-degrading basidiomycete, *Phanerochaete chrysosporium*, that produce lignin peroxidase in high nitrogen cultures. Mokuzai Gakkaishi. **33**: 821-823.

Kuwahara, M. J.K. Glenn, M.A. Morgan, and M.H. Gold. 1984. Separation and characterization of two extracellular H_2O_2-dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*. FEBS Lett. **169**: 247-250.

Lachenal, D. 1991. Full bleaching with a combination of ozone oxygen and peroxide should work. In Workshop on emerging pulping and chlorine-free bleaching technologies. Raleigh NC.

Lachenal, D. 1992. Status of pilot plant activities worldwide: the CTP bleaching pilot plant. Non chlorine bleaching conference. Hilton Head, SC. U.S.A., March 1992.

Lackner, R., E. Srebotnik, and K. Messner. 1991. Oxidative degradation of high molecular weight chlorolignin by manganese peroxidase of *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **178**: 1092-1098.

Law, K. 1959. A role of the laccase of wood-rotting fungi. Physiol. Plantarum **12**: 854-861.

Leatham, G.F. and T.K. Kirk. 1983. Regulation of ligninolytic activity by nutrient nitrogen in white-rot basidiomycetes. FEMS Microbiol. Lett. **16**: 65-67.

Leonowicz, A., G. Szklarz, M. Wojtas-Wasilewska. 1985. The effect of fungal laccase on fractionated lignosulphonates (Peritan Na). Phytochem. **24**: 393-396.

Liebergott, N. 1991. Bleaching with reductive agents. In Workshop on emerging pulping and chlorine-free bleaching technologies. Raleigh NC.

Liebergott, N., H.I. Bolker and D.W. Clayton. 1977. Gas-phase bleaching of softwood and hardwood kraft and kraft-oxygen pulps. Trans. Tech. Sect. CPPA: **3**: 16-22.

Liebergott, N. and B. van Lierop. 1981. Ozone delignification of black spruce and hardwood kraft, kraft-anthraquinone and soda-anthraquinone pulps. Tappi **64(6)**: 95-100.

Liebergott, N., H.G. Barclay, J. Merka, D.P.W. Pounds, H.I. Bolker, and D.W. Clayton. The Papribleach process-laboratory to pilot plant. Trans. Tech. Sect. CPPA **1(2)**: 49-60.

Lingappa, B.T., M. Prasad, Y. Lingappa, D.F. Hunt, and K. Biemann. 1969. Phenylethyl alcohol and tryptophol: Autoantibiotics produced by the fungus *Candida albicans*. **193**: 192-194.

Lockner, R., E. Srebotnik, and K. Messner 1991. Oxidative degradation of high molecular weight chlorolignin by manganese peroxidase of *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **178**: 1092-1098.

Ma, D., T. Hattori, Y. Akamatsu, M. adachi, and M. Shimada. 1992. Kinetic analysis of the noncompetitive inhibition of the lignin-peroxidase-catalyzed reaction by oxalic acid. Biosci. Biotech. Biochem. **56**: 1378-1381.

MacLeod, J.M. 1993. Extended delignification: Science, technology, and installations worldwide. MR248. Pulp and Paper Research Institute of Canada, Pointe Claire. p1-22.

Martin, F., C. Delaruelle, and J.-L. Hilbert. 1990. An improved ergosterol assay to estimate fungal biomass in ectomycorrhizas. Mycological Research **94**: 1059-1064.

Mason, J.C., M. Richards, W Zimmermann, and P. Broda. 1988. Identification of extracellular proteins from actinomycetes responsible for the solubilization of lignocellulose. Appl. Microbiol. Biotechnol. **28**: 276-280.

Massey, V. and G. Palmer. 1966. The existence of spectrally distinct classes of flavoprotein semiquinones. A new method for the quantitative production of flavoprotein semiquinones. Biochemistry **5**: 3181-3189.

Matcham, S.E., B.R. Jordan, and D.A. Wood. 1985. Estimation of fungal biomass in a solid substrate by three independent methods. Appl. Microbiol. Biotechnol. **21**: 108-112.

Matthews, C.H. 1974. Carbohydrate losses at high temperature in kraft pulping. Svensk Papperstid. **77**: 629-635.

