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*Full Length Research Paper*

# Optimization of laccase and manganese peroxidase production in submerged culture of *Pleurotus sajor-caju*

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**A white-rot fungus, *Pleurotus sajor-caju,* was isolated from coastal Tanzania and screened for crude lignolytic enzymes production using rhemazol brilliant blue R (RBBR) dye, 2,2-azino-bis (3-ethylbenzthiazoline)-6-sulfonate (ABTS) and guaiacol in a semi-solid medium. Laccase (Lac) and manganese peroxidase (MnP) were detected by α-napthol and pyrogallol solutions, respectively, on the guaiacol supplemented semi-solid media. The effect of temperature, pH, carbon, nitrogen, $Cu^{2+}$, 2,5-xylidine, ferulic acid, $Mn^{2+}$ and immobilization using *Luffa cylindrica* sponges in submerged culture fermentations were investigated for maximum enzymes production. After 7 days of incubation, 83 to 100% oxidation of RBBR, ABTS and guaiacol was observed. With optimized culture conditions, the fungal filtrate had maximum Lac and MnP activities of 80 and 0.94 U/ml, respectively compared to 0.62 and 0.0003 U/ml obtained with non-optimized ones; amounting to 129 and 3133 times increase in Lac and MnP activities, respectively. The improved crude enzymes activities, RBBR decolourization, ABTS and guaiacol oxidation capabilities of *P. sajor-caju* show its potential as a source of industrial enzymes for biotechnological applications.**

**Key words:** White-rot fungi, optimization, ***Pleurotus sajor-caju***, laccase, manganese peroxidase, submerged fermentation, immobilization.

## INTRODUCTION

White-rot fungi are wood decaying fungi that play an important role in the mineralization of lignin. Members of the genus ***Pleurotus*** are wood inhabiting white-rot basidiomycetes and grow on hardwoods, wood byproducts (such as wood chips, sawdust and paper products) and most agricultural wastes (Croan, 2000). These basidiomycetes are widely distributed in tropical forests and are associated with aggressive white-rot type decay of lignocellulosic biomass (Pointing et al., 2000). Most temperate species of ***Pleurotus*** including ***Pleurotus eryngii, Pleurotus sapidus, Pleurotus ostreatus and Pleurotus pulmonarius*** have been shown to produce laccase and MnP combination both under conditions of submerged fermentation (SF) and solid-state fermentation (SSF) (Orth et al., 1993; Stajic´ et al., 2006). There are few studies on the ligninolytic activities of tropical white-rot fungi such as ***Trametes*** species***, Lentinus velutinus, Pycnoporus sanguineus, Datronia concentrica, Irpex*** spp***, Creptidotus mollis*** (Tekere et al., 2001). However, the ligninolytic activities of some ***Pleurotus*** species example ***Pleurotus sajor-caju*** in particular, remain uninvestigated.

The high biodegradability of white-rot basidiomycetes is due to the presence of extracellular nonspecific and strong oxidative enzyme systems (Eichlerová et al., 2000; Tekere et al., 2001). The extracellular enzyme system includes lignin peroxidases (LiPs), manganese peroxidases (MnPs) and laccases (Lacs) (Eichlerová et al.,

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**Abbreviations: Lac,** Laccase; **RBBR,** rhemazol brilliant blue R dye; **MnP,** manganese peroxidase; **ABTS,** 2,2-azino-bis (3-ethylbenzthiazoline)-6-sulfonate.

2000; Tekere et al., 2001). These are secondary metabolic products, differing in chemical compositions and are often species-specific (Mtui and Nakamura, 2004; Dhouib et al., 2005). Rarely are these three enzymes present in the same organism, but can be produced in different combinations such as Lac-MnP-LiP, Lac-MnP, Lac-LiP or MnP-LiP (Hattaka, 1994).

Laccases are copper containing enzymes that catalyse the one electron oxidation of various aromatic compounds specifically phenols, anilines and their derivatives while reducing molecular oxygen ($O_{2)}$ to water (Gianfreda et al., 1999). Its low-substrate specificity and strong oxidative ability has made it to be significantly useful in pulp delignification, textile dye bleaching, effluent detoxification, bioremediation of soils, washing powder components, removal of phenolics from wines (Kiiskinen et al., 2004; Dhouib et al., 2005), enzymatic conversion of chemical intermediates and production of valuable compounds from lignin (Nyanhongo et al., 2002).

Manganese peroxidases are extracellular heme-containing glycoprotein produced only by ligninolytic (wood-rotting and litter-degrading) basidiomycetes, especially during the secondary metabolism (Rogalski et al., 2006). They catalyze the $H_2O_2$- dependent oxidation of $Mn^{2+}$ to a highly reactive $Mn^{3+}$ (Asgher et al., 2008). The latter, is stabilized by chelating with dicarboxylic acids (that is, lactate, oxalate, tartrate and malonate) to form $Mn^{3+}$-dicarboxylic acid complex (Asgher et al., 2008). The complex is a highly reactive oxidant that can freely diffuse away from the enzyme's active center because of its low molecular weight. Hence, it nonspecifically oxidizes a variety of phenolic and non-phenolic substances, including lignin and toxic pollutants (Rogalski et al., 2006).

The ligninolytic machinery in most basidiomycetes is highly regulated by nutrients such as nitrogen, copper and manganese. Their production is also affected by many typical fermentation factors such as medium composition, nature of carbon source, concentration of carbon source, pH of fermentation broth, fermentation temperature, amount and nature of nitrogen source and presence of inducers ($Cu^{2+}$, $Mn^{2+}$, 2, 5-xylidine, ferulic acid and veratryl alcohol) (Arora and Gill, 2001; Prasad et al., 2005; Asgher et al., 2010; Iqbal et al., 2011). However, no studies on optimization of culture conditions for production of laccase and MnP by tropical ***Pleurotus*** species particularly ***Pleurotus sajor-caju*** have been reported to date. This study reported for the first time the enzymatic profiles of tropical ***Pleurotus sajor-caju;*** a white-rot fungus isolated in coastal Tanzania. Hence, the effects of different concentrations of carbon, nitrogen and inducers ($Cu^{2+}$, $Mn^{2+}$, 2,5-xylidine, veratryl alcohol and ferulic acid) on the production of Laccase (Lac) and MnP by ***Pleurotus sajor-caju*** were investigated. Furthermore, this study evaluated the effect of immobilizing fungal mycelia by using ***Luffa cylindrica*** sponges on Lac and MnP production.

