PRODUCTION OF LACCASE BY THE WHITE-ROT FUNGUS *PYCNOPORUS SANGUINEUS*

by

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To absent friends...

"Lieve God, watzijnder al wonderen in soo een klein schepsel"

(Van Leeuwenhoek, Letter 76, October 15, 1693)

CONTENTS

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Above all- the Composer of life.

PREFACE

Sappi kraft mill, Ngodwana, SA.

The strength and rigidity of stems in higher plants are the result of production and deposit of cellulose, hemicellulose and lignin in the plant cell walls (Terashima & Atalla, 1995). Lignin and cellulose are both rather rigid organic polymers (Tuor *et al*., 1995), which have developed during evolution for construction and preservation purposes (Call & Mücke, 1997). The degradation of lignin in the pulping and bleaching processes is essential for the manufacturing of paper products. These compounds have to be exposed to harsh physiochemical conditions to modify or degrade their structure for utilisation in the pulp and paper industry (Coll *et al*., 1993). The problems caused by chemicals used in bleaching forced industry to consider alternative, more environmental friendly methods (Yang & Eriksson, 1992). Such a biological alternative to traditional bleaching was provided through the discovery of oxidative enzymes (Poppius-Levlin *et al*., 1997).

Defibrilation of wood is still based on inventions of the 19th century, which have been fine-tuned. New developments in the pulping processes include methods to reduce the kappa number before final bleaching. In the bleaching of pulp there were some dramatic changes in the last 30 years. Oxygen delignification was introduced in the 1980's as well as peroxide and ozone stages in the 1990s (Call & Mücke, 1997). At present, the kraft process is still the most common commercial delignification method (Kondo *et al*., 1996). In the bleaching of kraft pulp, a combination of chlorination and alkaline extraction is used to remove the residual lignin (Monteiro & De Carvalho, 1998). During the kraft pulping processes, the middle lamellae and wall lignin that bind the fibres in wood, as well as the embedded lignins are dissolved (Archibald *et al*., 1997). This results in a dark pulp due to the colour of the residual

modified lignin residues. These residues are removed during multistage bleaching using a combination of chlorination and alkaline-extraction steps (Kondo *et al*., 1996).

There is a tremendous effort to avoid the use of chlorine chemicals in bleaching due to stringent regulations and a rise in environmental concern (Grabner *et al*., 1997). This have compelled industry to research and develop cleaner processes and shift their main focus to the use of less polluting pulping and bleaching techniques (Luisa *et al*., 1996).

The use of biological bleaching provided one such option (Reid & Paice, 1994) using either hemicellulolytic or lignin degrading oxidative enzymes (Poppius-Levlin *et al*., 1997; Monteiro & De Carvalho, 1998). White-rot basidiomycetes and the components of the their ligninolytic system raised considerable interest (Kondo *et al*., 1996). The hemicellulolytic enzyme xylanase was extensively studied and applied on industrial scale with the effect of a higher pulp brightness resulting in a lower chemical input (Poppius-Levlin *et al*., 1997). Laccase and peroxidases also have potential in the bleaching industry (Grabner *et al*., 1997), namely to reduce the amount of chemicals and energy used during pulp bleaching (Semar *et al*., 1998).

There are only a few organisms in nature, belonging to the white-rot and brown-rot fungi, that are capable of degrading wood (Heinzkill *et al*., 1998). White-rot fungi are, therefore, at the moment of great interest for biological pulping and bleaching (Wall *et al*., 1993). White-rot fungi such as *Trametes (Coriolus) versicolor* (Wulf.: Fr.) Quél. and *Phanerochaete chrysosporium* Burdsall are known producers of

lignolytic enzymes that are involved in the natural delignification of wood (Call & Mücke, 1997; Poppius-Levlin *et al*., 1997).

White rot fungi are responsible for the destruction and decay of polysaccharides and lignins, whereas the brown-rot fungi mainly attack the polysaccharide portion of the wood and merely modify its lignin (Call & Mücke, 1997). White-rot fungi are the only known microorganisms that have evolved complex enzymatic systems that enable them to degrade lignin (Garzillo *et al*., 1998). Fungal attack on lignin involves various enzymes including lignin peroxidase (LiP), manganese peroxidase (Mn-P) and laccase (Buswell *et al*., 1995). Many efforts have been made to investigate the use of fungi for the removal of lignin in the pulping and bleaching process (Tuor *et al*., 1995).

The enzymatic machinery of wood degrading fungi was clarified to a large extent. The discovery of ligninase (lignin peroxidase) from *P. chrysosporium* triggered research on biodegradation of lignin (Tuor *et al*., 1995). The perception of lignin degradation was changed from an oxidative depolymerisation process caused by a single enzyme, to a process of intensive oxidative and reductive conversions in which different classes of enzymes can participate (Tuor *et al*., 1995). Many efforts have been made to investigate the application of these fungi for the removal of lignin in the pulping and bleaching process. It was first reported by Kirk and Yang (1979) that *P. chrysosporium* was able to partially delignify unbleached Kraft pulp.

The aim of this study was to select hypersecretory strains of white-rot fungi and to optimise cultivation conditions supporting high laccase yields. Initial work

focussed on the screening of a culture collection for of hypersecretory white-rot fungi (Chapter 2). Further selection of strains was based on the thermostability and optimum temperature of the laccases produced by the selected strains (Chapter 3). *Pycnoporus sanguineus* (SCC 108) was selected for the evaluation of different substrates, inducers and growth parameters to support production of laccase activity in conical flasks as well as different bioreactors (Chapter 4 to 6).

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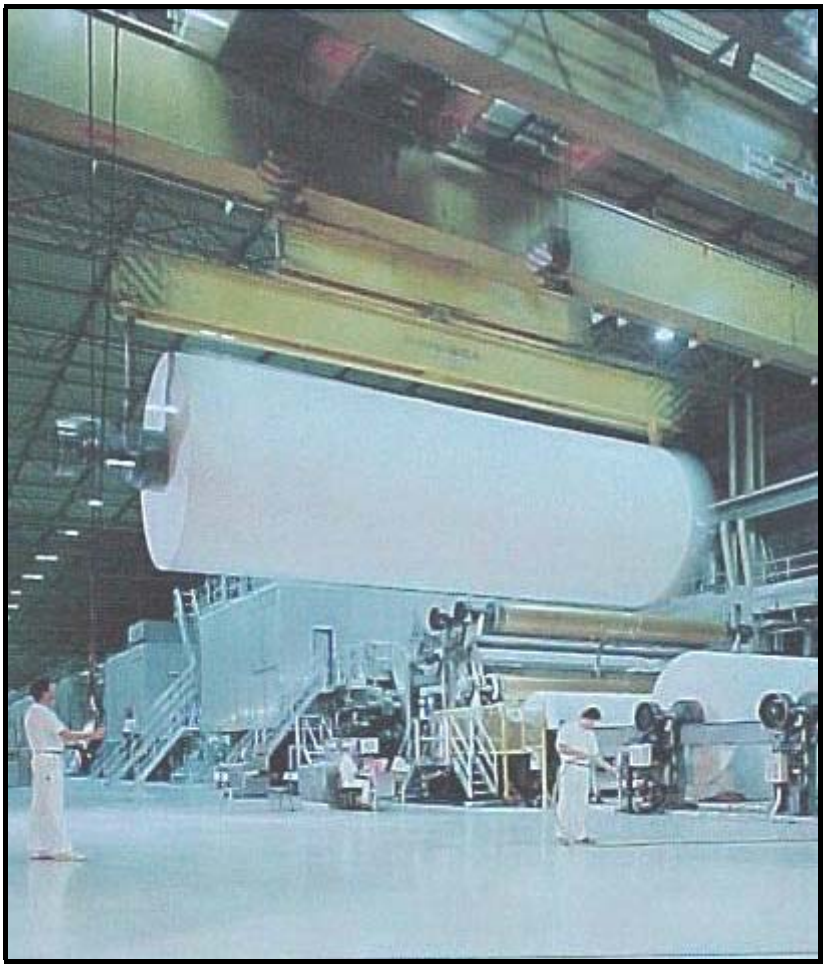
CHAPTER 1

LACCASE: ACTION, PRODUCTION AND APPLICATION

A LITERATURE REVIEW



Paper machine, Sappi Lanaken, Belgium.

ABSTRACT

White-rot fungi and their enzymes are receiving increasing attention for biotechnological applications in the pulp and paper industry as alternatives to conventional bleaching. Laccase has been identified as one of the enzymes that plays a major role in lignin degradation. Laccase only attacks phenolic subunits of lignin, but its substrate range can be extended to non-phenolic subunits by the inclusion of a mediator. The use of this enzyme was, therefore, not successful in pulp bleaching trials until the discovery of mediators. Although the existence of natural mediators has not been confirmed, various components have been identified that are able to act as mediators. Improved methods of laccase production could benefit the industrial utilisation of the enzyme. White-rot fungi constitutively produce low concentrations of laccase, but higher concentrations can be obtained with the inclusion of inducers in the cultivation media. The enzyme is mainly produced during the stationary growth phase of the fungi, but various factors such as glucose, nitrogen and pH can influence levels of laccase production. The enzyme does not only hold potential for biological pulp bleaching operations, but also has application in bioremediation, the textile dye industry as well as the food and beverage industries.

INTRODUCTION

Many efforts have been made to utilise enzymes for the degradation of lignin in the pulp and paper industry (Call & Mücke, 1997). One enzyme known to play a major role in natural delignification is laccase (EC 1.10.3.2; benzenediol:oxygen oxidoreductase) (Call & Mücke, 1997). The enzyme was first identified in the sap of the Japanese lacquer tree *Rhus vernicifera* (Thurston, 1994) and described in 1883 by Yoshida (as cited by Call & Mücke, 1997). Among the large blue copper containing enzymes, laccase is the most widely distributed (Leontievsky *et al*., 1997) and occurs in various plants and fungi (Bourbonnais *et al*., 1995). In the fungi, Deutromycetes, Ascomycetes and a wide range of Basidiomycetes are known producers of laccases, which are particularly abundant in many lignin-degrading white-rot fungi (Bourbonnais *et al*., 1995; Leontievsky *et al*., 1997; Thurston, 1994). Some of the best-studied and most important white-rot fungi are *Coriolus versicolor* (Wulf.: Fr.) Quel., *Dichomitus squalens* (Karsten) Reid, *Junghuhnia separabilima* (Pouzar) Ryvarden, *Phanerochaete chrysosporium* Burdsall, *Phlebia ochraceofulva* (Bourdot & Galzin) Donk, *Phlebia radiata* Fries and *Rigidoporus lignosus* (Klotzsch) Imazeki (Call & Mücke, 1997; Wall *et al*., 1993). There is only one bacterium, *Azospirillum lipoferum* (Beijerinck) Tarrand *et al*., in which laccase activity has been demonstrated (Thurston, 1994).

Laccases are mostly extracellular glyco-proteins (Archibald *et al*., 1997; Heinzkill *et al*., 1998) and are multinuclear enzymes (Gayazov & Rodakiewicz-Nowak, 1996) with molecular weights between 60 and 80 kDa (Heinzkill *et al*., 1998; Leontievsky *et al*., 1997; Thurston, 1994). Most monomeric laccase molecules contain four copper atoms in their structure that can be classified in three groups using

UV/visible and electron paramagnetic resonance (EPR) spectroscopy (Leontievsky *et al*., 1997). The type I copper (T1) is responsible for the intense blue colour of the enzymes at 600nm and is EPR detectable, the type II copper (T2) is colourless, but EPR detectable, and the type 3 copper (T3) consists of a pair of copper atoms that give a weak absorbance near the UV spectrum but no EPR signal (Palmieri *et al*., 1998). The T2 and T3 copper sites are close together and form a trinuclear centre (Leontievsky *et al*., 1997) that are involved in the catalytic mechanism of the enzyme (Palmieri *et al*., 1998).

MODE OF ACTION OF THE LACCASE ENZYME

Laccase only attacks the phenolic subunits of lignin, leading to Cα oxidation, Cα-Cβ cleavage and aryl-alkyl cleavage (Figure 1). Laccases are able to reduce one molecule of dioxygen to two molecules of water while performing one-electron oxidation of a wide range of aromatic compounds (Thurston, 1994), which includes polyphenols (Bourbonnais & Paice, 1996), methoxy-substituted monophenols and aromatic amines (Archibald *et al*., 1997; Bourbonnais *et al*., 1995). This oxidation results in an oxygen-centred free radical, which can then be converted in a second enzyme-catalysed reaction to quinone. The quinone and the free radicals can then undergo polymerisation (Thurston, 1994).

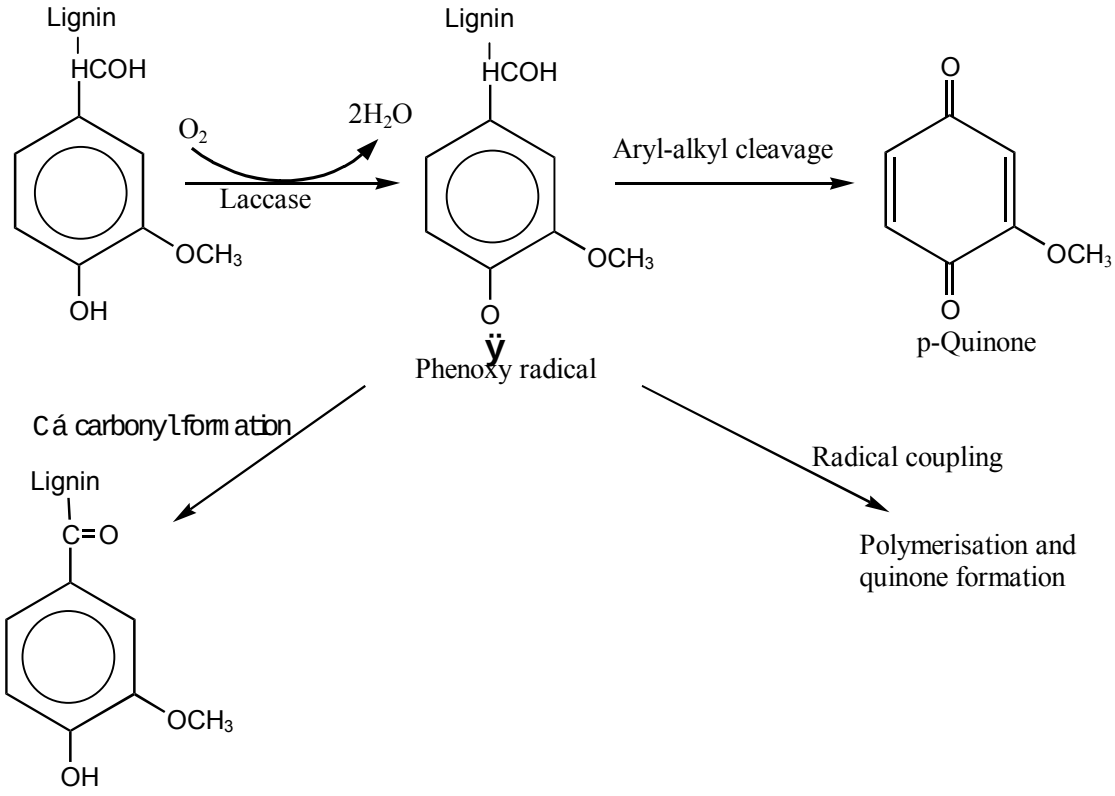


Figure 1. Oxidation of phenolic subunits of lignin by laccase (adapted from Archibald *et al*., 1997).

Laccases are similar to other phenol-oxidising enzymes, which preferably polymerise lignin by coupling of the phenoxy radicals produced from oxidation of lignin phenolic groups (Bourbonnais *et al*., 1995). Due to this specificity for phenolic subunits in lignin and its restricted access to lignin in the fibre wall, laccase has a limited effect on pulp bleaching (Bourbonnais & Paice, 1996). The substrate range of laccase can be extended to non-phenolic subunits of lignin by the inclusion of a mediator such as 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) (Figure 2).

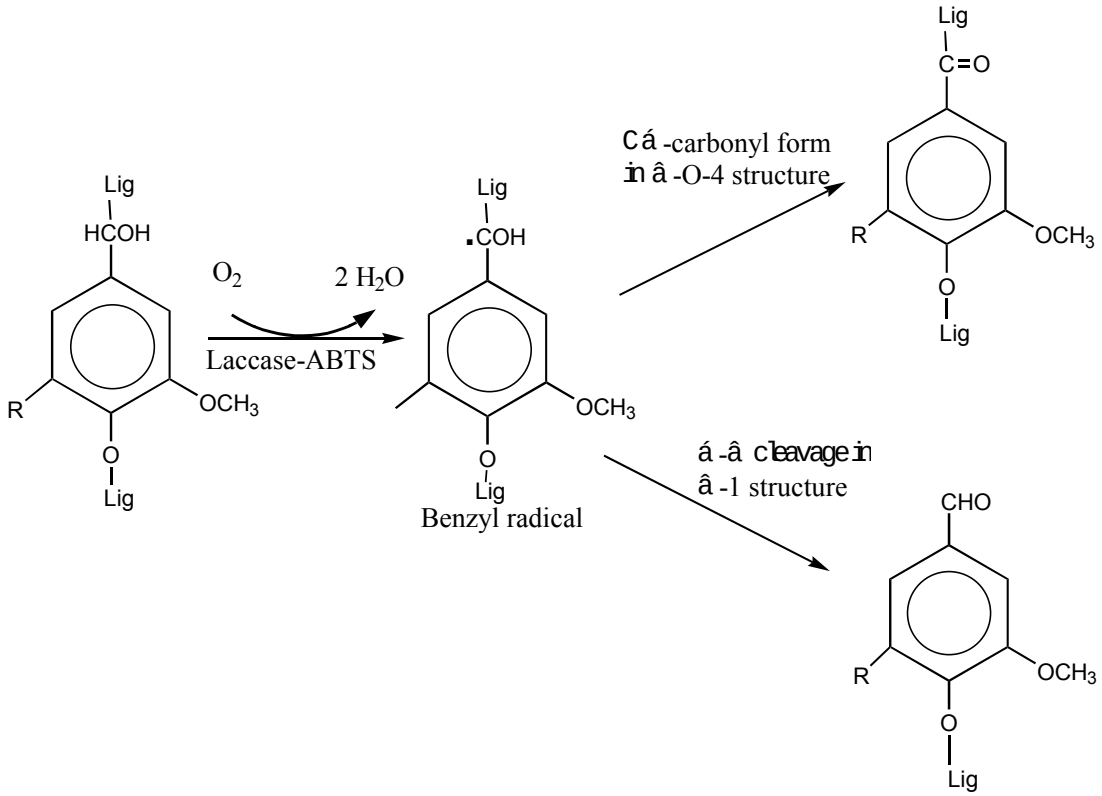


Figure 2. Oxidation of non-phenolic lignin subunits by laccase and ABTS (adapted from Archibald *et al.*, 1997).

In some fungi, the reactions of laccase are unrelated to ligninolysis (Thurston, 1994). Laccase plays a role in the morphogenesis and differentiation of sporulating and resting structures in basidiomycetes as well as lignin biodegradation of wood in white-rot fungi (Robene-Soustrade & Lung-Escarmant, 1997). Laccase is responsible for pigment formation in mycelia and fruiting bodies, improves cell-to-cell adhesion, assists in the formation of rhizomorphs and is also responsible for the formation of a polyphenolic glue that binds hyphae together (Thurston, 1994). Various plant pathogens also produce extracellular laccases that enable the fungus to overcome the immune response of the host (Thurston, 1994). The laccase also facilitates the detoxification of the plant tissue via the oxidation of antifungal phenols or

deactivation of phytoalexins (Assavanig *et al*., 1992; Robene-Soustrade & Lung-Escarmant, 1997).

Influence of pH on laccase activity

The pH optima of laccases are highly dependable on the substrate. When using ABTS as substrate the pH optima are more acidic and are found in the range between pH 3 and pH 5 (Heinzkill *et al*., 1998). In general, laccase activity has a bell shaped profile with an optimal pH that varies considerably. This variation may be due to changes to the reaction caused by the substrate, oxygen or the enzyme itself (Xu, 1997). The difference in redox potential between the phenolic substrate and the T1 copper could increase oxidation of the substrate at high pH values, but the hydroxide anion (OH^-) binding to the T2/T3 coppers results in an inhibition of the laccase activity due to a disruption of the internal electron transfer between the T1 and T2/T3 centres. These two opposing effects can play an important role in determining the optimal pH of the bi-phasic laccase enzymes (Xu, 1997). The role of the T1 copper in the pH optima of the enzyme was confirmed by Palmieri *et al*. (1998) who found that the T1 copper was absent in laccase enzymes exibiting more neutral pH optima.

Influence of temperature on laccase activity

The optimal temperature of laccase can differ greatly from one strain to another. The laccases isolated from a strain of *Marasmius quercophilus* (Farnet *et al*., 2000) were found to be stable for 1 h at 60 °C. Farnet *et al*. (2000) further found that pre-incubation of enzymes at 40 °C and 50 °C greatly increased laccase activity. Another technique that can be used to increase the stability of laccase is to immobilise the enzyme on glass powder by means of air-drying (Ruiz *et al*., 2000). This

technique also has potential for the enzyme to be used on the glass powder matrix in specific biotechnology applications where stability is required (Ruiz *et al*., 2000).

Isozymes

Many laccase producing fungi secrete isoforms of the same enzyme (Leontievsky *et al*., 1997). These isozymes have been found to originate from the same or different genes encoding for the laccase enzyme (Archibald *et al*., 1997). The number of isozymes present differ between species and also within species depending on whether they are induced or non-induced (Assavanig *et al*., 1992). They can differ markedly in their stability, optimal pH and temperature and affinity for different substrates (Assavanig *et al*., 1992; Heinzkill *et al*., 1998). Furthermore, these different isozymes can have different roles in the physiology of different species or in the same species under different conditions (Assavanig *et al*., 1992). Various laccase encoding gene sequences have been reported from a range of ligninolytic fungi. These sequences encode for proteins between 515 and 619 amino acid residues and close phylogenetic proximity between them is indicated by sequence comparisons (Bourbonnais *et al*., 1995).