Maxwell, D.P. and D.F. Bateman. 1968. Oxalic acid biosynthesis by *Sclerotium rolfsii*. Phytopathology **58**: 1635-1642.

McCarthy, A.J. and P. Broda. 1984. Screening for lignin-degrading actinomycetes and characterization of their activity against (^{14}C)-lignin-labelled wheat lignocellulose. J. Gen. Microbiol. **130**: 2905-2913.

McEldoon, J.P., and J.S. Dordick. 1991. Thiol and Mn^{2+} - mediated oxidation of veratryl alcohol by horseradish peroxidase. J. Biol. Chem. **266**: 14288-14293.

Michel, F.C. Jr., Dass, S.B., Grolke, E.A., and Reddy, C.A. 1991. Role of manganese peroxidases and lignin peroxidases of *Phanerochaete chrysosporium* in the decolorization of kraft bleach plant effluent. Appl. Environ. Microbiol. **57:** 2368-2375.

Miki, K., V. Renganathan, M.B. Mayfield and M.H. Gold. 1987. Aromatic ring cleavage of a β-biphenyl ether dimer catalyzd by lignin peroxidase of *Phanerochaete chrysosporium*. FEBS Lett. **210**: 199-203.

Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. **31**: 426-428.

Miller, G.L. 1963. Cellodextrins. In Methods in Carbohydrate Chemistry vol. 3 (R.L. Whistler, ed.). Academic Press. New York. 134-139.

Morohoshi, N., K. Fujita, H. Wariishi, and T. Haraguchi. 1987. Degradation of lignin by the extracellular enzymes of *Coriolus versicolor* V. Degradation of isolated lignin fractions by laccase I, II, and III. Mokuzai Gakkaishi. **33**: 310-315.

Morpeth, F.F. 1985. Some properties of cellobiose oxidase from the white-rot fungus *Sporotrichum pulverulentum*. Biochem J. **228**: 557-564.

Morpeth, F.F. and G.D. Jones. 1986. Resolution, purification and some properties of the multiple forms of cellobiose quinone dehydrogenase from the white-rot fungus *Sporotrichum pulverulentum*. Biochem J. **236**: 221-226.

Murata, S., R. Kondo, K. Sakai, Y. Kashino, T. Nishida, and Y. Kakahara. 1992. Chlorine-free bleaching process of kraft pulp using treatment with the fungus IZU-154. Tappi J. **75(12)**: 91-94.

Nagieb, Z.A., S.A. El-Meadaway, A.A. El-Gammal. 1988. Chemical characterization of native and degraded lignin from both rice straw and cotton stalks decayed by white rot fungi. Holzforsch Holzverwert **40**: 33-37.

Narayanan, T.K. and G. R. Rao. 1976. Production of β-(4-hydroxyphenyl)ethanol and β-(4-hydroxyphenyl)lactic acid by *Candida* species. Can. J. Microbiol. **22**: 384-389.

Narayanan, T.K. and G. R. Rao. 1974. Production of 2-phenethyl alcohol and 2-phenyllactic acid in *Candida* species. Biochem. Biophys. Res. Comm. **58**: 728-735.

Ni, Y., G.J. Kubes, and A.R.P. van Heiningen. 1990. A new mechanism for pulp delignification during chlorination. J. Pulp Paper Sci. **16**: 13-19.

Niemelä, K., R. Alén, and E. Sjöström. 1985. The formation of carboxylic acids during kraft and kraft-anthraquinone pulping of birch wood. Holzforschung **39**: 167-172.

Nishida, A., K.-E. Eriksson. 1987. Formation, pruification, and partial characterization of methanol oxidase, a H_2O_2-producing enzyme in P*hanerochaete chrysosporium.* Biotechnol. Appl. Biochem. **9**: 325-338.

Nobles, M.K. 1958. Cultural characteistics as a guide to the taxonomy and phylogeny of the *Polyporaceae*. Can. J. Bot. **36**: 893-926.

Odier, E., M.D. Mozuch, B. Kalyanaraman, T.K. Kirk. 1988. Ligninase-mediated phenoxy radical formation and polymerization unaffected by cellobiose:quinone oxidoreductase. Biochemie **70**: 847-852.