## MATERIALS AND METHODS

### Collection, screening and cultivation of the fungus

***Pleurotus sajor-caju*** was collected from decayed fallen wood from coastal Tanzania. The fungus was identified based on morphological and microscopic features (Buczacki, 1992; Härkönen et al., 2003) and confirmed by phylogenetic analysis of internal transcribed spacers containing rRNA gene sequence (Kamei et al., 2005). To obtain pure cultures, small fragments (about 1 mm diameter) from the inner flesh of the basidiocarp were plated onto 5% (w/v) malt extract agar (MEA). After 3 to 4 days, mycelia that emerged from the fragments were repeatedly transferred onto new MEA plates until pure cultures were obtained.

Rhemazol brilliant blue R (RBBR) dye, 2,2'-azino-bis (3-ethylbenzthiazoline)-6-sulfonate (ABTS) and guaiacol were used in screening as indicators for ligninolytic activities. The actively growing mycelial agar plugs (7-day old, 8 mm diameter) of the isolate was placed in the center of a 90 mm plate with 3% (w/v) MEA + 0.04% (w/v) RBBR, 3% (w/v) MEA+ 0.01% (w/v) guaiacol and modified Kirk and Farrell (1987) media (MKF). Duplicate plates were kept at 30°C in the dark. The decolorization or halo formation rate was calculated by measuring the RBBR decolorization or ABTS/guaiacol halo formation diameter (cm) of each isolate on daily bases.

### Optimization of culture conditions in the submerged culture fermentation

The modified Kirk's medium (Dhouib et al., 2005) and the modified Asther et al. (1988) media were used throughout the optimization strategies for laccase and manganese peroxidase production, respectively. The pH of the culture media were set at optimal pH values of 6.0 and 5.5 for Lac and MnP, respectively and these culture media were autoclaved at 121°C and 1 atmospheric pressure for 20 min before inoculating with 10 actively growing fungal mycelia disks that had been grown 7 days earlier on 3% MEA plates.

For all enzyme production optimization experiments, all fungal liquid culture media meant for laccase activities determination were done in 500 ml Erlenmeyer flasks and incubated with continuous agitation using a rotary shaker at 125 rpm while those for manganese peroxidases activities determination, 250 ml Erlenmeyer flasks were used and kept without agitation. All flasks were incubated at optimal temperatures obtained after optimization for each enzyme. Mycelial liquid cultures were collected after every 24 h into Eppendorf tubes and centrifuged using Eppendorf centrifuge (Hamburg, Germany) at 10,000 rpm for 10 min. The supernatants were analyzed for enzyme activities using UV-visible spectrophotometer (Thermospectronic, Great Britain).

### Incubation temperature and pH

Incubations were carried out at different temperatures ranging from 20 to 35°C at 5°C intervals, while medium pH was varied from 4.0 to 6.0 at 0.5 intervals.

### Effect of carbon sources at different concentrations

Glucose, cellulose and glycerol were used as carbon sources and each was studied independently. The effect of glucose was examined for laccase activity and thus, various glucose concentrations (5, 10, 15, 20, 25, 30 and 40 g/L) were added in the culture medium. Various amounts of glycerol (2, 4, 6, 8 and 10 g/L) were added in the culture medium to examine their effects on


**Figure 1.** (a) Dry fruits of loofa sponges after removing outer covering; (b) non-inoculated coarse LC matrices (1.2 $cm^3$); (c) inoculated LC matrices on the 3rd day of incubation.

manganese peroxidase activities. Various concentrations of cellulose (2, 4, 6, 8, 10, 15 and 20 g/L) were added in the culture medium separately, to test their effects on laccase and manganese peroxidase activities. In the culture medium containing glycerol or cellulose some glucose (4 $gl^{-1}$) was added as a simple utilizable sugar to enable initial growth of the fungal mycelia.

**Effect of inorganic nitrogen at different concentrations**

The effect of different concentrations of nitrogen on laccase and MnP production was studied in a medium with 2.7, 5.4, 10.9, 16.3, 21.7, 24.4 and 27.1 mM ammonium tartrate. These nitrogen concentrations were grouped as low N; 2.7 and 5.4 mM, medium N; 10.9 and 16.3 mM and high N; 21.7, 24.4 and 27.1 mM culture medium.

**Induction of laccase**

**Influence of copper on laccase production**

A sterile stock solution of copper sulfate was added in the actively growing fungal culture on the 3rd day of incubation to final concentrations of 0.1, 0.15, 0.2, 0.3, 0.4, 0.5, 0.6, 1.0 and 2.0 mM $CuSO_4$ in the culture medium. Control flasks were incubated without adding copper sulfate solution.

**Influence of 2, 5-xylidine on laccase production**

A filter-sterilized solution of 2,5-xylidine dissolved in 50% ethanol was added to the growing fungal cultures on the third day of incubation, until final concentrations of 0.1, 0.5, 1.0, 2.0, 3.0 and 4.0 mM were reached. The concentration of ethanol in the growth medium was always less than 0.5% and an equivalent amount of ethanol was added to the control flasks without 2,5-xylidine.

**Influence of ferulic acid on laccase production**

Additions of ferulic acid to the culture media were made during culture media preparation and before sterilization, to final concentrations of 0. 01, 0.05, 0.1, 0.5, 1.0, 1.5 and 2.0 mM. In the control experiment, no ferulic acid was added to the control flasks.

**Induction of MnP by $Mn^{2+}$ addition**

To determine the effect of $Mn^{2+}$ on MnP production, $MnSO_4 .H_2O$ was added to the fungal culture media during its preparation such that 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mM final concentrations in the culture media were obtained. Control culture media contained no $MnSO_4.H_2O$.

**Immobilization of fungal mycelia on Loofa (*Luffa cylindrica*) sponge**

Coarse sponges of ***L. cylindrica*** (LC) purchased at Kariakoo Market, Dar es Salaam, Tanzania, were used as immobilization material for the fungal mycelia. ***L. cylindrica*** is a natural material consisting of cellulose and lignin (1.4:2.9% of sponge dry weight) (Chang et al., 1995). The sponge is a highly porous and strong biomatrix, made of an open network of fibrous support from dry fruit of LC (Figure 1a and b). These were pretreated by cooking with boiling water to remove any other organic contaminants. After pretreatment, sponges were chopped into $2 \times 2 \times 0.3$ cm (1.2 $cm^3$) sizes, washed thoroughly with distilled water and sterilized by autoclaving at 121°C and 1.0 atm. for 15 min. Different numbers of the sponge chops (10, 20, 30, 40 cubes) were added to 500 ml Erlenmeyer flasks containing 100 ml culture medium that composed of the following: ammonium tartrate (optimal value = 0.5 g/L), $KH_2PO_4$ (2 g/L), $MgSO_4 .7H_2O$ (0.7 g/L), $CaCl_2 .2H_2O$ (0.14 g/L), $FeSO_4 .7H2O$ (0.07 g/L), $ZnSO_4 .7H_2O$ (0.046 g/L), $MnSO_4 .7H_2O$ (0.035 g/L), $CuSO_4.5H_2O$ (0.007 g/L), thiamine (0.0025 g/L), yeast extract (1 g/L), veratryl alcohol (0.067 g/L), Tween 80 (0.5 g/L), glucose (4 g/L) and peptone (5 g/L). Glycerol (2 g/L) was also added in the culture medium. Additions of these coarse LC matrices were made on the third day of fungal culture incubation, under stationary condition at 20°C for MnP production. The pH of the culture medium was adjusted to pH 5.5 for MnP production.