PRODUCTION OF FUNGAL LACCASE

White-rot fungi have been studied extensively for application in biological pulping and bleaching (Luisa *et al*., 1996b), because they are of the few organisms that are able to degrade lignin (Heinzkill *et al*., 1998). White-rot fungi, such as *Coriolus versicolor* and *Pycnoporus sanguineus* (L.:Fr.) Murr. are known producers of lignolytic enzymes that are involved in the natural delignification of wood (Call & Mücke, 1997; Poppius-Levlin *et al*., 1997). This group of fungi is the only known microorganisms that have evolved complex enzymatic systems that enable them to degrade lignin (Garzillo *et al*., 1998). Laccase has potential for industrial application, since laccase is able to degrade phenolic and nonphenolic lignin structures (Monteiro & de Carvalho, 1998). Laccases generally occur as extracellular glyco-proteins, which allow for rapid removal from fungal biomass (Heinzkill *et al*., 1998; Archibald *et al*., 1997). According to Galhaup *et al.* (2001) one of the major limitations for the large-scale applications of fungal laccases is the low production rates by both wild type and recombinant fungal strains.

White-rot fungi constitutively produce low concentrations of various laccases (Robene-Soustrade & Lung-Escarmant, 1997) when they are cultivated in submerged culture or on wood. Higher concentrations can be induced by the addition of various aromatic compounds such as xylidine and ferulic acid. High concentrations of laccase have also been observed in old non-induced cultures (Bourbonnais *et al*., 1995). The mechanisms of metabolism in microorganisms are used and controlled by its environmental conditions and medium composition (Monteiro & De Carvalho, 1998). There are various response element sites in the promoter regions of laccase genes.

These sites can be induced by certain xenobiotic compounds, heavy metals or heatshock treatment (Sannia *et al*., 2001).

Screening of fungal strains

Screening of laccase producing species and their variants is important for selecting suitable laccase producing strains (Herpoël *et al*., 2000). Screening for oxidative enzymes or mediators involves the investigation of many samples, as there are many parameters involved. For this reason one usually relies on the use of inexpensive, rapid and sensitive testing methods (Grabner *et al*., 1997). The screening strategy must aim to identify fungal strains and enzymes that will work under industrial conditions (Grabner *et al*., 1997; Monteiro & De Carvalho, 1998).

Cultivation

Laccases are generally produced during the secondary metabolism of white-rot fungi growing on natural substrate or in submerged culture (Gayazov & Rodakiewicz-Nowak, 1996). Various cultivation parameters that influence laccase production and activity have been described. These factors include carbon limitation, nitrogen source and concentration, and microelements. Gayazov and Rodakiewicz-Novak (1996) reported faster laccase production under semi-continuous production with high aeration and culture mixing compared to static conditions. When using conical flasks for cultivation it should be baffled to ensure a high oxygen transfer (Dekker *et al*., 2000). Xavier *et al*. (2001) found that the production of high titres of the laccase enzyme was not dependent on high biomass yields. The synthesis and action of the laccase are controlled during growth and can play an important role in pigment and fruiting body formation (Thurston, 1994). Nüske *et al.* (2001) successfully cultivated

the white-rot strains, *Nematoloma frowardii* (Spegazzini) Horak and *Clitocybula dusenii* (Singer) Maetrod, in 5-L, 30-L and 300-L stirred tank reactors for laccase production. Galhaup *et al.* (2001) were able to obtain 735 $U.ml^{-1}$ of laccase activity with *Coriolus pubescens* (Schum.: Fr.) Quél. during a fed-batch cultivation, but cultivation conditions were not described.

Influence of nitrogen on laccase production

Ligninolytic systems of white-rot fungi are mainly activated during the secondary metabolic phase of the fungus and are often triggered by nitrogen depletion (Keyser *et al*., 1978), but it was also found that in some strains nitrogen concentrations had no effect on ligninolytic activity (Leatham & Kirk, 1983). These contradictory observations were ascribed to differences between strains of *Phanerochaete chrysosporium* Burdsall and *Lentinus edodes* (Berk.) Sing. (Buswell *et al*., 1995). Monteiro and De Carvalho (1998) reported high laccase activity with semi-continuous production in shake-flasks using a low carbon to nitrogen ratio (7,8 $g.g^{-1}$). Buswell *et al*. (1995) found that laccases were produced at high nitrogen concentrations, although it is generally accepted that a high carbon to nitrogen ratio is required for laccase production. Laccase was also produced earlier when the fungus was cultivated in a substrate with a high nitrogen concentration and these changes did not reflect differences in biomass. Heinzkill *et al.* (1998) also reported a higher yield of laccase using nitrogen rich media rather than the nitrogen limited media usually employed for induction of oxidoreductases.

Influence of pH on laccase production

There is not much information available on the influence of pH on laccase production, but when fungi are grown in a medium of which the pH is optimal for growth (pH 5) the laccase will be produced in excess (Thurston, 1994). Most reports indicated initial pH levels set between pH 4,5 and pH 6,0 prior to inoculation, but the levels are not controlled during most cultivations (Arora & Gill, 2000; Fåhreus & Reinhammar, 1967; Pointing *et al.,* 2000; Vasconcelos *et al.*, 2000).

Influence of temperature on laccase production

It has been found that the optimal temperature for fruiting body formation and laccase production is 25 °C in the presence of light, but 30 °C for laccase production when the cultures are incubated in the dark (Thurston, 1994). In general the fungi were cultivated at temperatures between 25 °C and 30 °C for optimal laccase production (Arora & Gill, 2000; Fåhreus & Reinhammar, 1967; Pointing *et al.,* 2000; Vasconcelos *et al.*, 2000). When cultivated at temperatures higher than 30 °C the activity of ligninolytic enzymes was reduced (Zadrazil *et al*., 1999).

Induction of laccase production

Laccase production has been found to be highly dependent on the conditions for cultivation of the fungus (Heinzkill *et al*., 1998) and media supporting high biomass did not necessarily support high laccase yields (Xavier *et al.*, 2001). Ligninolytic systems of white-rot fungi were mainly activated during the secondary metabolic phase and were often triggered by nitrogen concentration (Buswell *et al*., 1995) or when carbon or sulfur became limiting (Heinzkill *et al*., 1998). Laccases were generally produced in low concentrations by laccase producing fungi

(Vasconcelos *et al*., 2000), but higher concentrations were obtainable with the addition of various supplements to media (Lee *et al*., 1999). The addition of xenobiotic compounds such as xylidene, lignin, and veratryl alcohol is known to increase and induce laccase activity (Xavier *et al.*, 2001).

Many of these compounds resemble lignin molecules or other phenolic chemicals (Marbach *et al.*, 1985; Farnet *et al.*, 1999). Veratryl (3,4-Dimethoxybenzyl) alcohol is an aromatic compound known to play an important role in the synthesis and degradation of lignin. The addition of veratryl alcohol to cultivation media of many white-rot fungi has resulted in an increase in laccase production (Barbosa *et al.,* 1996). Some of these compounds affect the metabolism or growth rate (Froehner & Eriksson, 1974) while others, such as ethanol, indirectly trigger laccase production (Lee *et al*., 1999).

The promoter regions of the genes encoding for laccase contain various recognition sites that are specific for xenobiotics and heavy metals (Sannia *et al*., 2001). These can bind to the recognition sites when present in the substrate and induce laccase production. White-rot fungi were very diverse in their responses to tested inducers for laccase. The addition of certain inducers can increase the concentration of a specific laccase or induce the production of new isoforms of the enzyme (Robene-Soustrade & Lung-Escarmant, 1997). Some inducers interact variably with different fungal strains (Eggert *et al*., 1996).

Eggert *et al.* (1996) found that the addition of xylidene as inducer had the most pronounced effect on laccase production. The addition of 10 μM xylidine after 24 h

of cultivation gave the highest induction of laccase activity and increased laccase activity nine-fold (Eggert *et al*., 1996). At higher concentrations the xylidene had a reduced effect, probably due to toxicity (Eggert *et al*., 1996). Laccase offers protection for the fungus against toxic phenolic monomers of polyphenols (Assavanig *et al*., 1992; Eggert *et al*., 1996).

Lee *et al*. (1999) investigated the inducing effect of alcohols on the laccase production by *Trametes versicolor*. The enhanced laccase activity was comparable to those obtained using 2,5-xylidine and veratryl alcohol (Mansur *et al*., 1997). It was postulated that the addition of ethanol to the cultivation medium caused a reduction in melanin formation. The monomers, when not polymerised to melanin, then acted as inducers for laccase production (Lee *et al*., 1999). The addition of ethanol as an indirect inducer of laccase activity offers a very economical way to enhance laccase production.

Lu *et al*. (1996) found that there is a strong correlation between hyphal branching and the expression and secretion of laccase. The addition of cellobiose can induce profuse branching in certain *Pycnoporus* species and consequently increase laccase activity (Lu *et al*., 1996). The addition of cellobiose and lignin can increase the activity of extracellular laccases without an increase in total protein concentration (Garzillo *et al.* 1998; Lu *et al*., 1996).

The addition of low concentrations of copper to the cultivation media of laccase producing fungi stimulates laccase production (Assavanig *et al*., 1992). Palmieri *et al*. (2000) found that the addition of 150 μM copper sulphate to the

cultivation media can result in a fifty-fold increase in laccase activity compared to a basal medium.

Inhibition

In general, laccases responds similarly to several inhibitors of enzyme activity (Bollag & Leonowicz, 1984). In a study conducted by Bollag and Leonowicz (1984) it was found that azide, thioglycolic acid and diethyldithiocarbamic acid all inhibited laccase activity, whereas EDTA affected laccase activity to a lesser extent (Bar, 2002).

It seems as if the use of excessive concentrations of glucose as carbon source in cultivation of laccase producing fungal strains has an inhibitory effect on laccase production (Eggert *et al*., 1996). In a study done by Monteiro and De Carvalho (1998), it was found that an increase in the amount of glucose in the media resulted in a delay of the laccase production. An excess of sucrose or glucose in the cultivation media can reduce the production of laccase, as these components allow constitutive production of the enzyme, but repress its induction (Bollag & Leonowicz, 1984). A simple but effective way to overcome this problem is the use of cellulose as carbon source during cultivation (Eggert *et al*., 1996).

APPLICATION OF LACCASE

Pulp bleaching

Lignin is a rigid phenylpropanoid polymer (Tour *et al*., 1995) that has evolved in plants for structural stability and protection (Call & Mücke, 1997). It is a three-dimensional polymer that is randomly synthesised from coniferyl, p-coumaryl and sinapyl alcohol precursors (Terashima & Atalla, 1995). These compounds have to be exposed to harsh physiochemical conditions to modify their structure for removal in the pulp and paper industries (Coll *et al*., 1993). The degradation of lignin in pulping and bleaching processes is essential for the manufacturing of paper products. The environmental problems caused by the chemicals used in the bleaching industry (Yang *et al*., 1992) compelled these industries to consider environmental friendly alternatives. There are only a few organisms in nature, most belonging to the subphylum Basidiomycotina, that are able to modify lignin (Call & Mücke, 1997). The discovery of their oxidative enzymes provided an alternative to traditional bleaching (Poppius-Levlin *et al*., 1997).

Fungal attack on lignin involves various enzymes including lignin peroxidase (LiP), manganese peroxidase (Mn-P) and laccase (Buswell *et al*., 1995). As knowledge about laccase and its lignin degrading ability increased, so has the interest for application in the pulp and bleaching industry (Poppius-Levlin *et al*., 1997). Although the main role of enzymes is aiding the biochemical reactions in the cell, it has also been established as a reliable and convenient processing aid in many industries (Call & Mücke, 1997).

The introduction of some white-rot strains (IZU-154) to kraft bleaching, made it possible to obtain bleached kraft pulp without the use of chlorine (Kondo *et al*., 1996). These bleached pulps had good optical and strength properties, but unfortunately the use of a fungal bleaching process is very slow and takes days instead of hours (Monteiro & De Carvalho, 1998). The direct use of an actively growing fungus for pulp bleaching is, therefore, not feasible for industrial processes due to the time constraints (Archibald *et al*., 1997) and the degradation of the cellulose caused by the cellulases secreted by the fungi (Monteiro & De Carvalho, 1998). The lignolytic enzymes rather than the fungus itself offer a faster and more direct attack on the lignin structure (Monteiro & De Carvalho, 1998).

The laccase mediator system

Non-chlorine bleaching of pulp with laccase was first patented in 1994 using an enzyme treatment to obtain a brighter pulp with low lignin content (Luisa *et al*., 1996a). Studies on biobleaching with lignin modifying enzymes were, however, not successful until the discovery of mediators (Luisa *et al*., 1996b). The laccase was only successful in reducing the lignin content of pulps in the presence of the living fungus (Call & Mücke, 1997), which indicated that the enzyme alone is not responsible for delignification. According to Call & Mücke (1997) the enzyme required an unknown substance present in the culture broth, which is probably some form of mediator. Although its mechanism is not yet fully understood it is known that kraft pulp is delignified by laccase only in the presence of a mediator such as 2,2′-azinobis (3-ethylbenzthiazoline-6-sulphonate) (ABTS), but never by the laccase enzyme alone (Bourbonnais *et al*., 1995). The substrate range of laccase could be extended to non-phenolic subunits with the inclusion of primary mediators such as

1-hydroxybenzotriazole (HBT) (Monteiro & De Carvalho, 1998) and ABTS (Poppius-Levlin *et al*., 1997).

The ABTS has the ability to act as a mediator for laccase (Figure 3), thereby enabling the oxidation of non-phenolic lignin compounds that are not laccase substrates (Thurston, 1994). This mediator was found to prevent and even reverse polymerisation of kraft lignin and promotes the delignification of kraft pulp by laccase (Bourbonnais *et al*., 1995). Laccase-ABTS treatment can delignify kraft pulp up to 40 % under similar conditions to those currently used in kraft bleaching (Bourbonnais & Paice, 1996). Although ABTS is an effective mediator it was originally developed for analytical purposes (Bourbonnais & Paice, 1996) and its implementation as a mediator does not seem feasible, as it is estimated that the price would be too high, even if manufactured in bulk (Archibald *et al*., 1997).

It is postulated that the mediator molecules are converted to a reduced state in the presence of laccase. The mediator functions as an electron carrier that is able to diffuse into the secondary wall of wood fibres and react directly with the lignin (Poppius-Levlin *et al*., 1997), while the relatively large size of the laccase prevents it from diffusing into the cell walls (Bourbonnais *et al*., 1995). The possible diffusion of the mediator (ABTS) into the secondary cell wall is illustrated in figure 3. This enables laccase to oxidise veratryl alcohol to veratryl aldehyde and non-phenolic β-1 and β-0-4 model compounds to be cleaved or oxidized at the Cα position (Archibald *et al*., 1997).

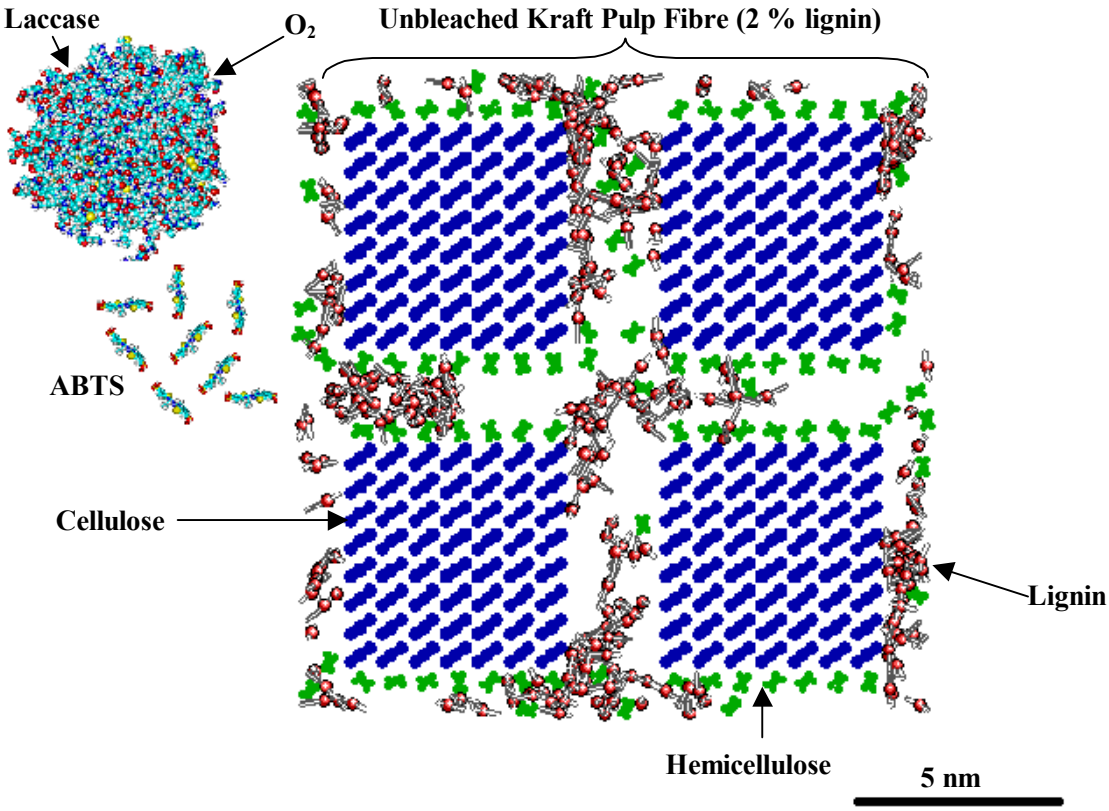


Figure 3. **Schematic representation of a cross section through a secondary wall of a wood fibre (Figure used with permission of L. Jurasek).**

1-hydroxybenzotriazole (HBT) is the most widely studied mediator for laccase delignification and has proven to be a very selective delignification agent in the presence of laccase. The HBT facilitates a high degree of delignification of kraft pulp while leaving the carbohydrates in the pulp intact and it is thus one of the most effective mediators in lignin degradation (Poppius-Levlin *et al.*, 1998). Unfortunately, HBT is known to have an inhibitory effect on the laccase protein. The mediator can be partially converted under delignification conditions to benzotriazole, which does not mediate delignification. The laccase-HBT combination has resulted in a promising improvement of totally chlorine free bleaching. Laccase-HBT has been

reported to activate the residual lignin of various pulps towards bleaching with alkaline and hydrogen peroxide (Poppius-Levlin *et al*., 1998).

It was postulated by Li & Eriksson (2001) that certain white-rot fungi make use of natural mediators for lignin degradation. Li & Eriksson (2001) obtained delignification results comparable to that of conventional mediators with the use of chemical components similar to that found in plant and fungal extracts. Biological bleaching processes will require substantial amounts of enzyme that have activity at relatively high pH values (Heinzkill *et al*., 1998). Obtaining large quantities of enzymes will not be a problem in the future, as the use of recombinant organisms and screening of natural producers on inexpensive carbon sources will render processes capable of high laccase production. The main area of focus in the future should, therefore, be on the development of more cost-effective mediators and optimising their reaction conditions in order to use less mediators (Luisa *et al*., 1996a).

Alternative applications

Laccases do not only show potential for biological delignification of pulp but also for other applications. Laccases can be applied for the treatment of and detoxification of soils containing phenolic pollutants as well as other polluted systems due to the broad substrate range of the enzyme (Filazzola *et al*., 1999; Jarosz-Wilkolazka *et al*., 2001). The application of laccase for dyeing of materials with sulfur and reduced vat dyes has been patented (Xu *et al.,* 2000). The use of laccase for the treatment of textile (Wong & Yu, 1999) and bleach-plant effluents (Manzanares *et al.*, 2001) has also been investigated with success. The use of laccase

for the production and treatment of beverages and as biosensor for the estimation of phenol or other enzymes in fruit juice has also been proposed (Gianfreda *et al.*, 1999).

CONCLUSIONS

The growing amount of information about white-rot fungi and the enzymes they produce has led to increased attempts to incorporate these enzymes in biotechnological applications (Call & Mücke, 1997; Filazzola *et al*., 1999). Laccase is one of the most important enzymes playing a role in lignin degradation (Call & Mücke, 1997) and the laccase mediator system provides a biological alternative to traditional chlorine bleaching processes (Poppius-Levlin *et al*., 1997). It is, however, important to realise that the pulp, mediator, laccase and the dosages play an important role in successful delignification of the pulp (Kandioler & Christov, 2001).

The laccase enzyme has a wide field of application including the pulp and paper industries, the treatment of various industrial effluents, enzymatic decolouring of material and bioremediation of soils (Duran & Esposito, 2000). One of the limitations to the large-scale application of the enzyme is the lack of capacity to produce large volumes of highly active enzyme (Galhaup *et al.*, 2001). These problems can be solved with the use of recombinant organisms or screening for natural hypersecretory strains (Luisa *et al*., 1996a). Environmental factors influence the ability of fungi to produce high titres of laccase and different strains react differently to these conditions (Robene-Soustrade & Lung-Escarmant, 1997). One should thus select a strain capable of producing high concentrations of a suitable enzyme and then optimise conditions for laccase production by the selected organism.

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CHAPTER 2

THE SELECTION OF WHITE-ROT FUNGI FOR THE PRODUCTION OF LACCASE

***Coprinus impatiens* (Fr.) Quél.**

ABSTRACT

Many efforts have been made to utilise enzymes for the degradation of lignin, especially in the pulp and paper industry. White-rot fungi are well-known producers of ligninolytic enzymes and laccase is one of the enzymes that is known to play a major role in delignification. The aim of this study was to develop a suitable screening technique and select fungal strains with the ability to produce large quantities of laccases. One hundred and twenty nine strains from the Sappi Culture Collection of wood-inhabiting fungi were evaluated during these trials. These strains included reference strains and negative controls. The fungi were cultivated for 10 days, using diluted molasses in conical flasks or test tubes. The enzyme activities of laccase in the supernatants were determined spectrophotometrically using 2,2′-azinobis(3-ethylbenzthiazoline-6-sulphonate) as substrate. The data were subjected to cluster analysis, and sixteen strains that produced similar or higher amounts of laccase to that obtained from a selected reference strain (*Coriolus versicolor*; 0,304 Units.ml^{-1}). *Agaricus bisporus* (SCC 173; 0,710 Units.ml^{-1}), a *Peniophora* sp. (SCC 199; 0,833 Units.ml^{-1}) and *Pycnoporus sanguineus* (SCC 108; 0,798 Units.ml^{-1}) produced significantly higher titres of laccase than any of the other screened strains.