Osswald, W.F., W. Höll, and E.F. Eltsener. 1986. Ergosterol as a biochemical indicator of fungal infection in spruce and fir needles from different sources. Zeitschrift fur Naturforschung. **41**: 542-546.

Paice, M.G., L. Jurasek, C. Ho, R. Bourbonnais, and F.S. Archibald. 1989. Direct biological bleaching of hardwood kraft pulp with the fungus *Coriolus versicolor*. Tappi J. **72**: 217-221.

Paice, M.G., I.D. Reid, R. Bourbonnais, F.S. Archibald, and L. Jurasek. 1993. Manganese peroxidase produced by *Trametes versicolor* during pulp bleaching demethylates and delignifies kraft pulp. Appl. Environ. Microbiol. **59**: 260-265.

Paszcynski, A., V.-B. Huynh, and R. Crawford. 1985. Enzymatic activities of an extracellular, manganese-dependent peroxidase from *Phanerochaete chrysosporium*. FEMS Microbiol. Lett. **29**: 37-41.

Pease, E.A. and M. Tien. 1992. Heterogeneity and regulation of manganese peroxidases from *Phanerochaete chrysosporium*. J. Bacteriol. **174**: 3532-3540.

Perera, I.R., E. Uzcategui, F. Hakansson, G. Brinkman, G. Pettersson, G. Johansson, and B.U.R. Sundquist. 1990. Rapid Comm. Mass. Spec. **4**: 285-289.

Perez, J. and T.W. Jeffries. 1993. Roles of organic acid chelators in manganese regulation of lignin degradation by *Phanerochaete chrysosporium*. Appl. Biochem. Biotechnol. **39/40**: 227-238.

Perez, J. and T.W. Jeffries. 1992. Roles of manganese and organic acid chelators in regulating lignin degradation and biosynthesis of peroxidases by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **58**: 2402-2409.

Périé, F.H. and M.H. Gold. 1991. Manganese regulation of manganese peroxidase expression and lignin degradation by the white rot fungus *Dichomitus squalens*. Appl. Environ. Microbiol. **57**: 2240-2245.

Pick, E., and Keisari, Y. 1980. A simple colormetric method for the measurement of hydrogen peroxide produced by cells in culture. J. Immunol. Methods **38**: 161-170.

Piontek, K., T. Glumoff, and K. Winterhalter. 1993. Low pH crystal structure of glycosylated lignin peroxidase from *Phanerochaete chrysoporium*. FEBS Lett. **315**: 119-124.

Popp, J.L., B. Kalyanaraman, and T.K. Kirk. 1990. Lignin peroxidase oxidation of Mn^{+2} in the presence of veratryl alcohol, malonic or oxalic acid, and oxygen. Biochemistry **29**: 10475-10480.

Poulos, T.L., S. Edwards, H. Wariishi, and M.H. Gold. 1993. Crystallographic refinement of lignin peroxidase at 2 Å. J. Biol. Chem. **268**: 4429-4440.

Ramachandra, M., D.L. Crawford, and G. Hertyl. 1988. Characterization of an extracellular lignin peroxidase of the lignocellulolytic actinomycete *Streptomyces viridosporus*. Appl. Environ. Microbiol. **54**: 3057-3063

Reeve, D.W. 1989. Delignification process variables. In Pulp and Paper manufacture, vol 5 alkaline pulping. T.M. Grace and E.W. Malcolm (eds). The Joint Textbook Committee of the Paper Industry. Montreal.

Reid, I.D. and Paice, M.G. 1994. Effect of residual lignin type and amount on biological bleaching of kraft pulp by *Trametes versicolor*. Appl. Environ. Microbiol. **60**: 1395-1340.

Reid, I.D., M.G. Paice, C. Ho, and L. Jurasek. 1990. Biological bleaching of softwood kraft pulp with the fungus *Trametes versicolor*. Tappi J. **73**: 149-153.

Renganathan, V., S.N. Usha, and F. Lindenburg. 1990. Cellobiose-oxidizing enzymes from the lignocellulose-degrading basidiomycete *Phanerochaete chrysosporium*: interaction with microcrystalline cellulose. Appl. Microbiol. Biotechnol. **32**: 609-613.

Renganathan, V. and M.H. Gold. 1986. Spectral characterization of the oxidized states of lignin peroxidase an exracellular heme enzyme from the white rot basidiomycete *Phanerochaete chrysosporium*. Biochem. **25**: 1626-1631.