**Enzyme activity assays**

Laccase activity was determined by the oxidation of ABTS as the substrate (Bourbonnais et al., 1995). The reaction mixture contained 0.5 mM ABTS, 0.1 M sodium acetate buffer (pH 5.0) and 10 to 100 Ul culture supernatant. Oxidation of ABTS was monitored spectrophotometrically by determining the increase in absorbance at 420 nm, ($A_{420nm}$) with a molar extinction coefficient, $\varepsilon_{420}$ = 36000

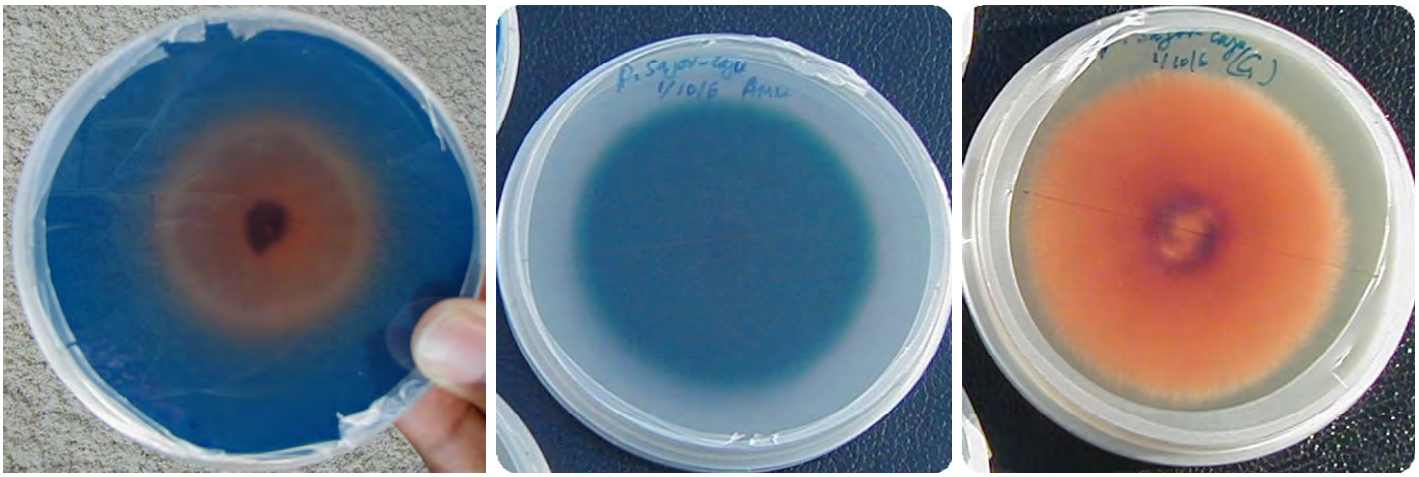


**Figure 2.** Halo formation as a result of: (a) RBBR decolorization; (b) ABTS oxidation; and (c) guaiacol oxidation by extracellular enzymes from ***Pleurotus sajor-caju*** grown on the semi-solid medium at 30°C for 7 days.

$M^{-1}cm^{-1}$.

Manganese peroxidase activity was determined by monitoring the oxidation of guaiacol (2-methoxyphenol) as the substrate at 465 nm with extinction coefficient, $\varepsilon_{465}$ = 12100 $M^{-1}cm^{-1}$ (Wunch et al., 1997). The reaction mixture contained 0.5 M sodium succinate buffer (pH 4.5), 4 mM guaiacol, 1 mM $MnSO_4$, 600 Ul of mycelia culture filtrate, and 1 mM $H_2O_2$. One unit (U) of enzyme activity was defined as the amount of enzyme oxidizing 1 Umole of substrate per min under assay conditions.

## RESULTS AND DISCUSSION

### Initial screening for Lac and MnP activities

In the initial screening, complete (100%) RBBR decolorization and ABTS oxidation was observed after seven days of incubation. Guaiacol (83%) was oxidized after 7 days of incubation. Oxidation of ABTS and guaiacol was confirmed by the formation of green and reddish-brown halo around the microbial growth, respectively, while colourless halo was for RBBR decolorization (Figure 2). The fungal filtrate exhibited maximum Lac and MnP activities of 0.62 and 0.0003 U/ml, respectively, in the submerged culture fermentations under non-optimized conditions. Dye decolorization and halo formation as a result of oxidation of coloured compounds is due to lignolytic enzymes production (Rodriquez et al., 2000; Robinson et al., 2001; Kiiskinen et al., 2004). It is an evidence of multi-enzymatic actions that could be applied in xenobiotic biodegradation studies as well as an indication of the physiological conditions of basidiomycetes during bioremediation process (Machado et al., 2005). The results of this study support previous studies that plate test is an efficient and simple method for bioprospecting fungi with novel lignolytic enzymes for industrial application purposes.

### Optimization of lignolytic enzymes production

### Effect of incubation temperature and initial medium pH

The optimum temperature for maximum laccase and MnP production by ***P. sajor-caju*** was found to be 30°C on day 9 with an activity of 0.2844 U/ml and 20°C on day 7 with an activity of 0.0052 U/ml, respectively. Very little lignolytic activities were observed at temperatures above 30°C probably due to the fact that increasing the temperature could have inhibited the fungal growth and hence, low/decreased enzyme activities. The same trend has also been demonstrated by Zadrazil et al. (1999) when ***Pleurotus*** specie and ***Dichomitus squalens*** were cultivated at temperatures higher than 30°C. Similar results have been reported by Nakamura et al. (1999) whereby, maximum lignolytic activity from cultures of ***B. adusta*** were attained at 30°C, but above 37°C there was no activity observed. Also, Iqbal et al. (2011) found substantial decrease in ligninolytic enzymes of ***Trametes versicolor*** IBL-04 when cultivated at temperatures higher than 30°C.

Maximum laccase and MnP produced were 0.300 and 0.0074 U/ml, respectively at pH 6.0 (day 7) and 5.5 (day 9), respectively. Activities in the most acidic medium (pH 3.5) were low compared to slightly acidic medium. These findings are in agreement with previous reports as most fungal enzymes, especially laccases, have maximum activity when the initial pH of the nutrient medium ranges from 4 to 6 (Galhaup et al., 2002; Jang et al., 2002; Chen et al., 2003).