INTRODUCTION

Lignin has to be exposed to harsh physiochemical conditions to modify or degrade its structure (Coll *et al*., 1993). Only a few organisms in nature, most of them belonging to the white-rot fungi, are able to delignify wood or even modify lignin to a significant extent (Kaal *et al.*, 1995). White-rot fungi are currently of great interest, as many species in this group have been described as producers of lignolytic enzymes that are involved in the natural delignification of wood (Archibald & Roy, 1992). Some of the more important enzymes responsible for lignin degradation in nature include lignin peroxidase (diaylpropane: oxygen, hydrogen peroxide oxidoreductase; EC 1.11.1.14), manganese peroxidase (Mn(II): hydrogen peroxide oxidoreductase; EC 1.11.1.13) and laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2.) (Call & Mücke, 1997; Heinzkill *et al*., 1998).

Laccase is regarded as one of the most active enzymes in lignin degradation (Ullah *et al*., 2000). This enzyme is also potentially suitable for industrial application, since laccase is able to degrade phenolic and nonphenolic lignin structures (Monteiro & De Carvalho, 1998). Laccases generally occur as extracellular enzymes which allow for rapid purification (Heinzkill *et al*., 1998)

Laccase production occurs in various fungi over a wide range of taxa (Bourbonnais *et al*., 1995). Fungi from the Deutromycetes, Ascomycetes as well as Basidiomycetes are known producers of laccases (Assavanig *et al*., 1992; Bollag & Leonowicz, 1984). The enzyme is particularly important in the large number of lignin-degrading white-rot fungi (Bourbonnais *et al*., 1995). *Coriolus versicolor* (Wulf.:Fr.) Quél., *Cerrena unicolor* (Bull.: Fr.) Murril*, Pleorotus ostreatus* (Jacq.: Fr.)

Kummer, *Pycnoporus sanguineus* (L.:Fr.) Murr. and *Pycnoporus cinnabarinus* (Jacq.: Fr.) Karst. have been described as producers of laccase (Pandey *et al.,* 1999; Poppius-Levlin *et al*., 1997). Many laccase producing fungi secrete isoforms of the same enzyme (Leontievsky *et al*., 1997). The number of isoforms present varies between species and also within species (Assavanig *et al*., 1992). Screening of a large number of white-rot fungi is, therefore, necessary to select strains that are able to produce high titres of laccases. Such a screening trial should, preferably, rely on the use of inexpensive, rapid and sensitive testing methods (Grabner *et al*., 1997) and the screening strategy must be compiled in such a manner as to identify fungal strains and enzymes that will work under industrial conditions (Grabner *et al*., 1997; Monteiro & De Carvalho, 1998).

The aim of this study was to evaluate the ability of Basidiomycetous strains from a collection of wood-inhabiting fungi to produce high titres of laccase. During this study, different cultivation techniques were also compared in order to facilitate efficient screening. The ability of some of these stains to produce cellulase under the selected cultivation conditions was also determined, as cellulase could be detrimental to the application of laccase on lignocellulose substrates.

MATERIALS AND METHODS

Determination of laccase activity

Oxidation of 2,2′-azinobis (3-ethylbenzthiazoline-6-sulphonate) (ABTS) (Aldrich, Steinheim, Germany) by laccases causes a blue discoloration of the substrate (Poppius-Levlin *et al.*, 1997). The activity of the enzyme can, therefore, be determined spectrophotometrically by following the oxidation of ABTS. Enzyme activity was expressed as international units, were one unit is defined as the amount of enzyme forming one μmole of product per minute. In this study, all assays were performed with 500 μM ABTS (Eggert *et al*., 1996) as substrate in 50 mM sodium acetate buffer at pH 4,5 (Coll *et al*., 1993). A 20 μl aliquot of the supernatant of each culture was added to 580 μl of the ABTS in disposable cuvettes and the change in absorbance (extinction coefficient: 36 $mM^{-1}.cm^{-1}$) monitored spectrophotometrically at 418 nm (Xu, 1996) and 25 °C for five minutes. The substrate without supernatant was used as a standard.

Inoculum production

Each of the fungal strains previously selected (Chapter 2) was grown on 1 % malt extract agar plates. The inoculum of each strain was produced by transferring approximately ten pieces (2mm x 2mm) of colonised agar to 100 ml of medium (1 % molasses in water) in a 500 ml conical flask and incubating it for ten days at 24 °C. After ten days the mycelial mat on the broth surface was homogenised (Heildolph DIAX 600 homogeniser, Kelheim, Germany) and the suspension transferred to the cultivation medium.

Optimisation of screening technique

All the cultivations of *P. sanguineus* (SCC 87) were done in 1-% molasses and from inoculum produced as above. The sugarcane molasses used in this study was obtained from Transvaal Sugar Limited (TSB), Malelane, South Africa. To find a suitable method for the cultivation of a large number of strains the following four techniques were evaluated:

1. Cultivation in 500 ml conical flasks with 100 ml of medium and incubated at 160 $r.min^{-1}$ on a rotary shaker.
2. Cultivation in 500 ml conical flasks with 100 ml of medium and incubated on a bench top without any agitation.
3. Cultivation in 22 ml test tubes with 5 ml of medium, incubated at a 30 ° angle on a tube roller at 2 $r.min^{-1}$.
4. Cultivation in 22 ml test tubes with 5 ml of medium, incubated at a 30 ° angle on a bench top without rolling.

The media in conical flasks were inoculated using 5 ml of inoculum and those in test tubes with 1 ml inoculum. All the cultures were incubated for 10 days at 25 °C after which the cultures were homogenised and the laccase activity determined in the filtered (0,45 μm nylon filter, Cameo, Osmonics, USA) supernatant. Each cultivation was replicated three times and the data subjected to one-way analysis of variance. Means of the different treatments were compared with Tukey's test at the 95 % level of confidence (Winer, 1971).

Cellulase activity

Stereum hirsutum (Wild.: Fr.) S.F. Gray (SCC 49), *P. sanguineus* (SCC 87) and the brown-rot fungus *Gloeophyllum sepiarium* (Wulf.: Fr) Karst. (SCC 49) were

used to investigate the potential production of cellulase. These fungi were cultured for 10 days at 25 °C in 1 % molasses. A 500 µl aliquot of each of the supernatants of these cultures was transferred to 3,0 ml buffer solution containing 1 % of carboxy methyl cellulose (CMC) (BDH, Poole, England) with citrate-phosphate buffer (pH 5,0) and incubated for one hour. Pure CMC was also included as a negative control. The activity of cellulase was determined quantitatively by determining an increase in the concentration of reducing sugars present in both the substrate and supernatant before and after mixing. The concentration of sugars present in the substrates was determined spectrophotometrically with a Technikon autoanalyser II (Technikon, Dublin). Xylose (100 $\mu g.ml^{-1}$) free of CMC or enzyme was used as a standard. The sugars from the cultivation medium were accounted for by subtracting the concentration of sugars present in the supernatants before addition of CMC.

Screening

The Sappi Culture Collection (SCC) of wood-inhabiting fungi is maintained at the Department of Microbiology and Biochemistry at the University of the Free State and contains more than 400 cultures of wood-inhabiting fungi. The ability of most of these fungi to produce laccase has previously been determined (Wolfaardt, 1999) using the drop-test method (Stalpers, 1978). These tests were, however, qualitative and these results were only used for making a preliminary selection of 129 strains of laccase-producing fungi and negative controls from the culture collection. Other strains with known potential for biotechnological application were included in this trial as reference strains, namely *Coriolus versicolor* (ATCC 20869), *C. versicolor* (52-J), *Ceriporiopsis subvermispora* (Pil.) Gilbertson & Ryv. (SS-3) and *C.*

subvermispora (CZ-3) (Archibald & Roy, 1992; Bajpai *et al*., 2001; Bourbonnais & Paice, 1992).

A completely randomised trial design with three replications was used. The data were subjected to analysis of variance and significantly different means were separated with the Scott-Knott procedure for cluster analysis (Gates & Bilbro, 1978).

RESULTS AND DISCUSSION

Optimisation of screening technique

The methods using shaking or rolling resulted in higher laccase activity than those without agitation, possibly because of enhanced oxygen transfer (Table 1). Although there was no significant difference ($p \leq 0,05$) in the laccase activities obtained using rolled test tubes and the shaked conical flasks, cultivation in test tubes had the benefit of using smaller volumes of media.

Table 1. Laccase activity in cultures of *Pycnoporus sanguineus* (SCC108), using different cultivation methods.

Cultivation conditions	Culture volume (ml)	Laccase activity (Units.ml^{-1})
Rolled test tubes	6,0	0,126 a
Shaked conical flasks	100,0	0,123 a
Stationary test tubes	6,0	0,072 b
Stationary conical flasks	100,0	0,063 c

a, b, c, d Values followed by the same letter do not differ significantly ($p \leq 0,05$) with Tukey's test.

Cellulase activity

The amount of sugars in the uninoculated molasses medium was approximately 133 $\mu g.ml^{-1}$. There was no change in the concentration of sugars in the carboxy methyl cellulose (CMC) solution before and after exposure to the supernatants of any of the cultures, indicating that none of the isolates produced significant amounts of cellulase when grown on molasses as substrate. The repression of the cellulase production was possibly due to the high sugar content of molasses (Macgillivray & Matic, 1970; Mathews & Van Holde, 1990).

Screening

Sixteen of the tested strains exhibited mean laccase activities that were higher or equal to that of the reference strain (*C. versicolor* ATTC20869) (Table 2 & Appendix A). Twelve of these strains produced significantly ($p \leq 0,05$) more laccase than the reference strain. The best strain (*Peniophora* sp. SCC 199) improved more than two-fold on the laccase production of the reference strain. The cluster of best strains included *Peniophora* sp. (SCC 199) and *P. sanguineus* (SCC 108).

Table 2. Fungal strains with the highest production of laccase after incubation in rolled tubes for 10 days at 25 °C.

Species	Strain number	Laccase activity (Units. ml^{-1})
Peniophora sp.	SCC 199	0.833 a
Pycnoporus sanguineus	SCC 108	0.798 a
Agaricus bisporus	SCC 173	0.710 b
Laetiporus sulphureus	SCC 180	0.511 c
Lenzites betulina	SCC 274	0.488 c
Lenzites betulina	SCC 20	0.443 d
Peniophora sp.	SCC 152	0.415 d
Pycnoporus sanguineus	SCC 126	0.414 d
Poria sp.	SCC 124	0.411 d
Lenzites betulina	SCC 29	0.380 d
Unidentified sp.	SCC 260	0.356 e
Coriolus hirsutus	SCC 79	0.350 e
Pycnoporus sanguineus	SCC 207	0.307 f
Coriolus versicolor	ATTC 20869	0.304 f
Pycnoporus sanguineus	SCC 94	0.295 f
Skeletocutis sp.	SCC 143	0.274 f
Hyphodontium sp.	SCC 155	0.270 f

a, b, c, d, e, f Values followed by the same letter do not differ significantly ($p \leq 0,05$) with the Scott Knott procedure.

The technique for quantitative screening provided more valuable information than previous qualitative studies (Wolfaardt, 1999; De Jong *et al*., 1992). Many of the strains differed significantly in the amount of laccase they produced within the same species. Herpoël *et al*. (2000) had a different screening strategy and focused on dikaryotic strains within one species. Different monokaryotic strains were then obtained from the best dikaryotic strain and subjected to a second screening. In their screening of dikaryotic strains, the highest activity was 4,03 Units.ml^{-1} (Herpoël *et al*., 2000). Although the laccase activities observed in our screening trial were much lower than this value, it is important to note that the conditions for cultivation were not optimised for the different strains. Different agitation methods, inducers and

media could interact with different strains, which could enhance the laccase production.

CONCLUSIONS

During this study, an inexpensive, repeatable and rapid screening technique was developed to evaluate white-rot fungi for laccase production. No cellulase activity was detected using this method and this was ascribed to possible repression of cellulase production by the high sugar concentration present in the molasses (Macgillivray & Matic, 1970; Mathews & Van Holde, 1990). These results indicated that molasses was a suitable feedstock for obtaining cellulase free supernatants from cultures of white-rot fungi. The screening trial was successful and strains were identified that were comparable to or better than the reference strain (*C. versicolor* ATCC 20869).

The ability to produce high titres of laccase would not be the sole factor in considering a strain for commercial laccase production. It would, for instance, be important to determine the optimal temperature and pH of laccases from hypersecretory strains. These screening experiments should, therefore, be considered as the first step in the selection of enzymes with specific characteristics and stability under harsh industrial conditions.

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CHAPTER 3

THERMOSTABILITY AND OPTIMUM TEMPERATURE OF LACCASES PRODUCED FROM SELECTED WHITE-ROT FUNGI

***Coriolus versicolor* (Wulf.: Fr.) Quél.**

ABSTRACT

The extreme conditions used in current pulp bleaching operations require enzymes that are able to withstand harsh physical conditions. It is, therefore, important to screen enzymes for pH and temperature optima and stability. This chapter describes the selection of laccases for thermostability, rather than pH stability, since pH is easier to manipulate under industrial conditions than temperature. In this study a fungal laccase was selected that displayed a high thermostability and optimum temperature. Culture supernatants from the 17 selected strains were exposed to temperatures between 25 °C and 75 °C for four hours. Enzyme activity was determined spectrophotometrically using 2,2′-azinobis (3-ethylbenzthiazoline-6-sulphonate) as substrate. The optimum temperatures for the enzymes were determined by exposure at temperatures of 25 to 95 °C. All the laccases exhibited some degree of thermostability up to 65 °C. At higher temperatures rapid denaturation of most enzymes occurred. Most of the selected strains had an optimum laccase activity at 65 °C. *Pycnoporus sanguineus* (SCC 108) and unidentified strain SCC 260 produced laccases that exhibited a high optimal temperature greater than 50 °C and a high thermostability, exhibiting half-lives greater than 3,5 h. These results compare favourably with previously published results and demontrated that this laccases have potential to be used in a pulp-bleaching environment.

INTRODUCTION

During Kraft bleaching, pulp is exposed to high temperatures of approximately 55 °C (Monteiro & De Carvalho, 1998). Stability at this temperature is thus an important requirement for the enzyme to be used in biobleaching (Poppius-Levlin *et al*., 1997). At high temperatures the enzymes can be denatured and rapidly lose catalytic activity (Mathews & Van Holde, 1990). The aim of this study was to select fungal strains with the ability to produce high titres of thermo-stable laccases that can potentially be applied for delignification by the pulp and paper industry.

A number of fungal isolates were screened for their ability to produce high titres of laccase with activity at 25 °C (Chapter 3). During the present study, the optimum temperature and thermostability of enzymes from 16 selected strains and a reference strain (*Coriolus versicolor* (Wulf.:Fr.) Quél. ATCC 20869) were evaluated.

The optimal temperature of laccase can differ greatly from one strain to another (Farnet *et al*., 2000). Monteiro and De Carvalho (1998) observed that a laccase from a *Trametes (Coriolus) versicolor* strain was stable at 55 °C and 65 °C. Stability at 65 °C was also regarded as high for a commercially available laccase (Tienzyme-^{TM} Website; http://www.tienzyme.com/laccase.htm). I consider this characteristic of laccase as the most important for commercial application of laccase in the pulping industry, due to the high temperatures involved in this process (Monteiro & De Carvalho, 1998).

MATERIALS AND METHODS

Fungal Strains

Sixteen strains and a reference strain were selected during the screening trial for further evaluation (Chapter 3). These strains were maintained at 4 °C on 2-% malt extract agar (MEA) (Biolab Diagnostics (Pty.) Ltd., Midrand).

Inoculum and cultivation

Inoculum of each strain was prepared in conical flasks (500 ml) by transferring 2 cm^2 agar (MEA) overgrown with fungal mycelium to 100 ml of medium consisting of 4-% molasses (Transvaal Sugar Limited, Malelane, South Africa). These cultures were incubated for 10 days at 25 °C on the bench top. After 10 days the mycelial mats were fragmented for 10 seconds at 3500 $r.min^{-1}$ with a homogeniser (Heildolph DIAX 600, Germany). The inoculum (5,0 ml) was transferred to 100 ml cultivation medium consisting of 4-% molasses (pH 5,5) in 500 ml conical flasks. The cultures were incubated for 10 days on a rotary shaker (160 $r.min^{-1}$) at 25 °C and the supernatant harvested to determine the thermostability and optimum temperature of the laccases.

Laccase activity

The laccase activity was determined spectrophotometrically at 418 nm using 2,2′-azinobis (3-ethylbenzthiazoline-6-sulphonate) (ABTS) (Aldrich, Steinheim, Germany) as substrate at pH 4,5 in 50 mM sodium acetate buffer (Xu, 1996). Supernatants (20 µl) of each culture were added to 580 µl of the ABTS. The change in absorbance (extinction coefficient: 36 $mM^{-1}.cm^{-1}$) was monitored with a

spectrophotometer (Genesis 5, Specronic® Genesys™, Milton Roy Company, USA). The substrate-containing buffer, without supernatant, was used as a standard.

Determination of the thermostability

Enzyme-containing supernatants were incubated at 25, 55, 65 and 75 °C in water baths for four hours to allow ample time for denaturation and accurate determination of the half-lives. These temperatures were selected to allow comparison with previous results (Monteiro and De Carvalho, 1998). The residual enzyme activity was determined every 10 minutes for one hour and then every 30 minutes for the next three hours at 25 °C. The half-lives of the enzymes were read from a plot of the activity of the enzymes against time.

Determination of the optimum temperature

The optimum temperature was determined for five strains that were selected on the basis of their thermostability. These strains were *Agaricus bisporus* (Lange) Imbach (SCC 173), a *Peniophora* sp. (SCC 199), *Pycnoporus sanguineus* (L.:Fr.) Murr. (SCC 108), *P. sanguineus* (SCC 207), an unidentified strain (SCC 260) and a reference strain *Coriolus versicolor* (ATCC 20869). The laccase activity was determined spectrophotometrically in a buffer heated to temperatures between 25 °C and 95 °C at 10 °C intervals.

RESULTS AND DISCUSSION

Thermostability of the laccases produced

Enzymes from all the strains were stable at 25 °C and 55 °C for more than four hours (Table 1) and no reduction in enzyme activity occurred. At 65 °C, 12 of the strains exhibited good stability with half-lives of more than 90 minutes. The best stability at 65 °C was observed from *Pycnoporus sanguineus* (SCC 108), *P. sanguineus* (SCC 207) and the unidentified strain (SCC 260) with half-lives of more than 3,5 hours (Table 1).

Table 1. Thermostability of selected fungal strains at different temperatures as reflected by half-lives.

Strain		Half-life (min.)			
		25 °C	55 °C	65 °C	75 °C
Agaricus bisporus	SCC 173	>240	>240	45	11
Coriolus hirsutus	SCC 79	>240	>240	150	8
Coriolus versicolor	ATCC 20869	>240	>240	96	12
Hyphodontium sp.	SCC 155	>240	>240	115	11
Laetiporus sulphureus	SCC 180	>240	>240	135	12
Lenzites betulina	SCC 20	>240	>240	168	15
	SCC 274	>240	>240	18	13
	SCC 29	>240	>240	45	10
Peniophora sp.	SCC 152	>240	>240	74	22
	SCC 199	>240	>240	105	14
Poria sp.	SCC 124	>240	>240	167	13
Pycnoporus sanguineus	SCC 108	>240	>240	238	45
	SCC 126	>240	>240	108	14
	SCC 207	>240	>240	230	34
	SCC 94	>240	>240	95	12
Skeletocutis sp.	SCC 143	>240	>240	40	10
Unidentified sp.	SCC 260	>240	>240	>240	30

At 75 °C, the enzymes were no longer stable and in most cases rapid denaturation of the enzymes occurred with most of the laccases having half-lives of less than 30 minutes (Table 1). The only exceptions were *Pycnoporus sanguineus* (SCC 108), *P. sanguineus* (SCC 207) and the unidentified strain (SCC 260) (Figure 1). The brief increase in activity of the *P. sanguineus* (SCC 207) laccase after 20 minutes was probably due to the unfolding of the protein structure (Mathews & Van Holde, 1990).