Roy, B.P. and F.S. Archibald. 1993. Effects of kraft pulp and lignin on *Trametes versicolor* carbon metabolism. Appl. Environ. Microbiol. **59**: 1855-1863.

Roy, B.P. and Archibald, F.S. 1994. An indirect free radical based assay for the enzyme cellobiose:quinone oxidoreductase. Anal. Biochem. **216**: 291-298.

Roy, B.P. and F.S. Archibald. 1991. Production and characterization of an extracellular cellobiose quinone oxidoreductase (CBQase) from *Trametes versicolor.* Can. Soc. Microbiol. General Meeting abstr. AM3. p38.

Roy, B.P., Paice, M.G., Archibald, F.S., S.K. Misra, and L. Misiak. 1994. Creation of metal complexing agents, reduction of manganese dioxide and the promotion of manganese peroxidase-mediated Mn(III) production by cellobiose:quinone oxidoreductase from *Trametes versicolor.* J. Biol. Chem. **269:** 19745-19750.

Roy-Arcand, L., and F.S. Archibald. 1991. Direct dechlorination of chlorophenolic compounds by laccases from *Trametes (Coriolus) versicolor.* Enzym. Microb. Technol. **13:** 194-203.

Sadana, J.C. and R.V. Patil. 1988. Cellobiose dehydrogenase from *Sclerotium rolfsii.* Methods in Enzymol. **160:** 448-454.

Sadana, J.C. and R.V. Patil. 1985. The purification and properties of cellobiose dehydrogenase from *Sclerotium rolfsii* and its role in cellulolysis. J. Gen. Microbiol. **131**: 1917-1923.

Sakurai, T. 1992. Kinetics of electron transfer between cytochrome c and laccase. Biochem. **31**: 9844-9847.

Samejima, M. K.-E.L. Eriksson. 1991. Mechanisms of redox interactions between lignin peroxidase and cellobiose:quinone oxidoreductase. FEBS Lett. **292**: 151-153.

Samejima, M., R.S. Phillips, and K.-E.L. Eriksson. 1992. Cellobiose oxidase from *Phanerochaete chrysosporium*. Stopped-flow spectrophotometric analysis of pH-dependent reduction. FEBS Lett. **306**: 165-168.

Samejima, M. and K.-E.L. Eriksson. 1992. A comparison of the catalytic properties of cellobiose:quinone oxidoreductase and cellobiose oxidase from *Phanerochaete chrysosporium*. Eur. J. Biochem. **207**: 103-107.

Sariaslani, F.S., J.M., Beale Jr., and J.P. Rosazza. 1984. Oxidation of rotenone by *Polyporus anceps* laccase. J. Nat. Products. **47**: 692-697.

Schmidhalter, D.R. and G. Canevascini. 1993. Isolation and characterization of the cellobiose dehydrogenase from the brown-rot fungus *Coniophora puteana* (Schum ex Fr.) Karst. Arch. Biochem. Biophys. **300**: 559-563.

Schmidt, O. and W. Liese. 1982. Bacterial decomposition of woody cell walls. Int. J. Wood Preserv. **2**: 13-19.

Schmidt, O., Y. Nagashima, W. Liese, and U. Schmitt. 1987. Bacterial wood degradation studies under laboratory conditions and in lakes. Holzforschung **41**: 137-140.

Schmidt, J.A. and C. Heitner. 1993. Use of UV-visible diffuse reflectance spectroscopy for chromophore research on wood fibers: a review. Tappi J. **76(2)**: 117-123.

Scott, W., and J.L. Foote. 1979. The inhibition of stearoyl-coenzyme A desaturase by phenyllactate and phenylpyruvate. Biochim. Biophys. Acta. **573**: 197-200.

Seitz, L.M., H.E. Mohr, R. Burroughs, and D.B. Sauer. 1977. Ergosterol as an indicator of fungal invasion in grains. Cereal Chem. **54**: 1207-1217.

Shigo, A.L. 1967. Successions of microorganisms in discoloration and decay of wood. Int. Rev. For. Res. **2**: 237-299.

Sixta, H., G. Gotzinger, A. Schrittwieser, and P. Hendel 1991. Ozone bleaching: medium consistency technology and mill experience. In Workshop on emerging pulping and chlorine-free bleaching technologies. Raleigh NC.