### Effects of nitrogen on laccase and MnP production

The highest laccase produced was 13.66 U/ml on day 10

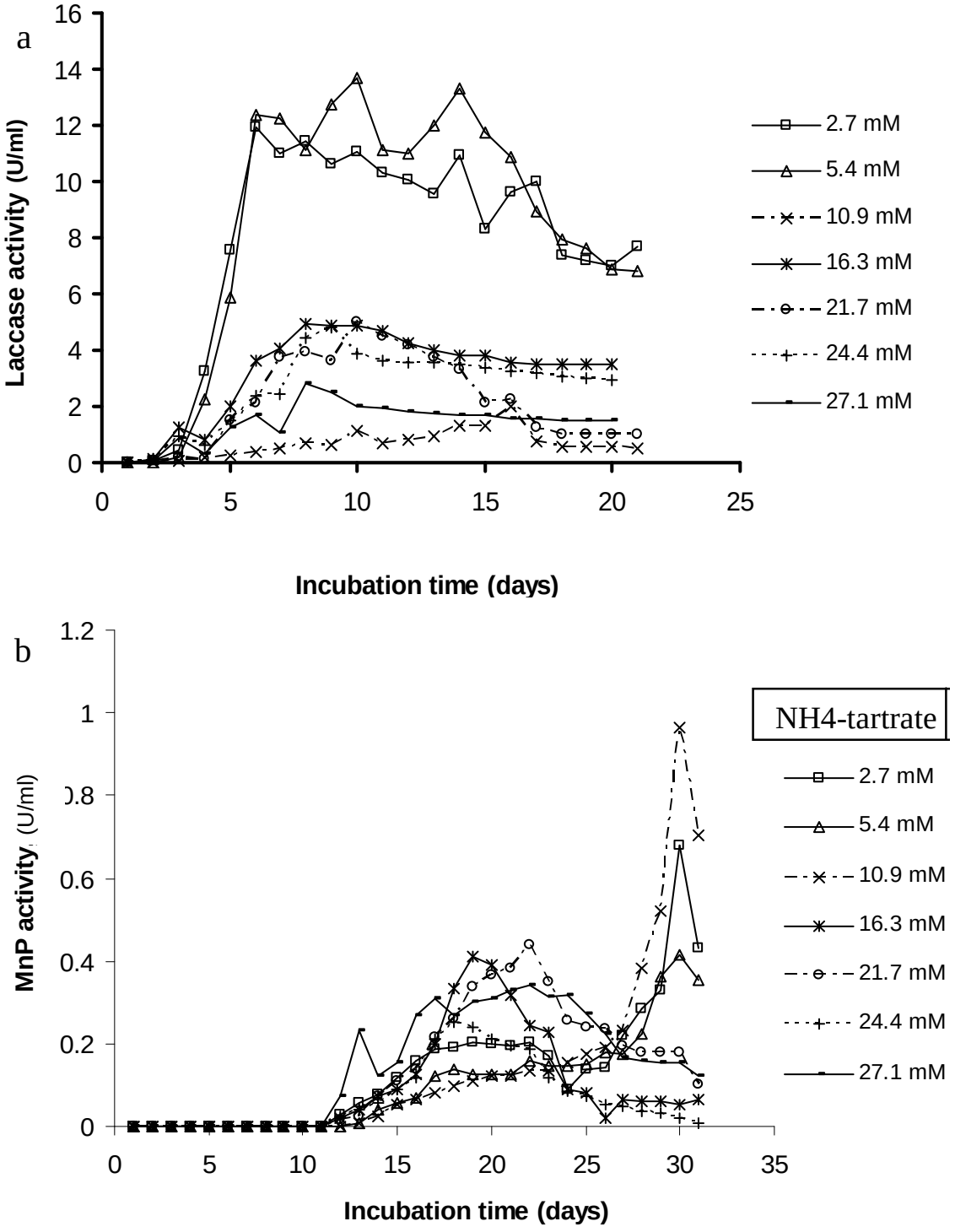


**Figure 3.** Lignolytic enzyme activities in ***Pleurotus sajor-caju*** under different concentrations of nitrogen during the submerged culture fermentation (a) laccase at pH 6.0, 30°C (b) manganese peroxidase at pH 5.5, 20°C.

and was observed in the culture medium with 5.4 mM ammonium tartrate categorized as low-N culture (Figure 3a). Such observed laccase activity was increased 46 times when compared to that obtained in the optimal initial medium pH (pH 6.0). Laccase production is known to be affected by the nitrogen concentration in media. High nitrogen levels are usually required for greater amounts of laccase to be produced (Gianfreda et al., 1999).

However, as a deviation in some fungi, nitrogen

limitation does not affect laccase production in the expected trend. While high nitrogen media gave the highest laccase activity in ***Lentinus edodes*** and ***Rigidoporus lignonus*** (Gianfreda et al., 1999), nitrogen-poor media enhanced the production of the enzyme in ***Pycnoporus cinnabarinus*** and ***Phlebia radiata*** (Gianfreda et al., 1999).

The highest MnP produced was 0.96 U/ml at day 30 and was observed in the culture media with 10.9 mM ammonium tartrate categorized as medium -N culture (Figure 3b). The activity observed was about 130 times higher than that obtained in the trial to optimize initial medium pH. While previous studies (Nakamura et al., 1999) showed that it is better to use N-limited conditions, this study shows that relatively high nitrogen conditions were more favorable for high MnP activities in the studied isolate.

High nitrogen conditions have the effect of increasing fungal growth and biomass yield, thus increased enzyme production could have been a result of increased fungal biomass. The results obtained here are consistent with some previous findings, for example, Levin and Forchiassin (2001) found high MnP production in the high N (40 mM-N) submerged culture of ***Trametes trogii***. Although, MnP production by most studied white rot fungi like ***P. chrysosporium***, ***Bjerkandera adusta*** is triggered in response to N limitation (Nakamura et al., 1999), some white rot fungi produce higher MnP enzymes in N-sufficient media as shown by Tekere et al. (2001) who found highest MnP production in ***T. versicolor***, ***L. velutinus*** and ***Irpex*** spp. cultures grown in high N containing media.