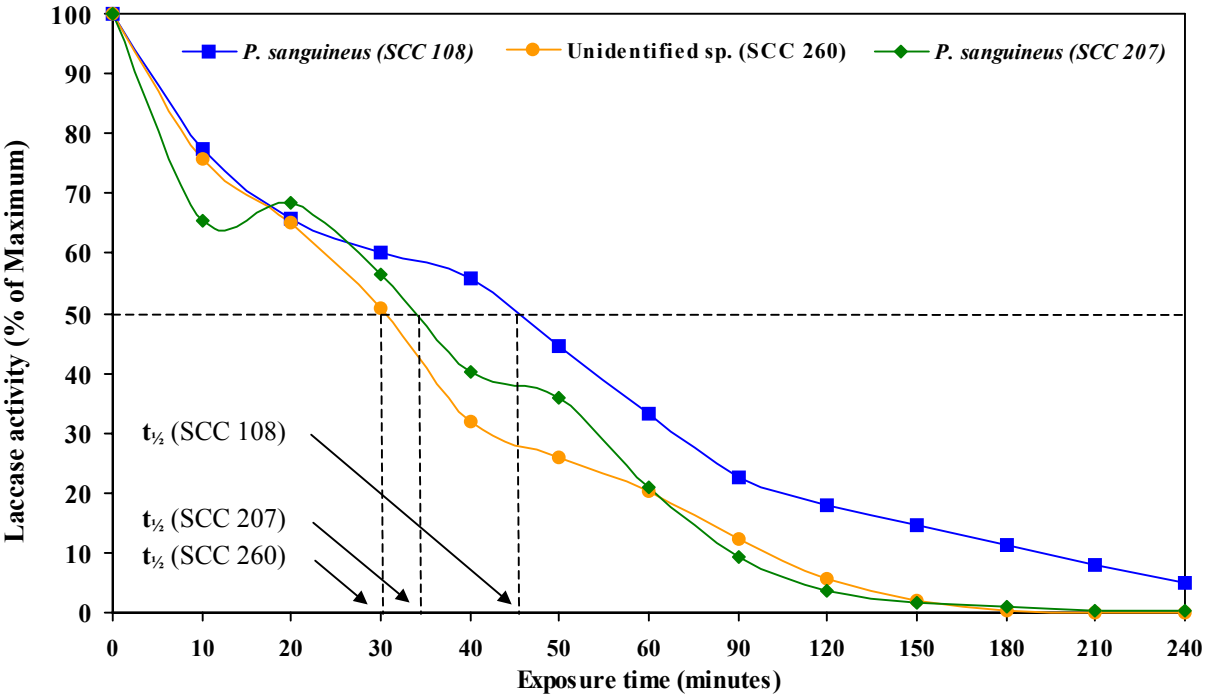

Figure 1. **Laccase activities of *Pycnoporus sanguineus* (SCC 108), *Pycnoporus sanguineus* (SCC 207) and the unidentified strain (SCC 260) after incubation at 75 °C.**

Optimal temperature

The laccase activity in the supernatant of the tested strains increased with an increase in temperature up to 65 °C (Figure 2), possibly due to the unfolding of the

protein structure as described by Mathews & Van Holde (1990). At higher temperatures rapid inactivation occurred due to denaturation of the enzyme.

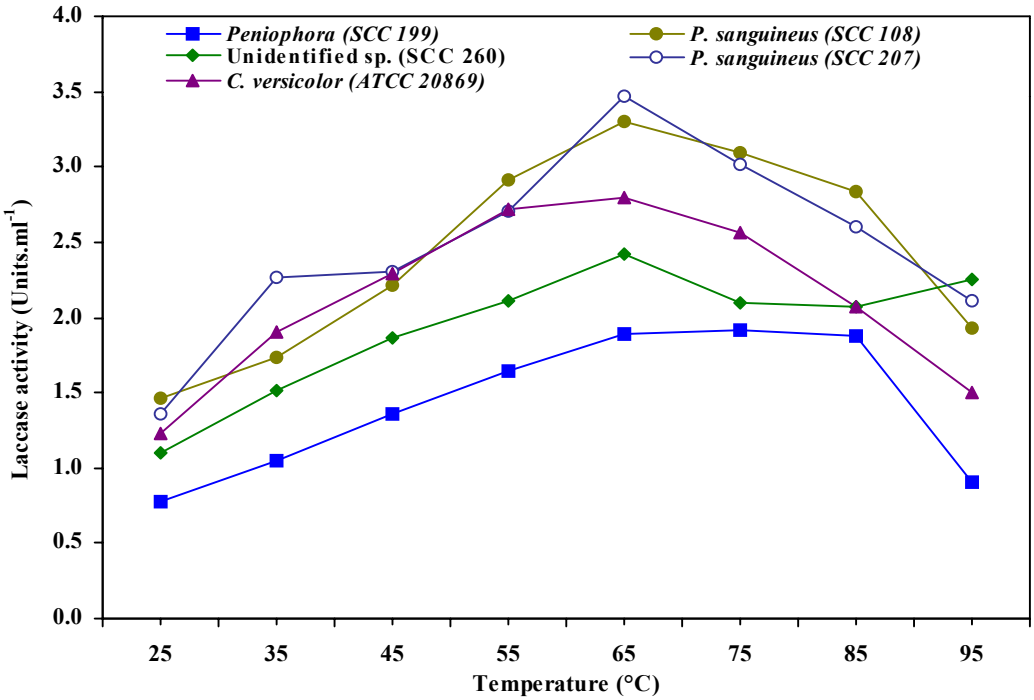

Figure 2. Activity of laccases from selected strains at different temperatures.

CONCLUSIONS

A number of strains that produced laccases with thermostability up to 65 °C were identified. *Pycnoporus sanguineus* (SCC 108) was selected as the most suitable strain for further study due to its ability to produce high titres (1,5 to 3,3 Units.ml^{-1}) of a laccase that was thermostable at 65 °C. This laccase exhibited higher thermostability than that of the reference strain *Coriolus versicolor* (ATCC 20869). The high optimal temperature of the *P. sanguineus* (SCC 108) enzyme is a benefit that could require less modification to current bleaching operations.

Pycnoporus sanguineus (SCC 108) and the unidentified strain (SCC 260) exhibited higher thermostability than obtained previously for *Trametes (Coriolus) versicolor* (Monteiro & De Carvalho, 1998). Stability at 55 °C and 65 °C is regarded as high (Monteiro & De Carvallo, 1998) and, therefore, several of the laccases tested in this trial should be regarded thermostable. The thermostability of the laccase evaluated in this study also compared well with commercially available enzymes from Tienzyme-^{TM} that are stable at 60° C (website, http://www.tienzyme.com.htm) and Novozymes that are stable for 30 minutes at 65 °C (Anon, 1997). The laccases isolated by Farnet *et al.* (2000) from a strain of *Marasmius quercophilus* were also stable at 60 °C.

Monteiro *et al.* (1998) evaluated laccases from *Coriolus versicolor* (ATCC20869) in pre-bleaching and bleaching steps at 55 °C and pH 8, with improved brightness, reduced lignin content and compatibility with industrial pulps. The laccases produced from *P. sanguineus* (SCC 108), *P. sanguineus* (SCC 207) and the unidentified strain (SCC 260) could be suitable for application in bleaching sequences because of their thermostability and high optimum temperatures. The stability of these enzymes can further be increased by pre-incubation at 40 °C and 50 °C as described by Farnet *et al*. (2000) and immobilisation of the enzyme on glass powder (Ruiz *et al*., 2000), but this need to be verified experimentally.

The ability of *Pycnoporus sanguineus* (SCC 108) to produce high titres of thermostable laccase makes it a suitable strain for laccase production and it will, therefore, be the subject of further study. These studies will focus on the specific growth and nutritional requirements to improve laccase production.

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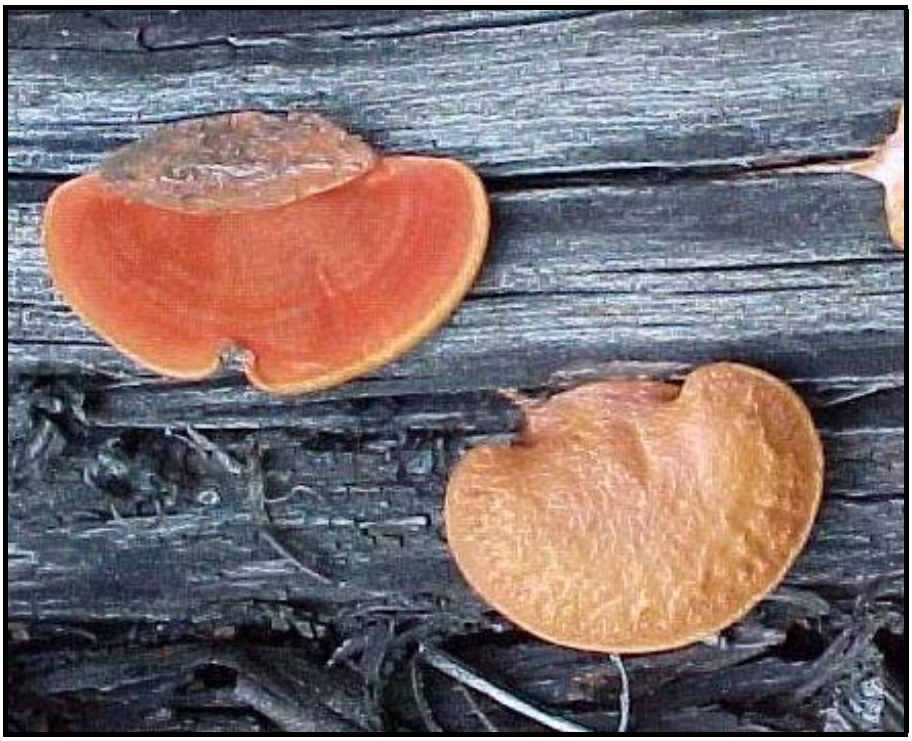
CHAPTER 4

EVALUATION OF MOLASSES AND MINERAL SALTS MEDIA FOR THE PRODUCTION OF LACCASE



***Pycnoporus sanguineus* (L.:Fr.) Murr.**

ABSTRACT

The aim of this study was to identify suitable substrates for cultivation of white-rot fungi to produce laccase. Previous studies indicated that diluted molasses could be used for cultivation of white-rot fungi and that it supported high levels of laccase production. In this study the production of laccase in diluted molasses was compared with that in supplemented molasses media and with that produced in different mineral salts media. The influence of acidic pre-treatment of molasses as a growth medium was also investigated. Higher biomass concentrations were obtained in 10 % molasses (17,4 $g.l^{-1}$) than in 4 % molasses (8,3 $g.l^{-1}$), but the laccase activities in 10 % molasses (0,655 $Units.ml^{-1}$) were significantly lower than that observed in 4 % molasses (1,657 $Units.ml^{-1}$). The acidic pre-treatment of 10 % molasses resulted in a 17 % increase in biomass obtained. Relatively high levels of laccase were obtained using molasses as a cultivation medium without the inclusion of an inducer. The addition of trace elements to diluted molasses increased laccase production by 26 %. None of the mineral salts media resulted in improved laccase or biomass production when compared to diluted molasses. I concluded that diluted molasses has potential as a cost effective substrate for the production of laccase.

INTRODUCTION

The metabolism of microorganisms, and consequently laccase production, is controlled by the physical environment and medium composition (Monteiro & De Carvalho, 1998). Various factors such as carbon to nitrogen ratio, carbon and nitrogen sources and microelements have been studied (Gayazov & Rodakiewicz-Nowak, 1996). A suitable medium is required to support sufficient growth and high laccase production, but at a reasonable cost for commercial production of the enzyme (Grabner *et al*., 1997). A molasses-based medium was considered to be an ideal substrate by Johnson *et al.* (1995), because of the general availability, high sugar content and low cost of molasses. Molasses has also been used successfully during previous experiments as substrate for the production of laccase by white-rot fungi (Chapters 2 & 3).

Molasses is a black liquor that is produced in large quantities as a by-product of the sugar-refining process (Johnson *et al.,* 1995; Macgillivray & Matic, 1970). More than 50 % of the world's molasses is used as animal feed or supplement (R & H Hall Website, http://rhhall.ie/print/issue3_1999.htm). This high-viscosity product contains nearly 50 % sugar and has potential to be employed as a low cost cultivation medium. Molasses is, however, a very complex substrate that can have large seasonal and annual variability due to differences in sugar concentration of the sugar cane and differences in the sugar extraction processes (Macgillivray & Matic, 1970). It is also known to contain certain inhibitors such as heavy metals (R & H Hall Website), which can be removed by either an acidic (Johnson *et al*., 1995) or alkaline pre-treatment (Van Zyl *et al*., 1988).

Due to the variable nature of molasses its influence on biomass and laccase production was compared to that of different mineral salts media. In this study the production of laccase in different concentrations of molasses was compared with that in supplemented molasses media and with that produced in different mineral salts media. One procedure for the pre-treatment of molasses was also evaluated. The objectives of this study were to identify a suitable mineral salts medium or to improve on the molasses medium used for the cultivation of *Pycnoporus sanguineus* (L.:Fr.) Murr. (SCC 108).

MATERIALS AND METHODS

Analytical procedures

In all experiments, the laccase activity was determined spectrophotometrically at 418 nm using 2,2′-azinobis (3-ethylbenzthiazoline-6-sulphonate) (ABTS) (Aldrich, Steinheim, Germany) as substrate at pH 4,5 and 25 °C (Xu, 1996). The biomass was removed from cultures using 4,7 cm glass fibre filters (MN GF-1, Macherey-Nagel, Düren, Germany) and determined gravimetrically after having been washed with distilled water and dried to constant mass at 105 °C.

Inoculum and cultivation conditions

Cultures of *Pycnoporus sanguineus* (SCC 108) were maintained on 2 % malt extract agar (Biolab, Midrand, South Africa) plates at 4 °C. The inoculum was prepared as described previously (Chapter 2) and 5 ml of the fragmented mycelium was transferred aseptically to 100 ml of the different cultivation media in 500 ml conical flasks. In each case the pH was adjusted to pH 5,5 with 3 N sulphuric acid

and 3 N potassium hydroxide prior to inoculation. The laccase activity and biomass concentration of the cultures were determined after cultivation for 10 days on a rotary shaker at 160 $r.min^{-1}$ and 25 °C.

Composition of molasses

A 20-l batch of "final molasses" was obtained from TSB (Transvaal Sugar Limited, Malelane, South Africa) and stored at 4 °C. The sugar and nitrogen concentrations of the molasses had been determined before it was evaluated as a substrate for laccase production. The sugar content of molasses was determined in 20,0 g samples of molasses, which were dissolved in 1000 ml distilled water and filtered through a 0,45 μm nylon filter (Cameo, Osmonics). Samples were diluted five-fold and the sugar content determined using high performance liquid chromatography with an Aminex®, Carbohydrate HPX 42C column (Bio-Rad). A differential refractometer (Waters R401, Waters Associates, Milford, Massachusetts, USA) was used as detector and double distilled water was used for the mobile phase. The total nitrogen content of molasses was determined using the Kjeldahl method described by Hesse (1971).

Supplementation of molasses with mineral salts

The biomass and laccase production by *P. sanguineus* (SCC 108) in 4 % diluted molasses were compared with two supplemented molasses media. The diluted molasses medium (MM) consisted of 40,0 g molasses in 1000 ml distilled water.

The molasses medium without yeast extract (MM-YE) contained ($g.l^{-1}$): molasses, 40,0; $(NH_4)_2SO_4$, 3,84; KH_2PO_4, 1,20; and citric acid, 0,25 as chelating agent.

The molasses medium with yeast extract (MM+YE) contained ($g.l^{-1}$): molasses, 40,0; yeast extract, 1,20; $(NH_4)_2SO_4$, 2,40; KH_2PO_4, 1,20; and citric acid, 0,25.

Acidic pre-treatment of molasses

The influence of acidic pre-treatment of 4 % and 10 % molasses on the laccase and the biomass production was evaluated. The pre-treatment involved a precipitation step where the pH of the molasses medium was reduced to pH 2,0 with 3 N sulphuric acid, boiling for 30 minutes, cooling and removing the precipitant by centrifugation (Johnson *et al.,* 1995).

Supplementation of molasses with trace elements

A trace elements solution developed by Du Preez and Van der Walt (1983) was added to 4 % molasses. Addition of 1 $ml.l^{-1}$ trace elements solution to the media, gave the following final concentrations ($mg.l^{-1}$): $FeSO_4.7H_2O$, 35,00; $MnSO_4.H_2O$, 7,00; $ZnSO_4.7H_2O$, 11,00; $CuSO_4.5H_2O$, 1,00; $CoCl_2.6H_2O$, 2,00; $Na_2MoO_4.2H_2O$, 1,30; H_3BO_3, 2,00; KI, 0,35; $Al_2(SO_4)_3$, 0,50.

Evaluation of mineral salts media

The biomass and laccase production by *P. sanguineus* (SCC 108) in three mineral-salts media were compared with those in diluted molasses (4 %). The mineral salts media contained 20,00 $g.l^{-1}$ sucrose, which is the equivalent amount to the total sugars present in 4 % molasses (Table 1).

The first mineral salts medium contained ammonium sulphate (3,00 $g.l^{-1}$) as sole nitrogen source at a high carbon to nitrogen ratio. It also contained (in $g.l^{1}$):

KH_2PO_4, 3,00; $MgSO_4.7H_2O$, 1,00; citric acid, 0,25; $CaCl_2.2H_2O$, 0,05 and 1,0 $ml.l^{-1}$ trace elements solution (Du Preez & Van der Walt, 1983).

The second medium contained ammonium sulphate (1,00 $g.l^{-1}$) and yeast extract (YE) (3,00 $g.l^{-1}$) as nitrogen sources at a high carbon to nitrogen ratio. It also contained (in $g.l^{-1}$): KH_2PO_4, 3,00; $MgSO_4.7H_2O$, 1,00; citric acid, 0,25; $CaCl_2.2H_2O$, 0,05 and 1,0 $ml.l^{-1}$ trace elements solution.

The third medium contained ammonium sulphate (4,50 $g.l^{-1}$) and yeast extract (YE) (3,00 $g.l^{-1}$) as nitrogen sources at a low carbon to nitrogen ratio: KH_2PO_4, 3,00; $MgSO_4.7H_2O$, 1,00; citric acid, 0,25; $CaCl_2.2H_2O$, 0,05 and 1,0 $ml.l^{-1}$ trace elements solution.

Trial design and statistical analyses

A completely randomised trial design was used for each of the experiments. Each treatment was replicated three times and the data subjected to one-way analysis of variance. Means of the different treatments were tested for significant differences with Tukey's test at a 95 % level of confidence (Winer, 1971).

RESULTS AND DISCUSSION

Composition of molasses

The sugar content of the tested molasses was 48,0 % (w/w) and was comprised of sucrose, fructose and glucose (Table 1). The total nitrogen content of the molasses was 0,58 %, giving a carbon to nitrogen ratio of 34 to 1 ($g.g^{-1}$).

Table 1. Composition of the molasses used in this study.

Components	Concentration ($g.l^{-1}$)
Carbohydrates	
Sucrose	420,0
Glucose	115,5
Fructose	136,5
Total	**672,0**
Nitrogen	
Total	**8,1**

Supplementation of molasses with mineral salts

Laccase production in molasses medium without yeast extract was significantly less than laccase activity in diluted molasses (Table 2). There was no significant difference between the amounts of biomass obtained from any of the three molasses-based media (Table 2). None of the supplements was, therefore, able to improve the laccase production or growth by the fungus in comparison to those obtained in diluted molasses.

Table 2 Laccase activity and biomass produced by *P. sanguineus* in different molasses based media.

Cultivation medium	Laccase activity (Units. ml^{-1})	Biomass ($g.l^{-1}$)
MM	1.657 a	8.3 a
MM-YE	1.023 b	9.9 a
MM+YE	1.408 ab	9.5 a

a, b Values in the same column, followed by the same letter do not differ significantly ($p \leq 0,05$) with Tukey's Test.

Acidic pre-treatment of molasses

Cultures growing in the 4-% molasses produced significantly higher titres of laccase activity than those growing in the 10-% molasses and the acidic pre-treatment had no significant effect on the laccase activity (Table 3). A higher biomass concentration was obtained from both the 10-% molasses media and the 10-% molasses, which had undergone the acidic pre-treatment, produced significantly more biomass than the untreated medium. This could possibly be ascribed to the removal of toxic components that were present in higher concentrations in 10-% molasses. Attention should, therefore, be paid to the presence of inhibitors when using higher concentrations of molasses for cultivation.

Table 3. Laccase activity and biomass concentration produced by *P. sanguineus* in pre-treated and untreated molasses media.

Molasses concentration	Pre-treatment	Laccase activity (Units.ml^{-1})	Biomass ($g.l^{-1}$)
4 %	Control	1,657 a	8,3 a
4 %	Acid	1,344 a	7,3 a
10 %	Control	0,655 b	17,4 b
10 %	Acid	0,717 b	20,4 c

a, b, c Values in the same column, followed by the same letter do not differ significantly ($p \leq 0,05$) with Tukey's Test.

Supplementation of molasses with trace elements

The inclusion of a trace elements solution in the 4-% diluted molasses had no significant influence on the growth of the fungus. The trace elements resulted in a significant ($p \leq 0,05$) increase (26 %) in laccase activity from 1,26 Units.ml^{-1} to 1,65 Units.ml^{-1}.

Evaluation of mineral salts media

Significantly higher titres of laccase were produced in the diluted molasses than in the mineral salts media (Table 4). This was possibly due to a natural inducer of laccase activity contained in molasses. None of the mineral salts media was able to support significantly more biomass than that obtained with 4-% molasses (Table 4). The mineral salts medium without yeast extract and a high carbon to nitrogen ratio had a much lower final biomass concentration than the mineral salts medium with a low carbon to nitrogen ratio.

Table 4 Laccase activity and biomass produced by *P. sanguineus* in diluted molasses and different mineral salts media.

Cultivation medium	Laccase activity (Units. ml^{-1})	Biomass (g.l^{-1})
Molasses medium (4 %)	1.02 a	7.3 ab
High C:N with ammonium sulphate	0.01 b	7.1 a
High C:N with YE and ammonium sulphate	0.28 b	8.6 ab
Low C:N with YE and ammonium sulphate	0.40 b	9.4 b

a, b Values in the same column, followed by the same letter do not differ significantly ($p \leq 0,05$) with Tukey's Test.

CONCLUSIONS

Molasses contains very high levels of sucrose, glucose and fructose that contribute to nearly 50 % of this substrate (wet weight). Molasses is a cost-effective feedstock for cultivation of fungi, as molasses also contains nitrogen, mineral salts and trace elements (Macgillivray & Matic, 1970). Relatively high levels of laccase were obtained using molasses as a cultivation medium without the inclusion of an inducer. The inducing effect of molasses on laccase production has, to my knowledge, not been reported. Molasses has, however, been used successfully for the cultivation of other fungi in industrial processes (Johnson *et al.* 1995).

Cultivations using diluted molasses (4 %) proved to be sufficient in obtaining high titres of laccase after 10 days of cultivation. The use of an acid pre-treatment proved to be unnecessary for 4-% molasses, although it did enhance growth in 10-% molasses, probably due to the presence of higher levels of inhibitors. The addition of mineral salts to the diluted molasses did not result in improvement of growth or laccase production. The low laccase activity observed in high concentrations of molasses was in agreement with results from Froehner & Erikson (1974), who showed that rapidly growing cultures of a *Neurospora* sp. did not produce laccase.