Spiker, J.K., D.L. Crawford, and E.C. Theil. 1992. Oxidation of phenolic and non-phenolic substrates by the lignin peroxidase of *Streptomyces viridosporus*. Appl. Microbiol. Biotechnol. **37**: 518-524.

Sterjiades, R., J.F.D. Jeffery, and K.-E.L. Eriksson. 1992. Laccase from sycamore maple (*Acer pseudoplatanus*) polymerizes monolignols. Plant Physiol. **99**: 1162-1168.

Stone, A.T. and J.J. Morgan. 1984. Reduction and dissolution of manganese (III) and manganese (IV) oxides by organics: 2. survey of the reactivity of organics. Environ. Sci. Technol. **18**: 617-624

Strebotnik, E. and K. Messner. 1990. Enzymatic attack of wood is limited by the inaccessibility of the substrate. In Biotechnology in pulp and paper manufacture, Applications and fundamental investigations (T.K. Kirk and H-M. Chang, eds.). Boston. Butterworth-Heinemann. pp 111-119.

Szklarz, G. and A. Leonowicz. 1986. Cooperation between fungal laccase and glucose oxidase in the degradation of lignin derivatives. Phytochem. **25**: 2537-2539.

Teuber, H.-J. and G. Staiger. 1955. Reaktionen mit nitrosodisulfonat. VIII. Mitteil.: ortho-benzoquinone und phenazine. Chem. Ber. **88**: 802-827.

Thompson, N.S., J.R. Peckham, and E.F. Thode. Studies in full chemical pulping. 1962. II Carbohydrate changes and related physical effects. Tappi J. **45**(6): 433-44x.

Tien, M., and T.K. Kirk. 1984. Lignin-degrading enzyme from *Phanerochaete chrysosporium*: purification, characterization and catalytic properties of a unique H_2O_2-requiring oxygenase. Proc. Nat. Acad. Sci. **81:** 2280-2284.

Tien, M., T.K. Kirk, C. Bull, and J.A. Fee. 1986. Steady-state and transient-state kinetic studies on the oxidastion of 3,4-dimethoxybenzyl alcohol catalyzed by the ligninase of *Phanerochaete chrysosporium* Burds. J. Biol. Chem. **261**: 1687-1693.

Tien, M. and T.K. Kirk. 1988. Lignin peroxidase of *Phanaerochaete chrysosporium.* Methods in Enzymology **161**: 238-249.

Tien , M. and T.K. Kirk. 1983. Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysoporium* Burds. Science **221**: 661-663.

Tuor, U., H. Wariishi, H.E. Shoemaker, and M.H. Gold. 1992. Oxidation of phenolic arylglycerol β-aryl ether lignin model compounds by manganese peroxidase from Phanerochaete chrysosporium: Oxidative cleavage of an alpha-carbonyl model compound. Biochem. **31**: 4986-4995.

Umezawa, T. and T. Higuchi. 1991. Chemistry of lignin degradation by lignin peroxidases. In Enzymes in Biomass Conversion (G.F. Leatham and M.E. Himmell, eds.) ACS Symp. Series 460. Washington. 236-246.

Umezawa, T., S. Kawai, S. Yokota, and T. Higuchi. 1986. Aromatic ring cleavage of various β-O-4 lignin model dimers by *Phanerochaete chrysosporium*. Wood Res. **73**: 8-17.

Van der Woude, M.W., K. Boominathan, and C.A. Reddy. 1993. Nitrogen regulation of lignin peroxidase and manganese-dependent peroxidase production is independent of carbon and manganese regulation in *Phanerochaete chrysosporium*. Arch. Microbiol. **160**: 1-4.

Vázquez, A., J. Tudela, R. Varón, and F. García-Cánovas. 1992. Determination of the molar absorptivities of phenothiazine cation radicals generated by oxidation with hydrogen peroxide/peroxidase Anal. Biochem. **202**: 245-248.

von Sonntag, C. 1979. Radiation chemistry of carbohydrates and of the sugar moeity in DNA. Stud. Phys. Theor. Chem. **6**: 85-98.