## Effect of different carbon concentrations on laccase and MnP production

Three different carbon sources with different concentrations were used; glucose (5 to 25 g/L), glycerol (2 to10 g/L) and cellulose (2 to 20 g/L). The optimum amount of glucose and cellulose required for maximum laccase production by ***P. sajor-caju*** in a submerged culture were 10 g/L glucose and 10 g/L cellulose (Figure 4a, b). The maximum activities (13.67 U/ml for glucose and 4.83 U/ml for cellulose) were reached on day 10 and 7, respectively. Glucose at 5 g/L concentration showed the least laccase activity as compared to other glucose concentrations used (Figure 4a). In Figure 4b, laccase production in all concentrations of cellulose tested, increased to a maximum value at day 5 and then declined sharply on day 6. However, its activity in all concentrations tested rose again and reached maximum at day 10 before it declined slowly on day 12. The sharp decline on day 6 could be due to the fact that the organism was switching from glucose to cellulose as alternative carbon source available after cessation of readily available carbon source that is, glucose in the culture medium.

Laccase activity obtained under optimum glucose concentration was enhanced by 0.1% compared to that measured with the optimum nitrogen concentration. With cellulose as carbon source, maximum laccase activity observed was 65% less as compared to that observed in the optimal amount of nitrogen and glucose concentration. These results suggest that glucose is the best carbon source for laccase production by this isolate when compared to cellulose. Lignin-degrading enzymes are secondary metabolites synthesized after the cessation of cell growth due to the limited amount of glucose.

Enzyme production increased with increase in the glucose or cellulose concentration up to 10 g/L at which their maximum values were reached and then declined starting at a concentration of 15 g/L. For manganese peroxidase production, glycerol (2 to 10 g/L) and cellulose (2 to 20 g/L) were used as carbon sources. 2 g/L of cellulose and 4 g/L glycerol were found to be the optimum amounts required for maximum MnP production (0.91 and 0.33 U/ml, respectively) as shown in Figures 5a and b.

Maximum production was reached on day 18 and day 13 for cellulose and glycerol containing culture media, respectively. The activities obtained at optimum cellulose and glycerol concentrations were 5 and 66% less, respectively when compared to those obtained in the optimized N culture. Generally, the results for MnP production under these two carbon sources showed that MnP expression by this isolate is highly favoured by low-carbon medium although, production was not higher than that of optimized N medium. High carbon medium resulted in the suppression of MnP production. It is well known from earlier studies that MnP production is a secondary metabolic event triggered by N and C limitation (Nakamura et al., 1999). This has also been reported by Tekere et al. (2001) where high MnP activities were obtained in ***L. velutinus*** and ***Irpex spp*** grown in low carbon culture medium.

### *Laccase induction*

The influence of different concentrations of $Cu^{2+}$, 2, 5-xylidine and ferulic acid on laccase production by ***P. sajor-caju*** was investigated. The highest laccase produced was 12.07 U/ml and was obtained in the culture medium containing 0.2 mM $Cu^{2+}$ on the 9th day of incubation (Figure 6a). The maximum laccase activity obtained at this concentration is 31 times higher compared to $Cu^{2+}$ free cultures (0.39 U/ml). Laccase activities (Figure 6a) increased in the culture media containing 0.1 to 0.2 mM $Cu^{2+}$.

However, further increase in copper sulfate concentration (0.3 to 2.0 mM) resulted in decrease in laccase activities. The optimal $Cu^{2+}$ concentration observed for this isolate was lower (2.0 mM) than that reported by Galhaup and Haltrich (2001) for submerged cultures of ***Trametes pubescens.*** However, this $Cu^{2+}$ concentration

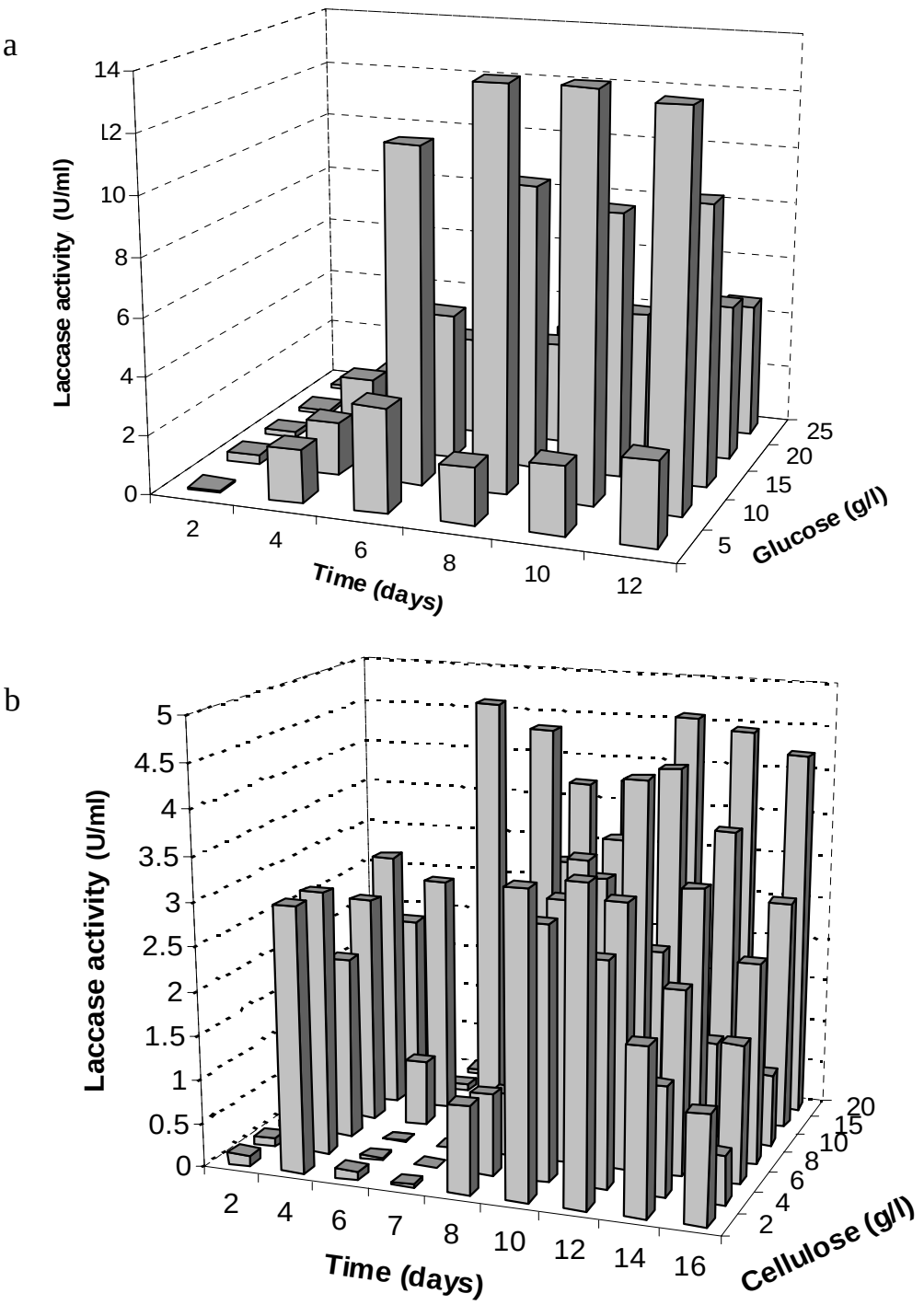


**Figure 4.** Time course of laccase production by ***Pleurotus sajor-caju*** under different concentrations of: (a) glucose and (b) cellulose, in submerged culture fermentation (pH 6.0, 30°C).