The addition of trace elements to the molasses resulted in a significant increase in laccase production without affecting the final biomass concentration. One of the reasons for this increase may be the copper contained in the trace elements solution, since copper can influence the metal response element sites in the promoter areas of various laccase genes, to induce laccase production (Sannia *et al*., 2001). Molasses was able to support production of high titres of laccase without the inclusion

of any inducers. This substrate will therefore be further investigated and compared to other inducer-containing media.

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CHAPTER 5

THE INFLUENCE OF DIFFERENT SUBSTRATES AND SUPPLEMENTS ON THE LACCASE AND BIOMASS PRODUCTION BY *PYCNOPORUS SANGUINEUS* (SCC 108)

***Pycnoporus sanguineus* (L.:Fr.) Murr.**

ABSTRACT

Many compounds that induce the production of the laccase protein have been described. These components can improve the laccase activity either directly or indirectly by affecting the metabolism of fungi. In this study, the influence of three potential inducers of laccase production by *Pycnoporus sanguineus* (SCC 108) was investigated. Ethanol, xylidene and veratryl alcohol were added to a molasses medium and a mineral salts medium and evaluated for their ability to enhance laccase production. Only the addition of ethanol and veratryl alcohol resulted in increased laccase production. Ethanol resulted in a three-fold increase in the laccase production by P. sanguineus in molasses medium. The highest increase of laccase activity (17-fold) was observed when *P. sanguineus* was cultivated in a mineral salts medium with 16 mM veratryl alcohol. Addition of these inducers, therefore, offers an affordable technique to enhance laccase production by *P. sanguineus*.

INTRODUCTION

The conditions for growth and cultivation play an important role in the laccase production by fungi, but the composition of media is equally important (Lee *et al*., 1999). Previous work demonstrated that high biomass yield did not necessarily result in high laccase activity (Chapter 4). These experiments and literature (Bourbonnais *et al*., 1995) indicated that the production of laccase increased during the stationary phase of growth. Many substrates have resulted in a positive effect on laccase production by various fungi (Aurora & Gill, 2001). Laccases are generally produced in low concentrations by white-rot fungi (Vasconcelos *et al*., 2000), but higher concentrations are obtainable with the addition of various supplements to media (Lee *et al*., 1999). Many of these supplements resemble lignin molecules or other phenolic compounds (Marbach *et al.*, 1985; Farnet *et al.*, 1999). Some of the compounds affect the metabolism or growth rate (Froehner & Eriksson, 1974) and others trigger laccase production indirectly (Lee *et al*., 1999). Many inducers that were able to increase the expression of laccase have been described (Assavanig *et al*., 1992; Garzillo *et al.* 1998; Lu *et al*., 1996). Production of certain laccases is inducible, since the promoter regions of the genes encoding for laccase contains various recognition sites that are specific for xenobiotics and heavy metals (Sannia *et al.*, 2001).

Many substrates have been described to improve laccase production in white-rot fungi, such as cellobiose (Lu *et al*., 1996), copper sulphate (Palmieri *et al*., 2000), gallic acid, cycloheximide, and ferulic acid (Bollag & Leonowicz, 1984). The effect of ethanol, xylidene and veratryl alcohol on laccase production by *Pycnoporus sanguineus* (L.:Fr.) (SCC 108) were investigated in this study. Lee *et al*. (1999) found that the addition of ethanol to the growth medium of *Trametes (Coriolus)*

versicolor (Wulf.:Fr.) Quél. had a stimulating effect on laccase production. The enhanced laccase activity was comparable to those obtained using 2,5-xylidine and veratryl alcohol (Mansur *et al*., 1997). It was postulated that the addition of ethanol to the cultivation medium caused a reduction in melanin formation. The precursors, when not polymerised to melanin, can then act as inducers for laccase production (Lee *et al*., 1999). The addition of ethanol as an indirect inducer of laccase secretion offers a potential economical way to enhance laccase production (Lee *et al*., 1999). Ethanol (Lee *et al*., 1999) was included in molasses medium, but due to the complex and variable character of molasses, it was decided to investigate the effects of the two other inducers using a mineral salts medium.

Veratryl (3,4-Dimethoxybenzyl) alcohol is an aromatic compound known to play an important role in the synthesis and degradation of lignin (Barbosa *et al.,* 1996). The addition of veratryl alcohol to cultivation media of many white-rot fungi has resulted in an increase in laccase production (Xavier *et al.*, 2001). Laccase offers protection to the fungus against toxic phenolic monomers of polyphenols (Assavanig *et al*., 1992). The increased production of laccase with the addition of xylidene has been described as a response by the fungus to break down a toxic component (Eggert *et al*., 1996). It was decided to investigate the effect of different concentrations of veratryl alcohol and xylidene on laccase production by *P. sanguineus* (SCC 108) only in the mineral salts medium as molasses itself had an inducing effect on laccase production (Chapter 4).

MATERIALS AND METHODS

Analytical procedures

In all experiments the laccase activity was determined spectrophotometrically at 418 nm using 2,2′-azinobis (3-ethylbenzthiazoline-6-sulphonate) (ABTS) (Aldrich, Steinheim, Germany) as substrate at pH 4,5 and 25 °C (Xu, 1996). The biomass was removed from 10-ml samples using 4,7 cm glass-fibre filters (MN GF-1, Macherey-Nagel, Düren, Germany) and determined gravimetrically after having been washed with distilled water and dried to constant mass at 105 °C.

Inoculum and cultivation conditions

Pycnoporus sanguineus (SCC 108) was identified as a suitable test strain, as it produced high titres of laccase that is stable at high temperatures (Chapter 2). Cultures were maintained on 2 % MEA plates at 4 °C. Inoculum of *P. sanguineus* was produced as described previously (Chapter 2). A volume of 5-ml inoculum was used to inoculate 100 ml cultivation media in 500 ml conical flasks. The pH was adjusted to pH 5,5 prior to inoculation using 3 N H_2SO_4 and 3 N KOH. The cultures had been cultivated for 10 days on a rotary shaker (160 rpm), where after the laccase activities and biomass concentrations were determined. Inducers were added to the cultures 24 h after inoculation due to the toxic nature of some of these compounds (Koroljova-Skorobogat'ko *et al*., 1998).

Cultivation media

The diluted molasses medium consisted of molasses (40,0 g) in 1000 ml distilled water. The mineral salts medium consisted of (in $g.l^{-1}$): sucrose, 20,00; yeast

extract, 3,00; $(NH_4)_2SO_4$, 4,50; KH_2PO_4, 3,00; $MgSO_4.7H_2O$, 1,00; citric acid, 0,25; $CaCl_2.2H_2O$, 0,05 and 1,0 $ml.l^{-1}$ of a trace elements solution (Du Preez & Van der Walt, 1983).

Influence of ethanol on laccase production

The effect of different concentrations of ethanol on biomass yield and laccase production was evaluated in the diluted molasses medium as well as a mineral salts medium. The ethanol (2 %, 3 %, 4 % & 5 %; v/v) was filter-sterilised and added to the media after the media was autoclaved (Lee *et al*., 1999). A control treatment was included in the trial that contained no ethanol.

Influence of veratryl alcohol on laccase production

In a trial to determine the optimal concentrations of veratryl alcohol for induction of laccase activity by *P. sanguineus*, veratryl alcohol was added to the mineral salts media to obtain final concentrations of 2 mM, 4 mM, 8 mM, 12 mM and 16 mM (Barbosa *et al*., 1996). As the highest activity was observed at the highest concentration of veratryl alcohol tested, the trial was repeated using a broader concentration range of 4 mM, 16 mM, 24 mM, 28 mM and 32 mM veratryl alcohol and a control treatment without veratryl alcohol.

Influence of xylidene on laccase production

Xylidene was added to the mineral salts medium to obtain final concentrations of 4 μM, 7 μM, 10 μM, 20 μM and 30 μM. The control treatment did not include xylidene.

Statistical analysis

A completely randomised trial design was used for each experiment. All treatments were replicated three times and the data subjected to one-way analysis of variance. Means of the different treatments were tested for significant differences with Tukey's test at a 95 % level of confidence (Winer, 1971).

RESULTS AND DISCUSSION

Influence of ethanol on laccase production

The addition of both 2 % and 3 % ethanol resulted in a significant increase in laccase activity compared to the control (Figure 1). Higher levels of ethanol (4 % and 5 %) did not cause changes to the amounts of laccase produced when compared to the control. Compared to the control, the addition of 2 % and 3 % ethanol to the cultivation medium did not influence the biomass production, but both 4 % and 5 % ethanol resulted in a significant decrease in biomass (Figure 2), possibly due to the toxicity of the ethanol (Lee *et al*., 1999). These lower biomass concentrations were probably the cause of the lower laccase activities at 4 % and 5 % ethanol. The laccase activity obtained with ethanol (2,94 Units.ml^{-1}) was higher than the 2,60 Units.ml^{-1} obtained by Lee *et al*. (1999) from *Trametes (Coriolus) versicolor*.

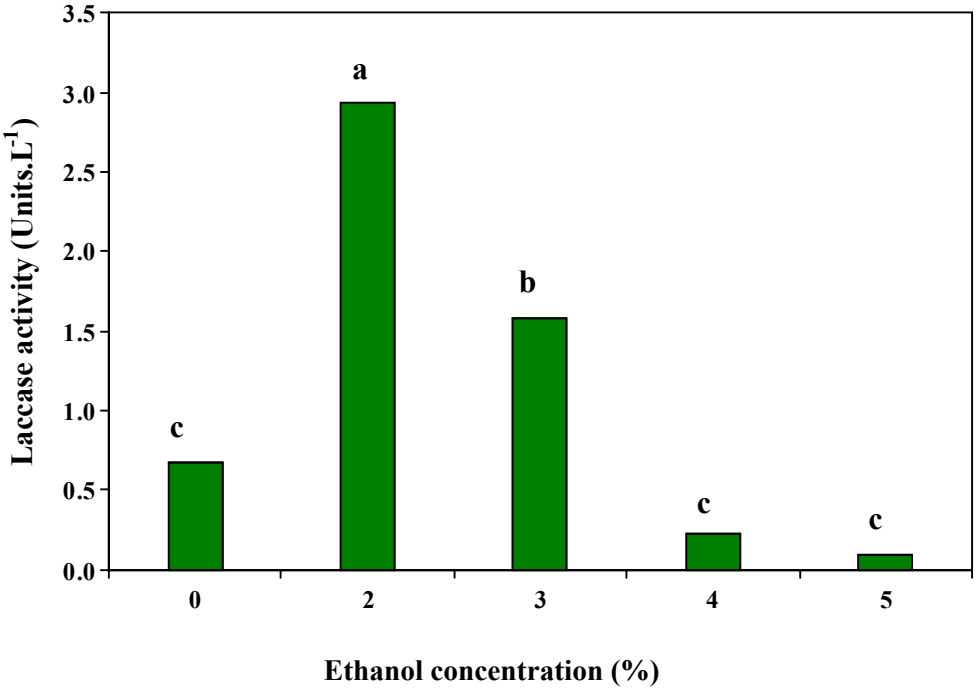

Figure 1. **Laccase activity produced by *P. sanguineus* after 10 days using different concentrations of ethanol as inducer in diluted molasses.**
Bars with the same letter do not differ significantly ($p \leq 0,05$) with Tukey's Test.

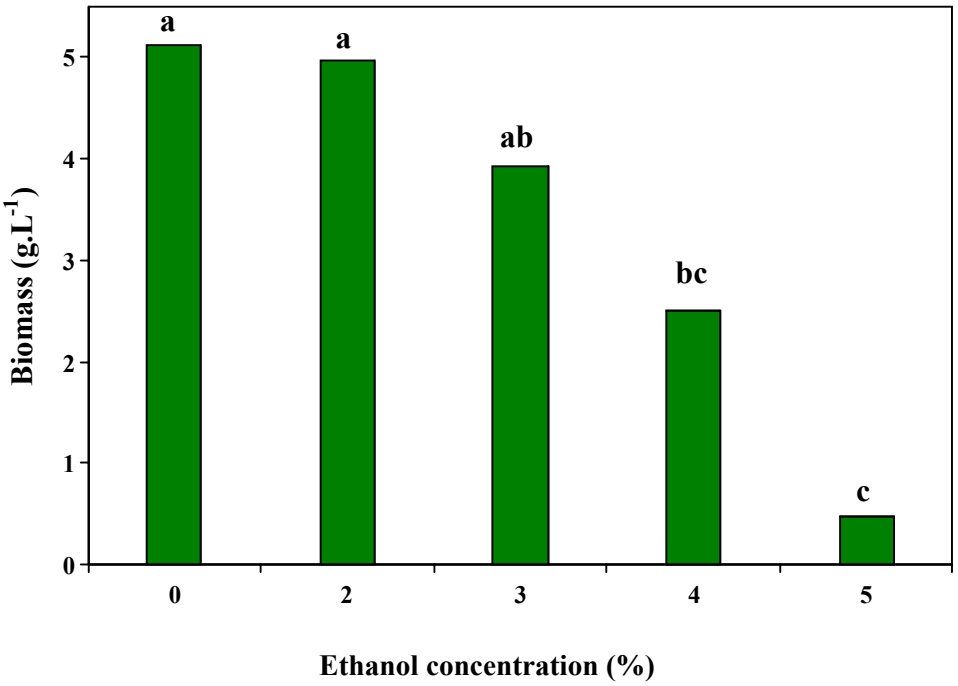

Figure 2. **Biomass produced by *P. sanguineus* after 10 days using different concentrations of ethanol as inducer in 4 % molasses.**
Bars with the same letter do not differ significantly ($p \leq 0,05$) with Tukey's Test.

Influence of veratryl alcohol on laccase production

Significantly higher laccase activity was obtained from all the concentrations of veratryl alcohol in comparison to the control, but the highest activity was obtained in the mineral salts medium with 16 mM veratryl alcohol (Figure 3).

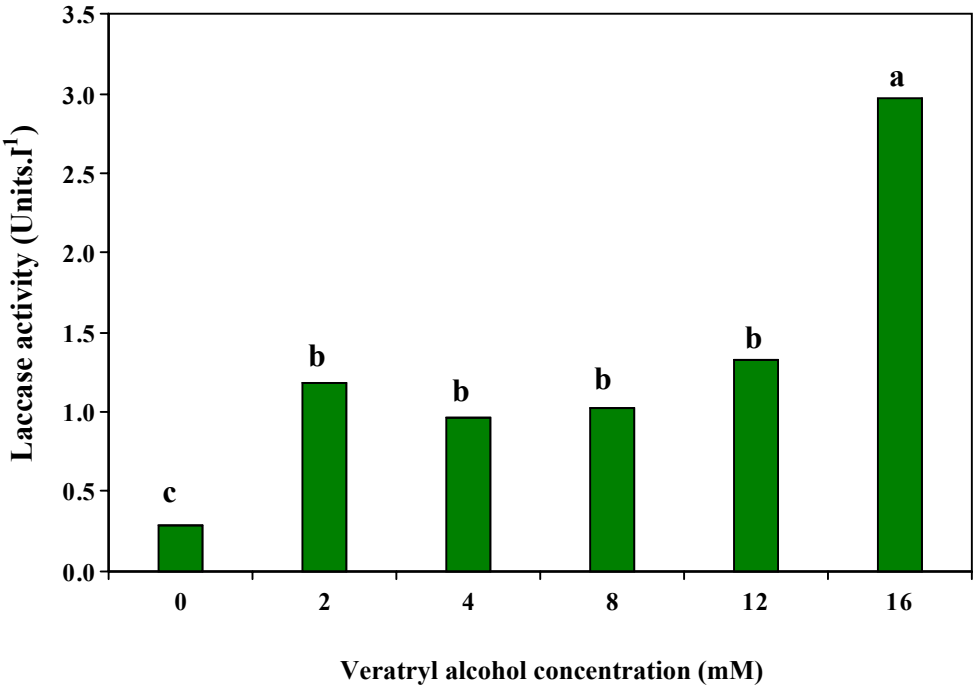

Figure 3. Laccase activity produced by *P. sanguineus* after 10 days using different concentrations of veratryl alcohol in a mineral salts medium. Bars with the same letter do not differ significantly ($p \leq 0,05$) with Tukey's Test.

At the broader substrate range, 16 mM veratryl alcohol resulted in significantly higher laccase activity than with any other concentration (Figure 4). The activity (3,99 Units.ml^{-1}) observed with veratryl alcohol was similar to that observed by Barbosa *et al*. (1996) in their study of laccase production by the Ascomycete, *Botryosphaeria* sp.

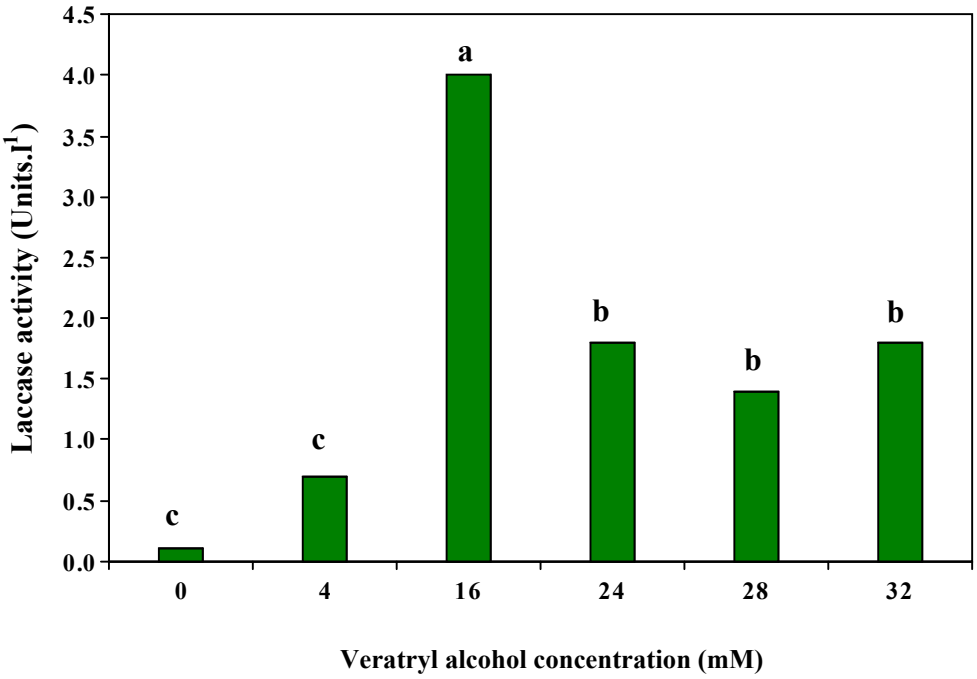

Figure 4. Laccase activity produced by *P. sanguineus* after 10 days using different concentrations of veratryl alcohol in a mineral salts medium.

Bars with the same letter do not differ significantly ($p \leq 0,05$) with Tukey's Test.

There was no significant difference in the amounts of biomass obtained from any of the veratryl alcohol induced cultures in the first trial (Figure 5). High concentrations of veratryl alcohol appear to be toxic to the fungus, as significantly lower concentrations of biomass were obtained at high concentrations of veratryl alcohol during the second trial (Figure 6). The laccase activity obtained with 16 mM veratryl alcohol was considerably higher in the second trial than the first trial (Figures 3 & 4). This can be ascribed to the higher concentration of biomass obtained at the same concentration during the second trial (Figures 5 & 6). The higher concentrations of biomass at 16 mM veratryl alcohol are probably due to problems encountered with the production of a consistent inoculum size.

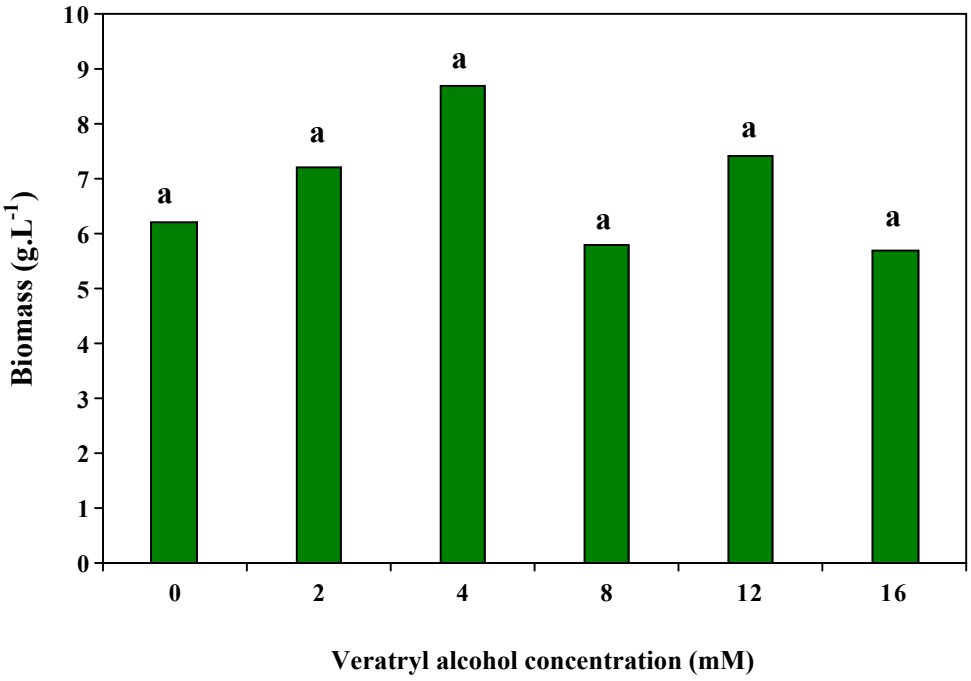

Figure 5. **Biomass produced by *P. sanguineus* after 10 days using different concentrations of veratryl alcohol in a mineral salts medium.**
Bars with the same letter do not differ significantly ($p \leq 0,05$) with Tukey's Test.