Waldner, R., Leisola, and A. Fiechter. 1988. Comparison of ligninolytic activities of selected fungi. Appl. Microbiol. Biotechnol. **29**: 400-407.

Wardrop, A.B. 1962. Fundamental studies in wood and fibre structure relating to pulping processes. Appita **16**: xv-xxx.

Wariishi, H., K. Valli, and M.H. Gold. 1992. Manganese(II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium.* Kinetic mechanism and role of chelators. J. Biol. Chem. **267**: 23688-23695.

Wariishi, H., K. Valli, and M.H. Gold. 1991. *In vitro* depolymerization of lignin by manganese peroxidase of *Phanerochaete chrysosporium.* Biochem. Biophys. Res. Commun. **176**: 269-275.

Wariishi, H., K. Valli, V. Renganathan, and M.H. Gold. 1989. Thiol-mediated oxidation of nonphenolic lignin model compunds by manganese peroxidase of *Phanerochaete chrysosporium*. J. Biol. Chem. **264**: 14185-14191.

Wariishi, H., K. Valli, and M.H. Gold. 1992. Manganese(II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*. J. Biol. Chem. **267**: 23688-23695.

Wariishi, H., K. Valli, and M.H. Gold. 1991. *In vitro* depolymerization of lignin by manganese peroxidase of *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **176**: 269-275.

Wariishi H. and M.H. Gold. 1989. Lignin peroxidase compound III. Formation, inactivation, and conversion to the native enzyme. FEBS Lett. **243**: 165-168.

Wariishi, H., H.B. Dunford, D. MacDonald, and M.H. Gold. 1989. Manganese peroxidase from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Transient state kinetics and reaction mechanism. J. Biol. Chem. **264**: 3335-3340.

Wariishi, H., K. Valli, and M.H. Gold. 1989. Oxidative cleavage of a phenolic diarylpropane lignin model dimer by manganese peroxidase from *Phanerochaete chrysosporium*. Biochemistry **28**: 6017-6023.

Waring, W.S., and C.H. Werkman. 1942. Growth of bacteria in an iron-free medium. Arch. Biochem. **1**: 303-310.

Westermark, U. and K.-E. Eriksson. 1974. Carbohydrate-dependent enzymic quinone reduction during lignin degradation. Acta Chem. Scand. **B28**: 204-208.

Westermark, U. and K.-E. Eriksson. 1974a. Cellobiose:quinone oxidoreductase, a new wood-degrading enzyme from white-rot fungi. Acta Chem. Scand. **B28**: 209-214.

Westermark, U. and K.-E. Eriksson. 1975. Purification and properties of cellobiose:quinone oxidoreductase from *Sporotrichum pulverulentum*. Acta Chem. Scand. **B29**: 419-424.

Wolfenden, B.S., and R.L. Willson. 1982. Radical cations as reference chromogens in kinetic studies of one-electron transfer reactions: pulse radiolysis studies of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate). J. Chem. Soc. Perkin Trans. **II**: 805-812.

Whistler, R.L. and J.N. BeMiller. 1963. α-D-isosaccharino-1,4-lactone. Action of lime water on lactose. In Whistler, R.L., Wolfrom, M.L. (eds) Methods in Carbohydrate Chemistry **2**. Academic Press, New York. pp. 477-479.

Wilson, M.T., N. Hogg, and G.D. Jones. 1990. Reactions of reduced cellobiose oxidase with oxygen. Is cellobiose oxidase primarily an oxidase? Biochem. J. **270**: 265-267.

Wood, J.D. and P.M. Wood. 1992. Evidence that cellobiose:quinone oxidoreductase from *Phanerochaete chrysosporium* is a breakdown product of cellobiose oxidase. Biochem. Biophys. Acta **1119**: 90-96.

Young, J. 1993. Solvent pulping symposium looks at current, future technology. Pulp Pap. **67(2)**: 117-119.

Yu, E.K.C., and Saddler, J.N. 1982. Enhanced production of 2,3-butanediol by *Klebsiella pneumoniae* grown on high sugar concentrations in the presence of acetic acid. Appl. Environ. Microbiol. **44**: 777-784.

Ziobro, G.C. 1989. The role of carbohydrates in the origin of kraft colour. In Sixth International Symposium of Wood and Pulping Chemistry. Raleigh. Proceedings of papers. 585-591.