was still within the range of 2 to 600 UM used in typical cultivation media for the production of laccase both in wild-type and recombinant strains of different basidiomycete fungi such as ***Marasmius quercophilus, P. ostreatus*** and ***Volvariella volvacea***, (Farnet et al., 1999; Palmieri et al., 2000; Chen et al., 2003). It has also been reported (Palmieri et al., 2000) that the induction of laccase in ***P. ostreatus*** occurred when the fungus is cultivated in a nutrient-rich medium supplemented with 150 UM $CuSO_4$ at the time of inoculation.

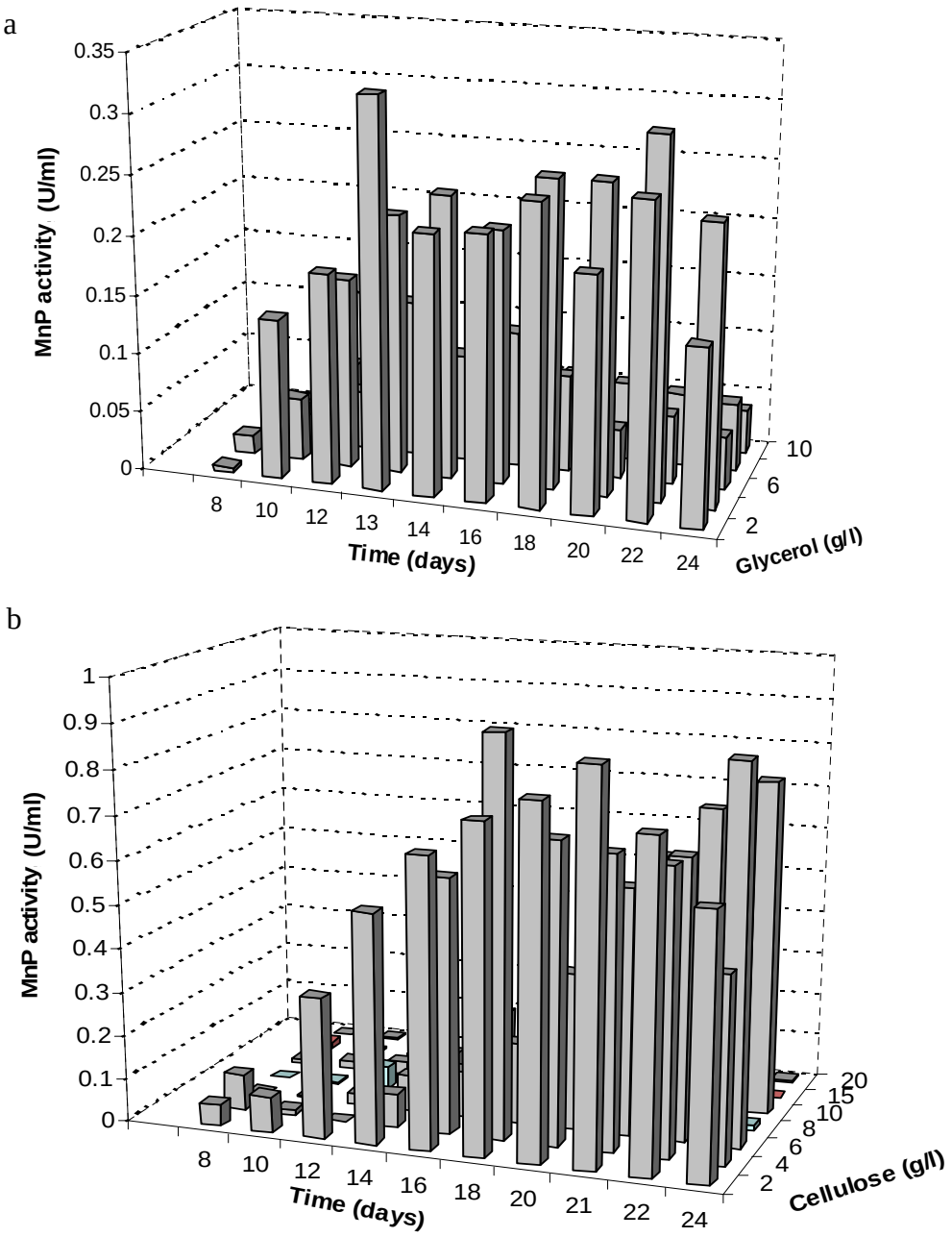


**Figure 5.** Time course of MnP production by ***P. sajor-caju*** under different concentrations of: (a) glycerol (b) cellulose during submerged culture fermentation.

Also, a $Cu^{2+}$ dose of 1.0 mM was required for enhancement of laccase synthesis by ***Trametes multicolor*** in bioreactor cultures (Hess et al., 2002). Therefore, it is clear that $Cu^{2+}$ is essential for inducing laccase production from basidiomycetes but there is an optimum amount required which is species specific. Hence, $Cu^{2+}$ above the optimum amount leads to reduction in laccase activity. This may be because at high concentrations, copper acts as a potent inhibitor of fungal growth (Chen et al., 2003).

2,5-xylidine, the most reported laccase inducer, enhanced laccase maximally in the culture media containing 2.0 mM. Maximum laccase activity (80 U/ml) under this concentration was obtained on the 8th day of incubation (Figure 6b). This activity is about 3.5 times higher than that of the control cultures which had only 22.70 U/ml (attained on day 12). The least laccase production (9.93 U/ml, on day 11) was observed in the culture media with 4.0 mM xylidine. Generally, xylidine concentrations of more than 2.0 mM had a detrimental effect on the organism and the laccase activities were below the control cultures. This may be because at very