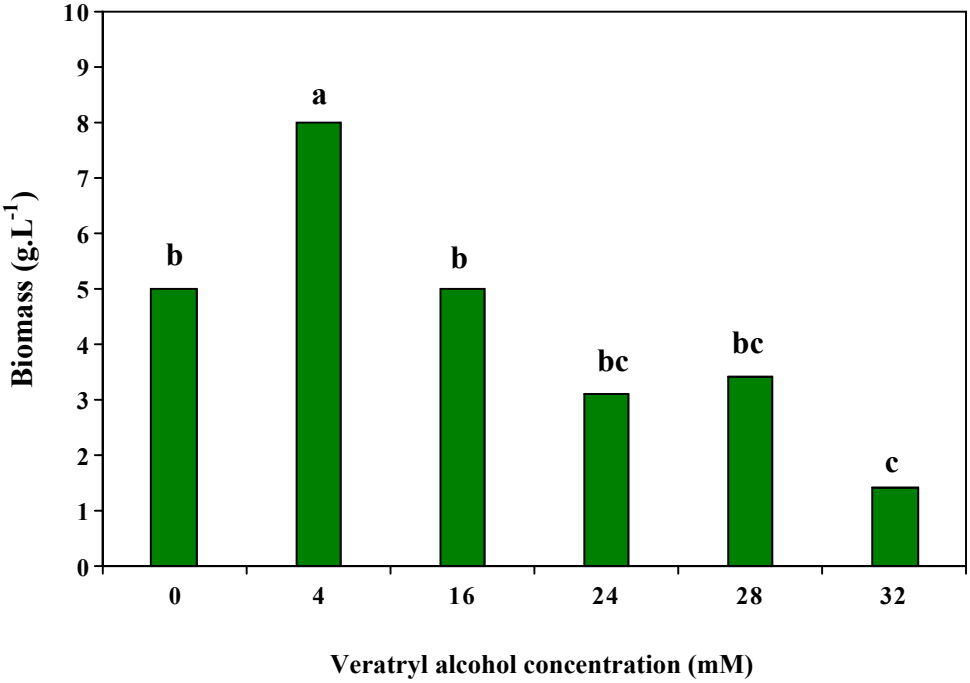

Figure 6. **Biomass produced by *P. sanguineus* after 10 days using different concentrations of veratryl alcohol in a mineral salts medium.**
Bars with the same letter do not differ significantly ($p \leq 0,05$) with Tukey's Test.

Influence of xylidene on laccase production

Xylidene did not have any significant effect ($p \leq 0,05$; Tukey's Test) on the laccase-producing ability of *P. sanguineus* (SCC 108). There was also no significant difference ($p \leq 0,05$) in the biomass concentrations obtained (Table 1). Although Pointing *et al.* (2000) successfully used xylidene as inducer with *P. sanguineus* (CY 788), xylidene had no inducing effect on *P. sanguineus* (SCC 108) in this study.

Table 1. Laccase and biomass produced by *P. sanguineus* after 10 days using different concentrations of xylidene in a mineral salts medium at 25 °C.

Xylidene concentration (μM)	Laccase activity (Units.ml^{-1})	Biomass (g.l^{-1})
0	0.125	6.9
4	0.202	7.3
7	0.121	7.1
10	0.128	6.6
20	0.213	6.3
30	0.199	7.7

CONCLUSIONS

Molasses offered a very cost-effective medium for the production of laccase and the addition of 2 % ethanol had a significant inducing effect on laccase production. Ethanol is less expensive and environmentally safer than most of the other potential inducers and, therefore, provides an option for more economical production of laccase.

Xylidene is generally regarded as the most effective inducer of laccase activity (Lee *et al*., 1999) and veratryl alcohol was used by Pointing *et al.* (2000) to induce

laccase activity in a *Pycnoporus sanguineus* strain. Xylidene had no effect on the ability of *P. sanguineus* (SCC 108) to produce laccase, but 16 mM veratryl alcohol induced higher laccase activity in the mineral salts medium. As the fungus seemed unresponsive to xylidene, further study will focus on the use of veratryl alcohol. The use of both ethanol and veratryl alcohol should be evaluated as inducers of laccase during cultivation of *P. sanguineus* in bioreactors.

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CHAPTER 6

BATCH CULTIVATION OF *PYCNOPORUS SANGUINEUS* FOR LACCASE PRODUCTION

15-L Braun bioreactor

ABSTRACT

Pycnoporus sanguineus (SCC 108) was selected for laccase production after extensive screening trials. Both a molasses medium and a mineral salts medium with 16 mM veratryl alcohol supported high laccase production in conical flasks. Very little is, however, known about the influence of different cultivation parameters on laccase production in fermentors. The aim of this study was to evaluate the influence of these parameters on growth and laccase production. In 2-l bioreactors, dense fungal growth occurred on the interior surface areas. This problem was overcome when a 15-l reactor was used. This may have been due to differences in reactor configuration and consequential differences in shear stress. The use of a mineral salts medium with 16 mM veratryl alcohol at a dissolved oxygen tension of 40 % of saturation, an uncontrolled pH and 25 °C proved to be the best set of conditions for high laccase production. It was also found that the inoculum size influenced the cultivation time before peak laccase activity. At this point it seems as if laccase production may be initiated by low pH levels.

INTRODUCTION

Laccase (benzenediol:oxygen oxidoreductase; EC 1.10.3.2) plays an important role in natural delignification processes (Call & Mücke, 1997). Laccase can also be applied in biobleaching through the laccase mediator system (Poppius-Levlin *et al*., 1997). Many white-rot fungi, most from the subphylum Basidiomycotina, produce laccases (Pointing *et al*., 2000). *Pycnoporus sanguineus* (L.:Fr.) Murr. is widely distributed on wood in tropical forests and is associated with severe white rot (Pointing *et. al*., 2000). White-rot fungi produce the laccase enzyme constitutively, but higher concentrations can be induced by certain cultivation factors (Aurora & Gill, 2000), such as dissolved oxygen tension, feedstock composition and concentration and the culture size (Burla *et al.*, 1992). *Pycnoporus sanguineus* (SCC 108) was selected as a suitable strain for further study based on its ability to produce high titres of thermostable laccase with a high optimum temperature (Chapters 2 & 3).

There are limitations to the use of shake-flasks in determining the growth kinetics of fungi. Limited information is available on submerged cultivation of *P. sanguineus* (Pointing *et al.*; 2000). The aim of this study was to investigate the influence of different parameters on the growth of and laccase production by *P. sanguineus* (SCC 108) during batch cultivation in 2-l and 15-l bioreactors under controlled conditions of dissolved oxygen tension, temperature and pH.

MATERIALS AND METHODS.

Fungal strain

Cultures of *Pycnoporus sanguineus* (SCC 108) were maintained on 2 % malt extract agar (Biolab, Midrand, South Africa) plates at 4 °C.

Analytical procedures

In all experiments, the laccase activity was determined spectrophotometrically at 418 nm using 2,2′-azinobis (3-ethylbenzthiazoline-6-sulphonate) (ABTS) (Aldrich, Steinheim, Germany) as substrate at pH 4,5 and 25 °C (Xu, 1996). The biomass was washed with distilled water and determined gravimetrically by filtration through 4,7 cm glass fibre filters (MN GF-1, Macherey-Nagel, Düren, Germany) under vacuum and drying to constant mass at 105 °C.

Determination of the sugar concentration

The sugar content in the samples was determined using a Sugar Analyser I high performance liquid chromatograph (Waters Associates, Milford, Massachusetts, USA) with an Aminex® Carbohydrate HPX 42C column (Bio-Rad, Richmond California, USA). Double distilled water served as the mobile phase and a differential refractometer (R401, Waters Associates) was used as detector.

Inoculum preparation

The fungal inoculum was prepared as described previously (Chapter 2) and the suspension of fragmented mycelium aseptically transferred to the 2-l and 15-l bioreactors.

Cultivation media

Diluted molasses and mineral salts media that were previously shown to support a high laccase activity or biomass production (Chapter 4) were used in this study. The molasses medium contained ($g.l^{-1}$): molasses, 40,0; citric acid as a chelating agent, 0,25 and 1 $ml.l^{-1}$ of a trace elements solution (Du Preez & Van der Walt, 1983). The mineral salts medium consisted of the following components ($g.l^{-1}$): Sucrose, 20,0; yeast extract (YE), 3,0; $(NH_4)_2SO_4$, 4,50; KH_2PO_4, 3,0; $MgSO_4.7H_2O$, 1,00; $CaCl_2.2H_2O$, 0,05; citric acid, 0,25 and 1,0 $ml.l^{-1}$ of the trace elements solution. The "final molasses" used in this trial was obtained from Transvaal Sugar Ltd, Malelane, South Africa. All media were adjusted to pH 5,5 with 3 N H_2SO_4 and 3 N KOH prior to inoculation.

Where indicated, veratryl alcohol (3,4-dimethoxybenzyl alcohol) (Fluka, Buchs, Switzerland) was used as an inducer (Barbosa *et al*., 1996; Vasconcelos *et al*., 2000) at a final concentration of 16 mM, as determined in Chapter 5. It was aseptically added to the medium 24 h after inoculation due to the potential toxicity of this compound (Koroljova-Skorobogat'ko *et al*., 1998).

Cultivation conditions

The growth of and laccase production by *P. sanguineus* (SCC 108) was initially evaluated in a Multigen F-2000 2-l bioreactor (New Brunswick Scientific Co., Edison, New Jersey, USA) fitted with three disk turbine impellers and using a working volume of 1,2 l. The reactors were autoclaved for 20 min prior to inoculation with 60 ml of the mycelial suspension. The dissolved oxygen tension (DOT) was monitored with a polarographic pO_2 electrode (Mettler Toledo, Urdorf, Switzerland)

and maintained at 40 % of saturation by manually controlling the stirring speed and aeration rate. Specific conditions of cultivation are presented in Tables 1 & 2.

A 15-l Biostat C bioreactor (B. Braun Biotech International, Melsungen, Germany) fitted with two disk turbine impellers and a single marine propeller, using a working volume of 8 l, was used for subsequent cultivations. The reactor was sterilised *in situ* for 20 min. at 121 °C prior to inoculation with 300 ml of the mycelial suspension. The DOT was monitored as above and automatically controlled at the desired level by variation of the aeration rate using a mass flow controller. The stirrer speed was maintained at 250 $r.min^{-1}$ during all cultivations. Specific conditions of cultivation are presented in Tables 3, 4 & 5.

In both reactors the pH was monitored with a pH electrode (Mettler Toledo) and, when desired, controlled by automatic titration with 3 N H_2SO_4 and 3 N KOH. The reactors were operated in batch mode and samples were aseptically removed every 12 h for analysis.

RESULTS AND DISCUSSION

Cultivation in 2-l bioreactors

The severe wall growth that occurred in the 2-l bioreactors rendered the determination of the culture parameters inaccurate and the experimental results questionable due to the non-homogeneous cultures. The data suggested that 25° C was more suitable for growth and laccase production than 30 °C and 35 °C (Table 1). In the cultivations without pH control (Figure 1) the pH decreased from the initial pH 5,5 to below pH 3,0.

Table 1. The influence of cultivation temperature and pH on the growth of and laccase production by *P. sanguineus* during batch cultivation in a 2-l bioreactor using a 4 % molasses medium.

Temperature (°C)	pH	μ_{max} [a] (h^{-1})	Biomass ($g.l^{-1}$)	Laccase ($U.ml^{-1}$)	Q_p [b] ($U.ml^{-1}.h^{-1}$)
25	4,5	0,02	9,7	0,192	0,064
30	4,5	0,02	10,9	0,080	0,036
35	4,5	0,01	9,0	0,042	0,023
25	3,5	0,03	7,6	0,334	0,094
25	5,5	0,04	7,6	0,085	0.000
25	uncontrolled	0,03	11,4	0,460	0,364

a μ_{max}: The maximum specific growth rate determined from the exponential region of the plot of biomass versus time.

b Q_p: Maximum volumetric laccase production rate, determined from the maximum slope of the plot of laccase activity versus time.

The highest growth rate was recorded at 25 °C and pH 5,5. The highest final biomass concentration was found at 25 and 30 °C at ph 4,5 as well as with no pH control (Table 1). The highest laccase activity and production rate was observed at 25 °C with no pH control (Table 1). In general, the suspended biomass in the 2-l reactors occurred as pellets of approximately 5 mm in diameter.

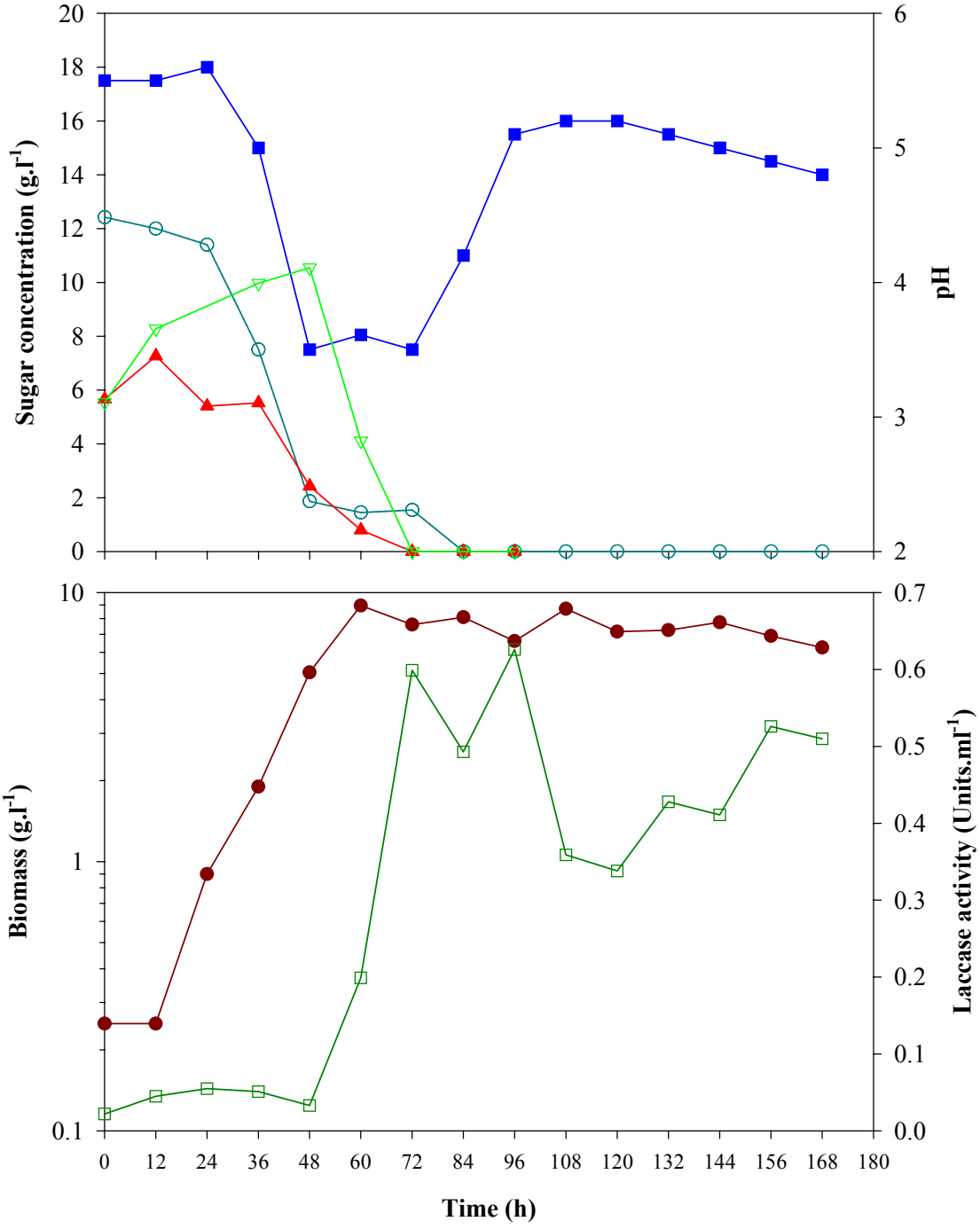

Figure 1. **Growth of and laccase production by *P. sanguineus* at 25 °C in a 15-l bioreactor with a 4 % molasses medium and pH levels controlled between pH 5,5 and pH 3,5. Symbols: biomass (●), laccase activity (□), pH (■), glucose (▲), sucrose (○), and fructose (▽).**

Cultivation in a 15-l bioreactor

Because cultivation without pH control appeared to be beneficial in terms of both laccase production and growth (Table 1), the pH was allowed to vary within a certain range. As 25 °C seemed to support both high laccase production and growth of the fungus (Table 1), all the cultivations in the 15-l reactor were performed at 25 °C.

The influence of different pH profiles on the growth of and laccase production by *P. sanguineus* in 4 % molasses was evaluated. Generally laccase activity increased after a 48 h cultivation period and the stationary growth phase was reached after 60 h (Figure 1). The fungus had the ability to hydrolyse sucrose and preferentially assimilated glucose (Figure 1). This resulted in a temporary accumulation of fructose that was consumed when the glucose concentration had decreased to below a certain threshold value. In all the cultivations in molasses medium without pH control, the pH decreased after 36 h and again started to increase after 72 h to 84 h (Figure 1). The highest final biomass concentration (8,95 $g.l^{-1}$) was obtained when the pH was allowed to fluctuate only between pH 5,5 to pH 3,5 (Table 2). The highest laccase activity (1,154 $Units.ml^{-1}$) and maximum specific growth rate (0,068 h^{-1}) was observed under conditions of no pH control (Table 2), whereas the highest volumetric production rate of laccase activity (0,094 $Units.ml^{-1}.h^{-1}$) was observed when the pH was allowed to naturally decrease from pH 5,5 and subsequently controlled at pH 3,5 upon reaching that level (Table 2).

Table 2. Influence of different pH profiles on the growth of and laccase production by *P. sanguineus* during batch cultivation in a 15-l bioreactor using a 4 % molasses medium at pH 4,5, 25 °C and a DOT of 40 %.

pH	μ_{max} [c] (h^{-1})	Biomass ($g.l^{-1}$)	$Y_{x/s}$ [d]	Laccase ($U.ml^{-1}$)	Q_p [e] ($U.ml^{-1}.h^{-1}$)
No pH control	0,068	5,53	0,255	1,154	0,064
5,5	0,054	7,79	0,453	0,286	0,036
Between pH 5,5 & 3,5 [a]	0,065	8,95	0,405	0,626	0,023
Decreased from pH 5,5 to 3,5 [b]	0,047	7,25	0,423	0,429	0,094

a pH was allowed to naturally fluctuate only between pH 5,5 to pH 3,5.

b Controlled at pH 5,5 after naturally decreasing from 5,5.

c μ_{max}: The maximum specific growth rate determined from the exponential region of the plot of biomass versus time.

d $Y_{x/s}$: Biomass yield, expressed as biomass produced per gram sucrose assimilated.

e Q_p: Maximum volumetric laccase production rate, determined from the maximum slope of the plot of laccase activity versus time.

In experiments to determine the influence of different DOT profiles on laccase production by and growth of *P. sanguineus,* the final biomass concentration and rates of laccase production were lower (Table 3) than the previous results (Table 2). Due to the variability of results obtained in the molasses medium, no clear conclusion could be drawn regarding the effect of the DOT. A mineral salts medium with 16 mM veratryl alcohol as inducer (as determined in Chapter 4 & 5) was used in further trials.

Table 3. Influence of different DOT levels on the growth of and laccase production by *P. sanguineus* during batch cultivation in a 15-l bioreactor at 25 °C and an uncontrolled pH in 4 % molasses medium.

DOT [a] % of saturation	μ_{max} [b] (h^{-1})	Biomass ($g.l^{-1}$)	Laccase ($U.ml^{-1}$)	Q_p [c] ($U.ml^{-1}.h^{-1}$)
40	0,080	3,13	0,205	0,008
5	0,065	3,64	0,140	0,006
1	0,036	4,36	0,613	0,006

a DOT: Dissolved oxygen tension.

b μmax: The maximum specific growth rate determined from the exponential region of the plot of biomass versus time.

c Q_p: Maximum volumetric laccase production rate, determined from the maximum slope of the plot of laccase activity versus time.

In the mineral salts medium, cultivation without pH control and without the inclusion of an inducer resulted in the highest final biomass concentration (Table 4). The highest growth rate was obtained at a controlled pH 5,5 (Table 4). A relatively high laccase activity (3,41 Units.ml^{-1}) was observed with the use of 16 mM veratryl alcohol under conditions of low dissolved oxygen tension (Figure 2). Although this high laccase activity was at first thought to be triggered by the low dissolved oxygen levels, a higher laccase activity of 7,82 Units.ml^{-1} was reached after 132 h at a DOT of 40 % (Figure 3). These results were repeatable in a cultivation under similar conditions that resulted in a laccase activity of 8,13 Units.ml^{-1} after 156 h (Figure 4). The difference of 24 h before laccase production peaked, was probably due to differences in the amount of biomass present in the inoculum (Figures 3 & 4). In comparison with the molasses medium, the pH of the mineral salts medium decreased at a slower rate. This effect can possibly be ascribed to the slower growth observed in the mineral salts medium. During all the cultivations in the 15-l reactor, the fungus grew as pellets of less than 1,5 mm in diameter.

The experimental culture data are presented in Appendix B.

Table 4. Influence of different pH profiles and 16 mM veratryl alcohol on the growth of and laccase production by *P. sanguineus* (SCC 108) during batch cultivation in a 15-l bioreactor at 25 ° C using a mineral salts medium.

Cultivation conditions			μ_{max} [c]	Biomass	$Y_{x/s}$ [d]	Laccase	Q_p [e]
pH	**DOT [a]**	**Veratryl alcohol (mM)**	**(h^{-1})**	**($g.l^{-1}$)**		**($U.ml^{-1}$)**	**($U.ml^{-1}.h^{-1}$)**
uncontrolled	40 %	none	0,045	5,66	0,354	0,316	0,010
5,5	40 %	16	0,094	5,18	0,323	0,234	0,008
uncontrolled	40 % to 0,5 %[b]	16	0,052	3,95	0,282	3,410	0,082
uncontrolled	40 %	16	0,076	4,13	0,256	7,816	0,237
uncontrolled	40 %	16	0,063	4,24	0,280	8,131	0,168

a DOT: Dissolved oxygen tension.

b The DOT was maintained at ≥ 40 % saturation for the first 48 h where after it was decreased to 0,5 % saturation by decreasing the aeration rate.

c μ_{max}: The maximum specific growth rate determined from the exponential region of the plot of biomass versus time.

d $Y_{x/s}$: Biomass yield, expressed as biomass produced per gram sucrose assimilated.

e Q_p: Maximum volumetric laccase production rate, determined from the maximum slope of the plot of laccase activity versus time.

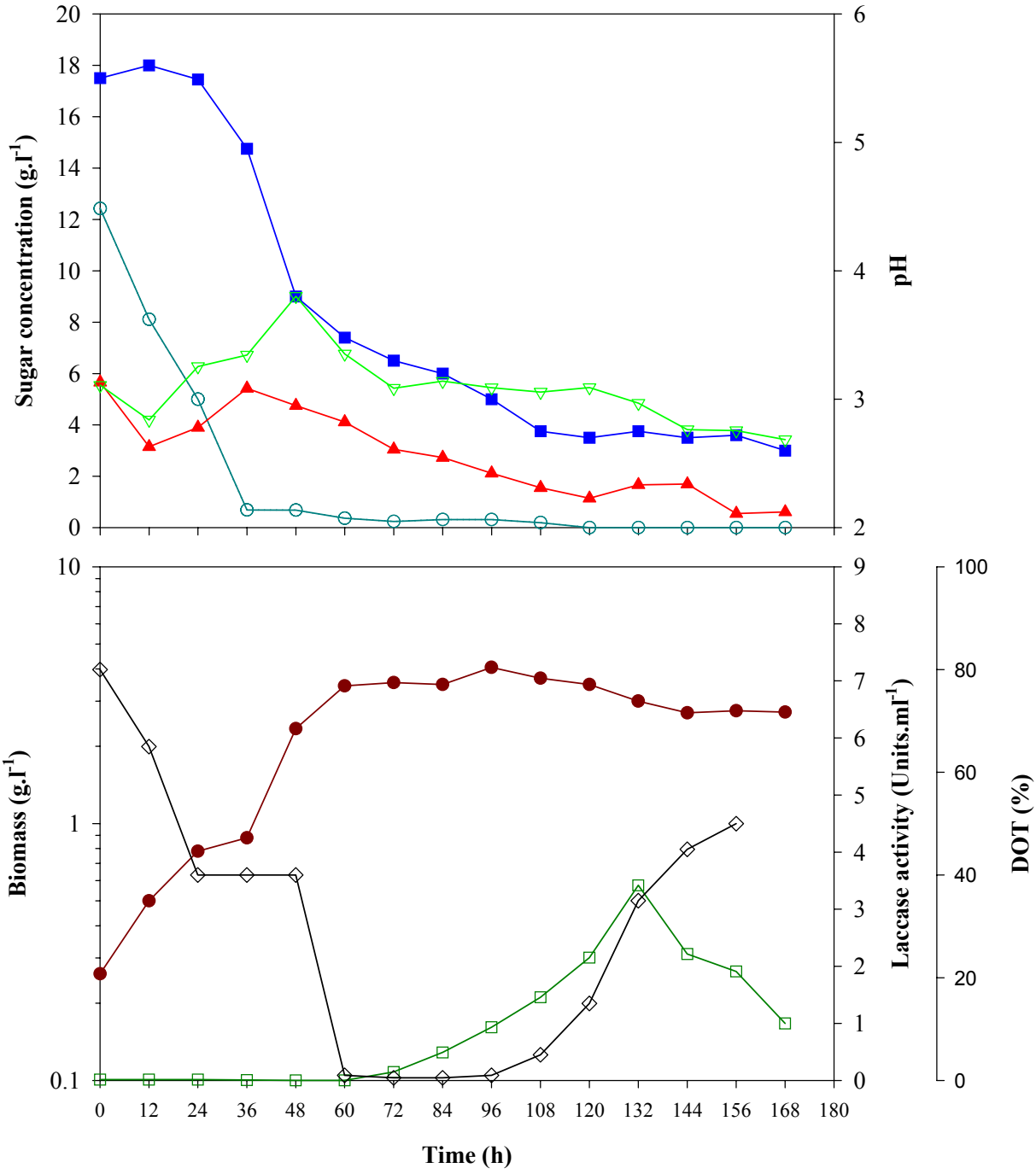

Figure 2. **Growth of and laccase production by *P. sanguineus* in mineral salts medium with 16 mM veratryl alcohol at 25 °C and without pH control. The DOT was controlled at 40 % saturation for the first 48 h and then decreased to 0,5 % by decreasing the aeration rate. Symbols: biomass (●), laccase activity (□), DOT (◇), pH (■), glucose (▲), sucrose (○) and fructose (▽).**

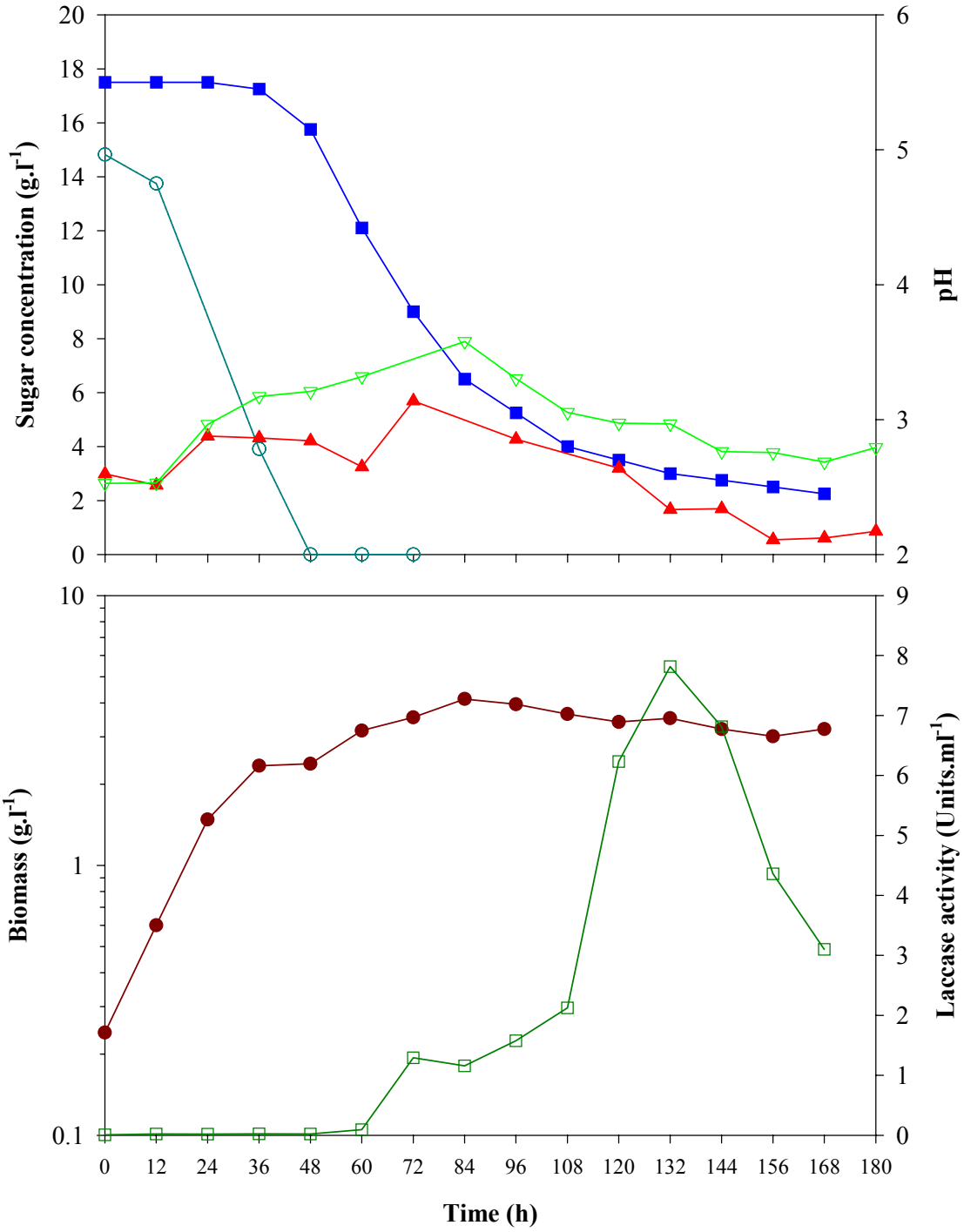

Figure 3. **Growth of and laccase production by *P. sanguineus* in mineral-salts medium with 16 mM veratryl alcohol, at 25 °C and without pH control. The DOT was controlled at 40 % saturation by automatic regulation of aeration rate. Symbols: biomass (●), laccase activity (□), pH (■), glucose (▲), sucrose (○), and fructose (▽).**

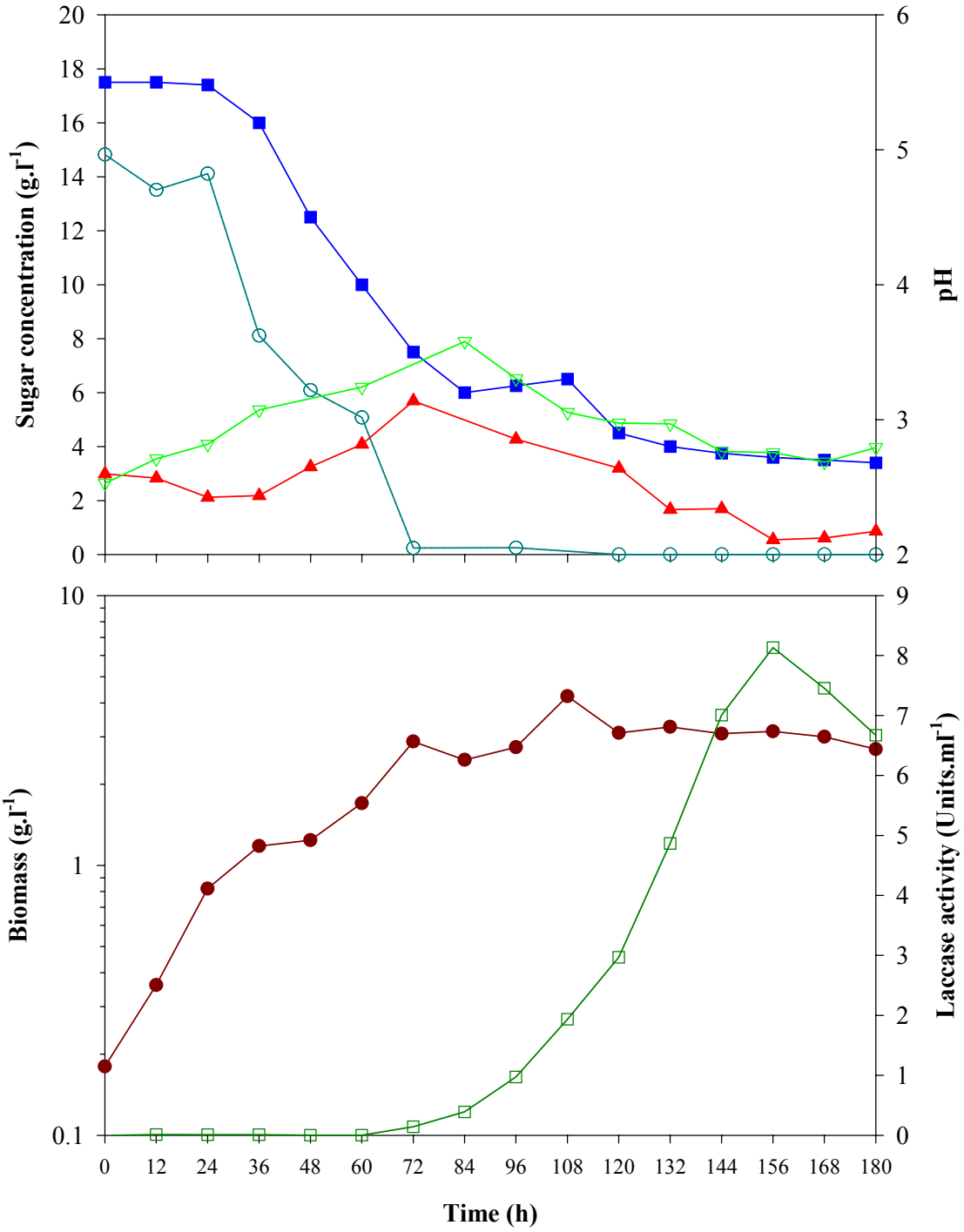

Figure 4. **Growth of and laccase production by *P. sanguineus* in mineral-salts medium with 16 mM veratryl alcohol, at 25 °C and without pH control. The stirrer speed was 250 r.min^{-1} and the DOT controlled at 40 % saturation by automatic regulation of aeration rate. Symbols: biomass (●), laccase activity (□), pH (■), glucose (▲), sucrose (○), and fructose (▽).**

CONCLUSIONS

There are few studies using *Pycnoporus* species for the production of laccase. There have, however, been reports of only a few strains capable of producing laccase activities higher than recorded here using *P. sanguineus* (SCC 108). The laccase activity obtained with *P. sanguineus* (SCC 108) in this study was eight-fold higher than the values reported in a study by Pointing *et al.* (2000) with *P. sanguineus* (CY 788). These authors were successful in employing xylidene as inducer and had only limited success with veratryl alcohol. Xylidene had no inducing effect on *P. sanguineus* in this study (Chapter 5), but high titres of laccase were observed after the addition of veratryl alcohol. The use of a mineral salts medium with 16 mM veratryl alcohol at a dissolved oxygen tension of 40 % of saturation, with no pH control and at 25 °C proved to be the best set of conditions for high laccase production, whereas the highest growth rate was found at a controlled pH of 5,5.

The composition of medium, pH control and inducer are of particular importance for obtaining high titres of laccase activity (Arora & Gill, 2000; Homolka *et al*., 1997). The cultivation of *P. sanguineus* (SCC 108) in 2-l bioreactors proved to be problematic due to severe wall growth. However, in a 15-l reactor wall growth was minimal. Different process parameters, e.g. stirrer speed, reactor type and medium composition, are known to influence the growth and morphology of fungi and their product formation (Schügerl *et al.,* 1997). This was evident from the different pellet sizes obtained in the different reactors as well as the varying amounts of biomass and laccase activity produced by *P. sanguineus* under different cultivation conditions.

It appears that a low pH level may trigger laccase production, since none of the cultivations at a controlled pH of 5,5 resulted in significant laccase activities. The pH in the mineral salts medium decreased slower than in the diluted molasses during cultivation due to the slower growth rates obtained in the mineral salts medium. This could explain why laccase production in molasses commenced earlier in the cultivation than in the mineral salts medium.

Molasses is a very complex substrate (Macgillivray & Matic, 1970) and possibly contained a natural inducer that allowed the production of high laccase activities without the inclusion of artificial inducers. Further investigation of this feedstock and its inducing effect on laccase activity is required, because of the inconsistent results obtained in this investigation with a molasses-based medium.

The inoculum is another factor that should also be studied further, as variation in inoculum size influenced the time required for peak laccase production. Fåhraeus & Reinhammar (1967) also observed faster laccase production in cultures with a large inoculum size and emphasized the influence of this parameter.

Although high titres of laccase activity were observed during this study, more attention should be given to the mechanism that triggers laccase production and the development of a technique for the production of a less variable inoculum. Although the use of molasses gave variable results in the bioreactor cultivations, this substrate constitutes a low cost feedstock for the large-scale production of laccase.

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

One of the factors limiting the large-scale application of laccase is the lack of capacity to produce large volumes of highly active enzyme (Galhaup *et al.*, 2001). Different strains of white-rot fungi react differently to the environmental factors influencing the ability of these fungi to produce high titres of laccase (Robene-Soustrade & Lung-Escarmant, 1997). During the present study the aim was to select a strain with the ability to produce high titres of laccase and then to optimise the cultivation conditions for improved laccase production. A repeatable, inexpensive and rapid screening technique was developed to evaluate white-rot fungi for laccase production. The screening trial was successful and strains were identified that were comparable to or better than the reference strain (*C. versicolor* ATCC 20869).

The ability to produce high titres of laccase activity was not the sole factor in considering a strain for commercial laccase production. A number of strains that produced laccases with thermostability up to 65 °C were identified. *Pycnoporus sanguineus* (SCC 108) was identified as the most suitable strain due to its ability to produce high titres (1,5 to 3,3 Units.ml^{-1}) of a laccase that was thermostable at 65 °C. The high optimal temperature of the laccase produced by *P. sanguineus* (SCC 108) is a benefit that could require less modification in order to incorporate it into current pulp bleaching operations.

Molasses contains high levels of sucrose, glucose and fructose and was found to be a cost-effective feedstock for cultivation of fungi, as it also contains nitrogen, mineral salts and trace elements (Macgillivray & Matic, 1970). High production of laccase activity was obtained using molasses as a cultivation medium without the inclusion of any inducers. This substrate was, therefore, further evaluated and compared to other inducer-containing media. Due to the variability in results obtained during trials a mineral salts media was evaluated and compared to molasses. The inclusion of veratryl (16 mM) alcohol induced the highest laccase activity in the mineral salts medium.

Pycnoporus species were used in only a few studies for the production of laccase and reports indicated that only a few strains were capable of producing laccase activities higher than recorded here with *P. sanguineus* (SCC 108) (Pointing *et al.,* 2000). Laccase activity obtained with *P. sanguineus* (SCC 108) was eight times higher than the values reported in by Pointing *et al.* (2000) with *P. sanguineus* (CY 788). Pointing *et al.* (2000) were unsuccessful in employing veratryl alcohol as inducer and only had success with xylidene. In the present study, xylidene had no inducing effect on *P. sanguineus*, but induction of high titres of laccase activity were observed with the addition of veratryl alcohol. The best production of laccase in submerged cultivation was achieved with the use of a mineral salts medium with 16 mM veratryl alcohol at a dissolved oxygen tension of 40 % of saturation, with no pH control and at 25 °C, whereas the highest growth rate was found at a controlled pH of 5,5. Indications are that laccase production is initiated by low pH levels, since none of the cultivations at a controlled pH of 5,5 resulted in production of high laccase activities.

In future more attention should be given to the mechanisms that induces laccase production and production of constant inoculum size and concentration. Although the use of molasses gave variable results in the bioreactor cultivations, this substrate should be considered a low-cost feedstock for the large-scale production of laccase.

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SUMMARY

KEYWORDS: Laccase, white-rot fungi, optimisation, batch cultivation, inducers, *Pycnoporus sanguineus,* phenoloxidase, enzyme, biomass.

Many efforts have been made to utilise enzymes for the degradation of lignin in paper pulp. One enzyme known to play a major role in delignification is laccase (EC 1.10.3.2; benzenediol:oxygen oxidoreductase). Laccase can potentially be applied in biobleaching processes through the laccase mediator system. White-rot fungi are well-known producers of laccase, but little is known about submerged cultivation of these fungi. It is, therefore, necessary to determine the influence of various parameters on laccase and biomass production by these fungi when grown in submerged culture.

The aim of this study was firstly to select fungal strains capable of producing high titres of thermostable laccase and secondly to optimise conditions for laccase production. Thirdly, the aim was to determine the kinetics for growth of and laccase production by *Pycnoporus sanguineus* (L.:Fr.) Murr. (SCC 108).

The ability of most fungi in our culture collection to produce lignin-degrading enzymes was determined previously. These results were, however, qualitative and the activity and quantity of laccases produced by these fungi had to be determined. Laccase-producing strains as well as reference strains and negative controls were included in quantitative screening experiments to identify hypersecretory strains. Enzyme activity was determined spectrophotometricaly using 2,2′-azinobis(3-ethylbenzthiazoline-6-sulphonate) as substrate. Sixteen strains and a reference strain,

Coriolus versicolor (Wulf.: Fr.) Quél. (ATCC 20869) were selected for further evaluation of thermostability and optimum temperature of the laccases. Some laccases in the crude extract displayed stability at temperatures as high as 65 °C. At 75 °C, the laccases from *P. sanguineus* (SCC 108), *P. sanguineus* (SCC 207) and the unidentified strain (SCC 260) exhibited half-lives of more than 30 minutes.

Molasses was a suitable medium for the cultivation of *P. sanguineus* and the production of laccase since high laccase activity and biomass were obtained in shake flasks with the use of 4-% molasses, even without any pre-treatment. Molasses contained compounds which resulted in enhanced laccase production when compared to mineral salts media with equivalent amounts of nutrients. Molasses could be considered as a low-cost carbon feedstock for the cultivation of *P. sanguineus,* but lacked some supplements for optimal laccase production. Increased laccase activity was obtained with the inclusion of certain xenobiotic components in both molasses and a mineral salts medium. High carbon to nitrogen ratios improved the production of laccase.

When the fungus was cultivated in a 15-l bioreactor the stationary growth phase of the fungus was reached after more than 60 h of cultivation. Laccase activity increased after 48 h, indicating that laccase production started in the exponential phase of growth and continued into the stationary phase. The cultivation of *P. sanguineus* in a mineral salts medium with 16 mM veratryl alcohol without pH control and dissolved oxygen tension at 40 % resulted in the highest laccase production (8,131 Units.ml^{-1}). Indications are that laccase production is activated by low pH values.

This study of the cultivation of *P. sanguineus* has contributed to our understanding of the physiology and laccase production by white-rot fungi. These results could be applied in industrial processes for laccase production and may also be relevant to laccase production by other strains of white-rot fungi.

OPSOMMING

SLEUTELWOORDE: Lakkase, witvrotfungi, optimalisering, lotkweking, induseerders, *Pycnoporus sanguineus,* phenoloksidase, ensiem, biomassa.