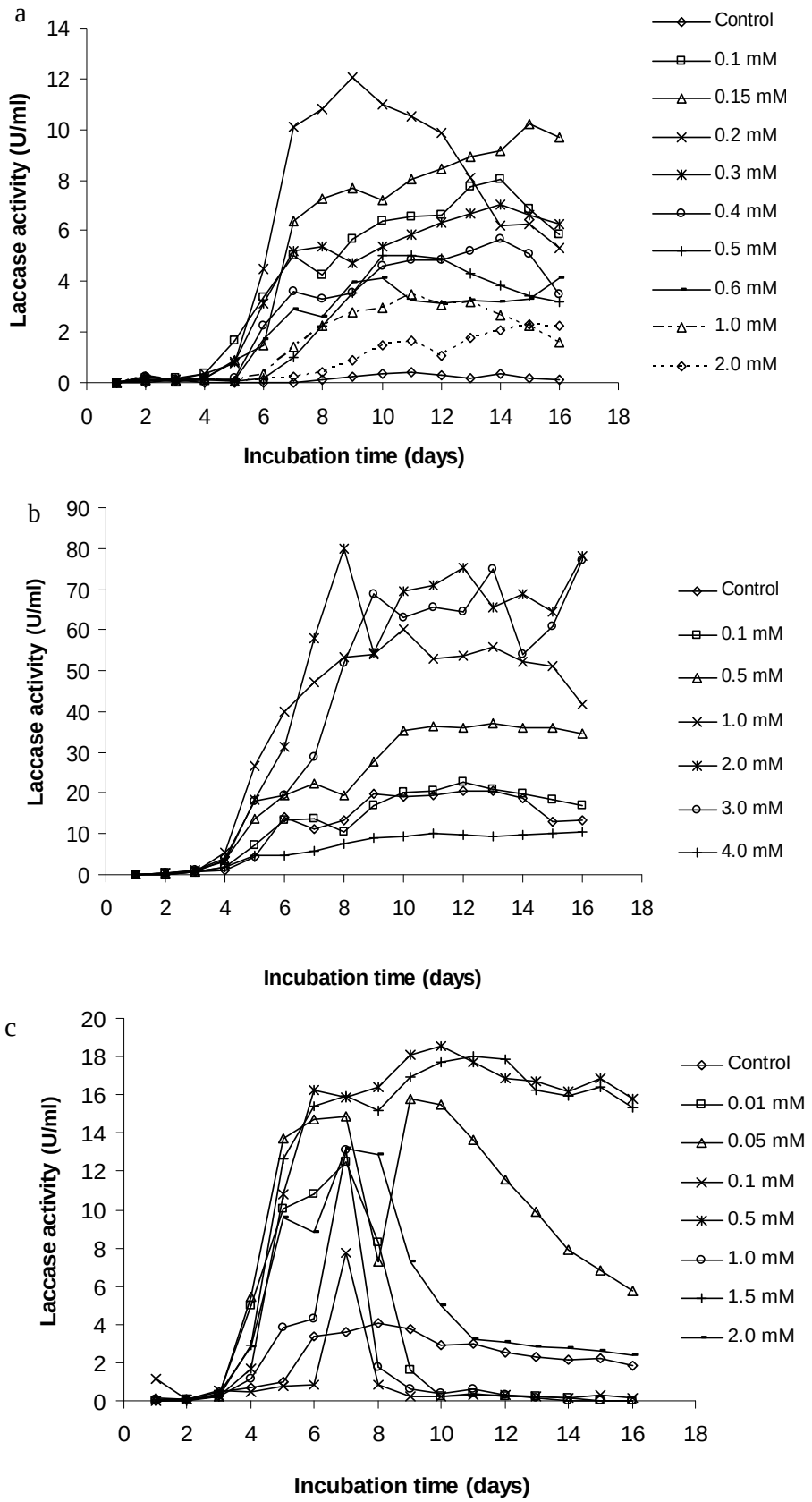


**Figure 6.** Laccase activities in ***Pleurotus sajor-caju*** under different concentrations of (a) $Cu^{2+}$ (b) xylidine and (c) ferulic acid, in the submerged culture fermentation.

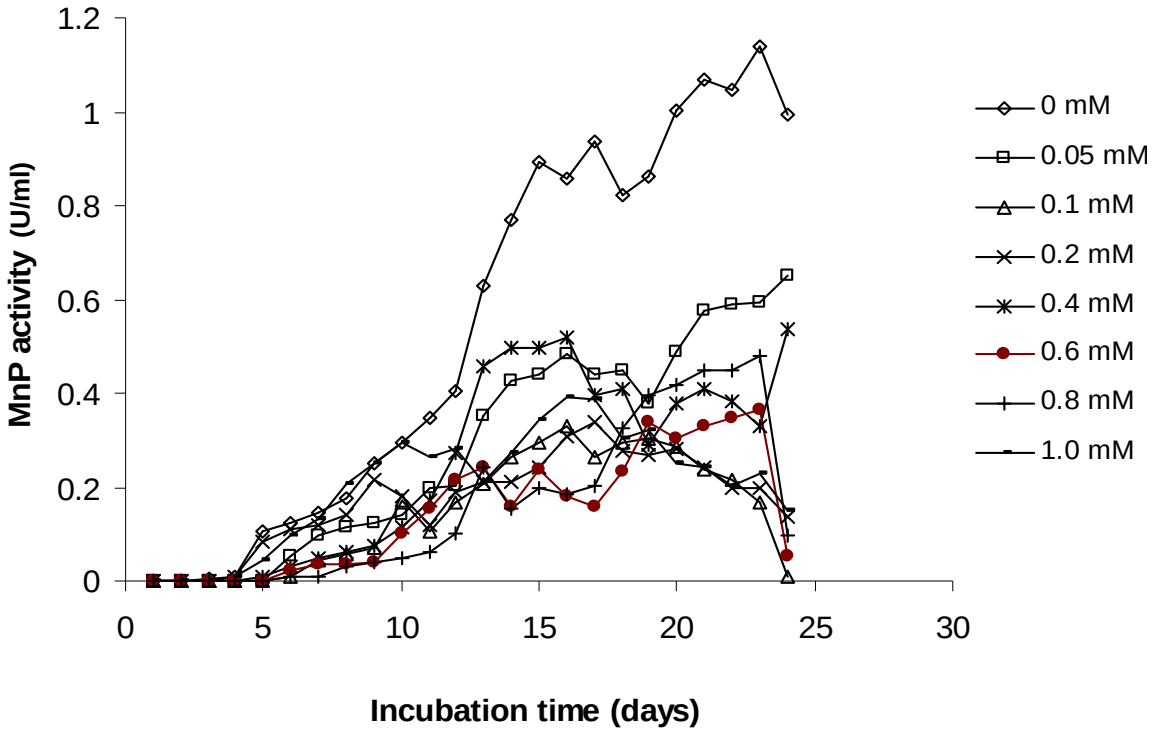


**Figure 7.** Effects of different concentrations of $Mn^{2+}$ on MnP production by ***Pleurotus sajor-caju.***

high concentrations, 2,5-xylidine could be toxic to the organisms, leading to the reduction in cell growth and enzyme production (Janusz et al., 2006). However, this study is consistent with other studies carried out by different research groups (Galhaup and Haltrich, 2001; Jang et al., 2002; Nyanhongo et al., 2002; Chen et al., 2003; Rancano et al., 2003), that xylidine enhanced laccase production in various ***Trametes*** sp and ***V. volvacea***. Eggert et al. (1996) also found that addition of xylidine as inducer had the most pronounced effect on laccase production by ***P. cinnabarinus***.