Talle pogings is reeds aangewend om ensieme vir die afbreking van lignien in papierpulp te gebruik. Een ensiem wat bekend is vir die belangrike rol wat dit in die afbreking van lignien speel, is lakkase (EC 1.10.3.2; benseendiol:suurstof oksidoreduktase). Lakkase kan moontlik aangewend word in biobleikprosesse deur middel van die lakkase-mediatorstelsel. Witvrotfungi is welbekende produseerders van lakkase, maar daar is min bekend oor die kweking van hierdie fungi in vloeistofkultuur. Dit is, daarom, noodsaaklik om die uitwerking van verskillende parameters op lakkase en biomassaproduksie deur hierdie fungi vas te stel, wanneer hulle in vloeistofkultuur gekweek word.

Die doel van hierdie studie was eerstens om fungus isolate te kies wat in staat is om hoë konsentrasie van hittestabiele lakkase te produseer en tweedens om die toestande vir die vervaardiging van lakkase te optimaliseer. Derdens was die doel om die kinetika vir die groei van en lakkasevervaardiging deur *Pycnoporus sanguineus* (L.:Fr.) Murr. (SCC 108) vas te stel.

Die vermoë van die meeste fungi in ons kultuurversameling om lignienafbrekende ensieme te vervaardig, is voorheen bepaal. Hierdie resultate was egter kwalitatief en die aktiwiteit en hoeveelheid lakkases wat deur hierdie fungi vervaardig is, moes bepaal word. Isolate wat lakkase vervaardig sowel as verwysingsisolate en negatiewe kontroles is by die kwantitatiewe

siftingseksperimente ingesluit om hiperproduserende isolate te identifiseer. Ensiemaktiwiteit is spektrofotometries bepaal deur van 2,2' –azinobis (3-etielbenstiazolien-6-sulfonaat) as substraat gebruik te maak. Sestien isolate en 'n verwysingsisolaat, *Coriolus versicolor* (Wulf.:Fr.) Quél. (ATCC 20869) is gekies om die hittestabiliteit en optimale temperature van die lakkase verder te evalueer. Sommige van die lakkases in die rou ekstrak het stabiliteit by temperature so hoog soos 65 °C vertoon. By 75 °C het die lakkase van *P. sanguineus* (SCC 108), *P. sanguineus* (SCC 207) en die ongeidentifiseerde isolaat (SCC 260) half-leeftye van meer as 30 min. vertoon.

Molasse was 'n geskikte medium vir die kweking van *P. sanguineus* en die vervaardiging van lakkase aangesien 'n hoë vlak van lakkase-aktiwiteit en biomassa in skudflesse verkry is met 4 % molasse, selfs sonder enige voorafbehandeling. Molasse het bestandele bevat wat verhoogde lakkasevervaardiging tot gevolg gehad het indien dit met minerale soutmedia met gelykstaande hoeveelhede voedingstowwe vergelyk word. Molasse kon as 'n lae koste koolstofvoedingsbron vir die kweek van *P. sanguineus* beskou word, maar daar was 'n tekort aan sommige aanvullings vir optimale lakkasevervaardiging. Verhoogde lakkase-aktiwiteit is verkry deur die insluiting van sekere xenobiotiese bestanddele in beide die molasse en 'n mineralesout medium. Hoë koolstof- tot stikstofverhoudings het die produksie van lakkase verhoog.

Toe die fungus in 'n 15-l bioreaktor gekweek is, is die stasionêre groeifase daarvan na meer as 60 h se kweking bereik. Lakkase-aktiwiteit het na 48 h vermeerder, wat aandui dat lakkasevervaardiging in die eksponensiële groeifase begin

het en in die stasionêre fase voortgegaan het. Die kweking van *P. sanguineus* in ‘n mineralesout medium met 16 mM veratriel alkohol sonder pH-beheer en ‘n opgeloste suurstofspanning van 40 %, het die hoogste lakkasevervaardiging (8,13 Eenhede.ml^{-1}) tot gevolg gehad. Daar is aanduidings dat lae pH vlakke lakkase produksie aktiveer.

Hierdie studie van die kweking van *P. sanguineus* het bygedra tot ons kennis van die fisiologie en lakkasevervaardiging deur witvrotfungi. Hierdie resultate sou in industriële prosesse vir die vervaardiging van lakkase toegepas kon word en mag ook van toepassing wees by die lakkasevervaardiging van ander isolate van witvrotfungi.

APPENDIX A

Laccase activities in supernatants of fungal cultures.

Species	Strain nr.	Laccase activity (Units.ml^{-1})
Peniophora sp.	SCC 199	0.833 a
Pycnoporus sanguineus	SCC 108	0.798 a
Agaricus bisporus	SCC 173	0.710 b
Laetiporus sulphureus	SCC 180	0.511 c
Lenzites betulina	SCC 274	0.488 c
Lenzites betulina	SCC 20	0.443 d
Peniophora sp.	SCC 152	0.415 d
Pycnoporus sanguineus	SCC 126	0.414 d
Poria sp.	SCC 124	0.411 d
Lenzites betulina	SCC 29	0.380 d
Unidentified sp.	SCC 260	0.356 e
Coriolus hirsutus	SCC 79	0.350 e
Pycnoporus sanguineus	SCC 207	0.307 f
Coriolus versicolor	ATCC 20869	0.304 f
Pycnoporus sanguineus	SCC 94	0.295 f
Skeletocutis sp.	SCC 143	0.274 f
Hyphodontium sp.	SCC 155	0.270 f
Coriolus versicolor	SCC 221	0.252 g
Coriolus glabrescens	SCC 90	0.243 g
Coriolus versicolor	SCC 30	0.228 g
Pycnoporus coccineus	SCC 41	0.226 g
Lenzites betulina	SCC 10	0.222 g
Coriolus versicolor	SCC 176	0.217 g
Peniophora sp.	SCC 160	0.216 g
Pycnoporus sanguineus	SCC 253	0.213 g
Coriolus sp.	SCC 71	0.212 g
Skeletocutis sp.	SCC 121	0.210 g
Coriolus versicolor	SCC 139	0.205 g
Coriolus hirsutus	SCC 150	0.201 g
Lenzites betulina	SCC 88	0.201 g
Bjerkandera adusta	SCC 136	0.197 g
Lentinus velutinus	SCC 299	0.195 g
Coriolus hirsutus	SCC 2	0.193 g
Skeletocutis sp.	SCC 113	0.189 g
Trametes glabrescens	SCC 89	0.183 g
Hyphodontium sp.	SCC 154	0.180 g
Stereum hirsutum	SCC 153	0.165 h
Coriolus pubescens	SCC 44	0.155 h
Odontia sp.	SCC 103	0.151 h
Pycnoporus sanguineus	SCC 4	0.147 h
Coriolus versicolor	SCC 101	0.138 h
Skeletocutis sp.	SCC 115	0.137 h

Species	Strain nr.	Laccase activity (Units.ml^{-1})
Coriolus zonatus	SCC 53	0.137 h
Fomes sp.	SCC 290	0.117 h
Pycnoporus sanguineus	SCC 241	0.110 h
Phellinus Gilvus	SCC 135	0.105 i
Ceriporiopsis subvermispora	SCC 179	0.100 i
Stereum illudens	SCC 31	0.093 i
Phellinus sp.	SCC 147	0.093 i
Coriolus versicolor	SCC 59	0.090 i
Pycnoporus sanguineus	SCC 164	0.089 i
Phellinus sp.	SCC 130	0.087 i
Pycnoporus sanguineus	SCC 78	0.086 i
Pycnoporus coccineus	SCC 97	0.086 i
Stropharia rugosoannulata	SCC 175	0.086 i
Ceriporiopsis subvermispora	CZ-3	0.079 i
Pleurotus sajor-caju	SCC 172	0.078 i
Ganoderma lucidum	SCC 285	0.078i
Pycnoporus sanguineus	SCC 87	0.077 i
Coriolus versicolor	SCC 123	0.075 i
Skeletocutis sp.	SCC 159	0.073 i
Pleurotus pulmonarius	SCC 171	0.073 i
Coriolus hirsutus	SCC 278	0.066 i
Pycnoporus coccineus	SCC 81	0.065 i
Phellinus sp.	SCC 198	0.060 i
Bjerkandera adusta	SCC 80	0.058 i
Phellinus sp.	SCC 280	0.053 i
Coriolus sp.	SCC 118	0.047 i
Coriolus hirsutus	SCC 289	0.044 j
Unidentified sp.	SCC 215	0.042 j
Coriolus sp.	SCC 192	0.040 j
Pycnoporus sp.	SCC 190	0.039 j
Phellinus gilvus	SCC 83	0.039 j
Pycnoporus sanguineus	SCC 226	0.034 j
Lentinus stupeus	SCC 275	0.033 j
Lentinus villosus	SCC 231	0.032 j
Stereum illudens	SCC 222	0.030 j
Stereum hirsutum	SCC 27	0.028 j
Phellinus gilvus	SCC 141	0.027 j
Stereum australe	SCC 23	0.026 j
Ganoderma applanatum	SCC 111	0.025 j
Stereum hirsutum	SCC 9	0.023 j
Stereum hirsutum	SCC 213	0.022 j
Stereum hirsutum	SCC 102	0.022 j
Phellinus sp.	SCC 3	0.022 j
Stereum rimosum	SCC 25	0.020 j

Species	Strain nr.	Laccase activity (Units.ml^{-1})
Unidentified sp.	SCC 39	0.018 j
Stereum hirsutum	SCC 70	0.017 j
Chondrostereum purpureum	SCC 48	0.014 j
Schizophyllum commune	SCC 158	0.013 j
Stereum ostrea	SCC 304	0.013 j
Phellinus gilvus	SCC 163	0.013 j
Gloeophyllum sepiarium	SCC 186	0.010 j
Coriolus versicolor	SCC 148	0.010 j
Stereum australe	SCC 17	0.007 j
Fomitopsis lilacino-gilva	SCC 122	0.005 j
Gymnopilus sp.	SCC 242	0.005 j
Stereum ostrea	SCC 295	0.005 j
Stereum illudens	SCC 75	0.005 j
Hypholoma fasciculare	SCC 64	0.004 j
Ganoderma applanatum	SCC 182	0.004 j
Stereum sanguinolentum	SCC 35	0.003 j
Stereum australe	SCC 7	0.003 j
Chondrostereum purpureum	SCC 58	0.003 j
Crepidotus sp.	SCC 301	0.002 j
Ganoderma lucidum	SCC 28	0.001 j
Grifola sp.	SCC 67	0.001 j
Phellinus sp.	SCC 297	0.001 j
Fomitopsis lilacino-gilva	SCC 47	< 0.001 j
Cylindrobasidium laeve	SCC 282	< 0.001 j
Coriolus versicolor	52-J	< 0.001 j
Unidentified sp.	SCC 42	< 0.001 j
Bjerkandera adusta	SCC 51	< 0.001 j
Stereum lobatum	SCC 24	< 0.001 j
Coriolus sp.	SCC 303	< 0.001 j
Pycnoporus sanguineus	SCC 200	< 0.001 j
Trametes nivosa	SCC 214	< 0.001 j
Gloeophyllum trabeum	SCC 38	< 0.001 j
Fomitopsis lilacino-gilva	SCC 287	< 0.001 j
Gloeophyllum sepiarium	SCC 49	< 0.001 j
Nigroporus vinosus	SCC 195	< 0.001 j
Ceriporiopsis subvermispora	SS-3	< 0.001 j
Daedalea quercina	SCC 45	< 0.001 j
Phellinus sp.	SCC 133	< 0.001 j
Skeletocutis sp.	SCC 138	< 0.001 j
Laetiporus sulphureus	SCC 174	< 0.001 j
Phanerochaete chrysosporium	SCC 178	< 0.001 j
Bjerkandera adusta	SCC 169	< 0.001 j

a, b, c, …j **values followed by the same letter do not differ significantly ($p \leq 0,05$) with the Scott Knott procedure.**

Appendix B

The experimental culture data of batch cultivations in the 15-l bioreactor.

Cultivation conditions	Time (h)	pH	DOT[a]	Sucrose ($g.l^{-1}$)	Glucose ($g.l^{-1}$)	Fructose ($g.l^{-1}$)	Biomass ($g.l^{-1}$)	Laccase ($Units.ml^{-1}$)
No pH control; DOT ≥ 40; diluted molasses.	0	5,5	95	12,42	5,66	5,54	0,75	0,039
	12	5,5	95	8,87	0,94	6,61	0,85	0,058
	24	5,7	75	5,47	2,00	2,80	1,08	0,067
	36	5	35	3,43	1,55	1,67	2,50	0,055
	48	3	75	2,29	0,96	1,17	5,53	0,054
	60	2,5	78	2,31	1,41	3,51	5,53	0,606
	72	2,6	80	0,91	0,73	2,38	4,15	1,154
	84	2,7	85	0,58	0,00	1,40	4,90	0,336
	96	2,8	90	0,00	0,00	0,22	5,30	0,394
	108	2,9	90	0,00	0,00	0,00	3,15	0,424
	120	3	90	0,00	0,00	0,00	2,75	0,324
pH 5,5; DOT ≥ 40 %; diluted molasses.	0	5,5	94	7,94	4,38	4,89	0,20	0,009
	12	5,5	88	7,47	3,49	5,11	1,00	0,010
	24	5,5	84	6,93	3,60	11,19	2,10	0,011
	36	5,5	78	0,64	0,43	7,27	2,50	0,021
	48	5,5	75	0,00	0,40	3,37	4,,0	0,041
	60	5,5	60	0,00	0,25	0,00	7,65	0,108
	72	5,5	55	0,00	0,00	0,00	7,20	0,247
	84	5,5	60	0,00	0,00	0,00	7,95	0,286
	96	5,5	30	0,00	0,00	0,00	4,90	0,251
	108	5,5	30	0,00	0,00	0,00	6,40	0,179
	120	5,5	30	0,00	0,00	0,00	8,70	0,129

Cultivation conditions	Time (h)	pH	DOT[a]	Sucrose ($g.l^{-1}$)	Glucose ($g.l^{-1}$)	Fructose ($g.l^{-1}$)	Biomass ($g.l^{-1}$)	Laccase ($Units.ml^{-1}$)
pH between 5,5 & 3,5; DOT ≥ 40 %; diluted molasses.	0	5,5	95	12,42	5,66	5,54	0,25	0,022
	12	5,5	95	10,98	6,38	7,03	0,25	0,045
	24	5,6	85	13,92	7,27	8,28	0,90	0,055
	36	5,0	60	11,40	5,40	6,13	1,90	0,051
	48	3,5	50	7,51	5,52	9,97	5,05	0,033
	60	3,5	60	1,86	2,43	10,55	8,95	0,199
	72	3,5	55	1,45	0,80	4,11	7,60	0,599
	84	4,2	65	1,54	0,00	0,00	8,10	0,493
	96	5,1	60	0,00	0,00	0,00	6,60	0,626
	108	5,2	95	0,00	0,00	0,00	8,70	0,359
	120	5,2	95				7,15	0,338
pH contolled at pH 3,5 after naturally decreasing from pH 5,5; DOT ≥ 40 %; diluted molasses.	0	5,5	95	9,36	4,30	3,71	0,10	0,032
	12	5,5	93	7,94	4,38	4,89	0,25	0,061
	24	5,7	90	7,47	3,49	5,11	1,30	0,047
	36	4,5	70	6,93	3,60	11,19	2,65	0,036
	48	3,5	60	0,64	0,43	7,27	4,25	0,101
	60	3,5	55	0,00	0,40	3,37	7,35	0,429
	72	3,5	50		0,25	0,00	7,25	0,331
	84	3,5	55	0,00	0,00	0,00	6,75	0,342
	96	3,5	40	0,00	0,00	0,00	5,00	0,286
	108	3,5	45	0,00	0,00	0,00	5,95	0,265
	120	3,5	30	0,00	0,00	0,00	5,35	0,271

Cultivation conditions	Time (h)	pH	DOT[a]	Sucrose ($g.l^{-1}$)	Glucose ($g.l^{-1}$)	Fructose ($g.l^{-1}$)	Biomass ($g.l^{-1}$)	Laccase ($Units.ml^{-1}$)
DOT = 40 %; pH uncontrolled; diluted molasses.	0						0,3	0,062
	12						0,7	0,072
	24						1,8	0,089
	36						2,9	0,085
	48						2,6	0,060
	60						2,1	-0,003
	72						2,1	0,000
	84						2,5	0,025
	96						2,5	0,205
	108						3,1	0,052
	120						2,7	0,017
DOT = 5 %; pH uncontrolled; diluted molasses.	0						0,34	0,000
	12						0,19	-0,002
	24						0,52	0,085
	36						1,63	0,140
	48						2,51	0,135
	60						3,64	0,038
	72						3,53	0,003
	84						2,68	0,000
	96						2,24	0,000
	108						2,58	0,000
	120						2,88	0,000

Cultivation conditions	Time (h)	pH	DOT[a]	Sucrose ($g.l^{-1}$)	Glucose ($g.l^{-1}$)	Fructose ($g.l^{-1}$)	Biomass ($g.l^{-1}$)	Laccase ($Units.ml^{-1}$)
DOT = 5 %; pH uncontrolled; diluted molasses.	0						0,65	0,102
	12						0,09	0,105
	24						0,46	0,134
	36						0,45	0,160
	48						0,51	0,219
	60						0,78	0,316
	72						0,93	0,292
	84						1,21	0,277
	96						1,43	0,406
	108						1,84	0,491
	120						3,14	0,539
	132						4,36	0,613
	144						2,00	0,558
	156						2,47	0,538
	168						2,19	0,601
pH uncontrolled; DOT ≥ 40 %; mineral salts medium.	0						0,06	0,047
	12						0,28	0,047
	24						0,46	0,053
	36						1,94	0,047
	48						4,42	0,234
	60						5,00	0,002
	72						3,32	0,000
	84						5,18	0,000
	96						4,04	0,000
	108						5,40	0,000

Cultivation conditions	Time (h)	pH	DOT[a]	Sucrose ($g.l^{-1}$)	Glucose ($g.l^{-1}$)	Fructose ($g.l^{-1}$)	Biomass ($g.l^{-1}$)	Laccase ($Units.ml^{-1}$)
	120						8,36	0,032
pH 5,5; DOT ≥ 40%; mineral salts medium with 16mM veratryl alcohol.	0	5,5					1,02	-0,002
	12	5,5					1,04	0,000
	24	5,5					0,98	0,008
	36	5,5					1,08	0,058
	48	5,5					1,44	-0,003
	60	5,5					3,16	0,000
	72	5,5					2,46	-0,002
	84	5,5					2,86	0,008
	96	5,5					5,66	0,068
	108	5,5					2,84	0,237
	120	5,5					-	0,316
DOT ≥ 40% for 48h where after it was lowered to 0,5%; pH uncontrolled; mineral salts medium with 16mM veratryl alcohol.	0	5,5	40	12,42	5,66	5,54	0,01	0,014
	12	5,5	40	12,00	7,27	8,28	0,26	0,013
	24	5,5	40	11,40	5,40		0,50	0,015
	36	4,7	40	7,51	5,52	9,97	0,78	0,007
	48	3,5	1	1,86	2,43	10,55	0,88	0,001
	60	3,3	1	1,45	0,80	4,11	2,34	0,002
	72	3,0	1	1,54	0,00	0,00	3,44	0,147
	84	2,7	2	0,00	0,00	0,00	3,54	0,490
	96	2,5	8	0,00	0,00	0,00	3,48	0,933
	108	2,4	12	0,00			4,06	1,457
	120	2,3	15	0,00			3,68	2,152
	132	2,3	20	0,00			3,48	3,418
	144	2,3	25	0,00			3,78	2,215
	156	2,3	30	0,00			3,60	1,909

Cultivation conditions	Time (h)	pH	DOT[a]	Sucrose ($g.l^{-1}$)	Glucose ($g.l^{-1}$)	Fructose ($g.l^{-1}$)	Biomass ($g.l^{-1}$)	Laccase ($Units.ml^{-1}$)
Run 1. DOT ≥ 40; pH uncontrolled; mineral salts medium with 16mM veratryl alcohol.	0	5,5		14,82	2,99	2,64	0,24	0,007
	12	5,5		13,75	2,57	2,65	0,60	0,022
	24	5,5			4,38	4,82	1,48	0,019
	36	5,5		3,90	4,32	5,85	2,34	0,024
	48	5,2		0,00	4,21	6,04	2,38	0,021
	60	4,4		0,00	3,25	6,59	3,16	0,095
	72	3,8		0,24	5,69		3,54	1,293
	84	3,3				7,90	4,14	1,159
	96	3,1		0,25	4,27	6,52	3,96	1,574
	108	2,8				5,27	3,64	2,125
	120	2,7		0,95	3,20	4,87	3,40	6,234
	132	2,6		0,00	1,66	4,84	3,51	7,816
	144	2,6		0,00	1,69	3,81	3,20	6,816
	156	2,5		0,00	0,55	3,77	3,01	4,360
	168	2,5		0,00	0,61	3,42		0,007
Run 2. DOT ≥ 40, pH uncontrolled; mineral salts medium with 16mM veratryl alcohol.	0	5,5		14,82	2,99	2,64	0,18	-0,002
	12	5,5		13,51	2,83	3,55	0,36	0,011
	24	5,5		14,11	2,12	4,09	0,82	0,010
	36	5,2		8,11	2,18	5,36	1,18	0,008
	48	4,5		6,09	3,25		1,24	0,002
	60	4		5,08	4,08	6,20	1,70	0,001
	72	3,5		0,24	5,69		2,88	0,144
	84	3,2				7,90	2,46	0,389
	96	3,3		0,25	4,27	6,52	2,74	0,972
	108	3,3				5,27	4,24	1,935
	120	2,9		0,95	3,20	4,87	3,10	2,970

Cultivation conditions	Time (h)	pH	DOT[a]	Sucrose ($g.l^{-1}$)	Glucose ($g.l^{-1}$)	Fructose ($g.l^{-1}$)	Biomass ($g.l^{-1}$)	Laccase ($Units.ml^{-1}$)
	132	2,8		0,00	1,66	4,84	3,26	4,867
	144	2,8		0,00	1,69	3,81	3,08	7,009
	156	2,7		0,00	0,55	3,77	3,14	8,131
	168	2,7		0,00	0,61	3,42	3,00	7,455
	180	2,7		0,00	0,86	3,96	2,70	6,671

a DOT: Dissolved oxygen tension.