Ferulic acid was another laccase inducer tested in this study. On the 10th day of inoculation, 18.58 U/ml laccase was produced in the cultures with 0.5 mM ferulic acid (Figure 6c). This activity is 4.7 times higher than that obtained in cultures without ferulic acid (4.06 U/ml). Hence, these findings are consistent with the results obtained by Herpoel et al. (2000) where laccase production was enhanced from 9.5 to 29 U/ml when the culture was supplemented with ferulic acid.

## Effects of manganese ions ($Mn^{2+}$) concentration on MnP production

$Mn^{2+}$ supplementation in the culture media did not increase MnP activities among all tested $Mn^{2+}$ concentrations when compared to control cultures (Figure 7). The highest MnP activity observed was 0.65 U/ml in the culture media containing 0.05 mM $Mn^{2+}$ on the 24th day of cultivation (Figure 7). However, the activity was 43% less than the maximum activity (1.14 U/ml) obtained in the control cultures.

Increasing $Mn^{2+}$ is known to increase manganese peroxidase activity. $Mn^{2+}$ regulates production of MnP by inducing gene transcription and this fact was demonstrated in ***P. chrysosporium*** (Tekere et al., 2001). Levels of MnP mRNA, MnP protein and MnP activity in ***P. chrysosporium*** increased with increasing concentration of $Mn^{2+}$ (Tekere et al. 2001). On the contrary, the addition of $Mn^{2+}$ to ***P. eryngii***, ***P. ostreatus*** and ***P. radiate*** cultures did not increase MnP activity (Tekere et al., 2001). In this study, the activity of MnP was highest in culture media without $Mn^{2+}$ for ***P. sajor-caju.*** It is therefore evident that while some white rot fungi need $Mn^{2+}$ for maximum MnP expression, others do not require it.

## Effects of LC-immobilized fungal mycelia on MnP production

The fungal mycelia were grown in culture media with different numbers of coarse ***L. cylindrica*** (LC) cubes. The highest MnP attained was 0.94 U/ml in the culture media with 20 LC cubes on the 20th day of incubation (Figure 8). This activity is about 2.2 times higher than that obtained in control cultures (0.43 U/ml, in day 20). At least there was an increase in MnP activity for every culture media with LC cubes compared to cultures without LC cubes. A static fungal mycelia immobilization

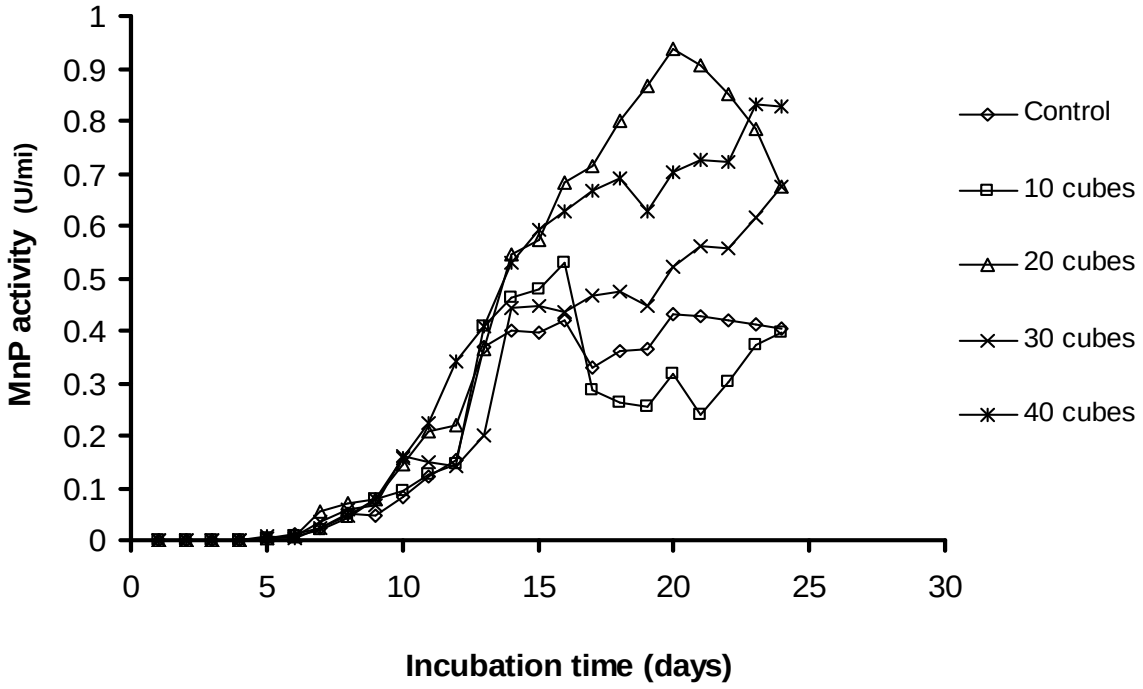


**Figure 8.** MnP production by the mycelia of ***P. sajor-caju*** immobilized on different numbers of LC cubes.

has been reported to enhance lignolytic enzymes production in white rot fungi especially peroxidases (Nakamura et al., 1999). This is due to the fact that it allows the contact area between cells and oxygen to be increased without shear stress (Wang et al., 2005). Also, it reduces protease activity and a pH change does not affect the organism directly (Wang et al., 2005). These findings are in agreement with previous findings by Mazmanci and Unyanyar (2005) who found 99% dye decolorization in the ***Funalia trogii*** culture immobilized on ***L. cylindrica*** sponges as compared to control cultures that had 2% dye decolorization.

## Conclusion

This study attempted to optimize culturing conditions in order to improve Lac and MnP activities in submerged culture of ***P. sajor-caju***. Varying the physicochemical parameters such as incubation temperature and initial medium pH improved the amounts of enzymes produced. Furthermore, altering the media compositions including addition of inducers such as $Cu^{2+}$, $Mn^{2+,}$ xylidine, ferulic acid and fungal mycelia immobilization enhanced the enzyme yields.

When ***P. sajor-caju*** was cultured in the medium with combination of all optimum factors, Lac and MnP activities of 80 and 0.94 U/ml, respectively were obtained. Thus, it was found that combination of all optimized operational parameters in the submerged fermentation increased Lac and MnP activities by 129 and 3133 times compared to that observed in non-optimized conditions. This work provides baseline information on growth parameters optimization for ***P. sajor-caju*** under submerged culture conditions. Future studies will focus towards purifying the enzymes as well as testing them in industrial and environmental biotechnology.

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