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OPTIMIZED PRODUCTION OF LIGNIN PEROXIDASE, MANGANESE PEROXIDASE AND LACCASE IN SUBMERGED CULTURES OF *TRAMETES TROGII* USING VARIOUS GROWTH MEDIA COMPOSITIONS

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ABSTRACT

A white-rot fungus, Trametes trogii*, 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. Lignin peroxidase (LiP), manganese peroxidase (MnP) and Laccase (Lac) were detected by pyrogallol and α-napthol solutions, respectively on the guaiacol supplemented solid media. The effect of temperature, pH, carbon, nitrogen,* Cu^{2+}*, 2,5-xylidine, ferulic acid, varatryl alcohol and* Mn^{2+} *in submerged culture fermentations were investigated for maximum enzymes production. After 7 days of incubation, 72-100% oxidation of RBBR, ABTS and guaiacol was observed. With optimized culture conditions, the fungal filtrate had maximum LiP, MnP and Lac activities of 0.18, 4.44 and 593 U/ml, respectively compared to 0.0011, 0.0054 and 2.3 U/ml obtained with non-optimized ones, amounting to 16,264%, 82,122% and 25,683% increase in LiP, MnP and Lac activities, respectively. The enhanced crude enzymes activities, RBBR decolorization and ABTS guaiacol oxidation capabilities of T. trogii show its potential as a source of industrial enzymes for biotechnological applications.*

Key words: Optimization, *Trametes trogii*, lignin peroxidase, manganese peroxidase, laccase, fermentation, submerged.

INTRODUCTION

Lignolytic fungi are filamentous group of wood decaying fungi that play an important role in the mineralization of lignin. These are the only organisms that are known to have evolved complex enzymatic machinery to degrade lignin, the non-hydrolysable part of wood, to any extent (Revankar and Lele, 2006). They are considered to be the most promising group of microorganisms because they produce lignolytic enzymatic complex that are composed of at least three enzymes: lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac). These are extracellular enzymes that are secondary metabolic products, differing in chemical compositions and are often species-specific (Härkönen *et al*. 2003; Mtui and Nakamura, 2004; Dhouib *et al*. 2005). These enzymes have broad substrate specificity, ability to form reactive radicals and have strong oxidative mechanisms which enable them to degrade a wide variety of pollutants such as textile and pulp mill effluents, organochloride agrochemicals and other synthetic aromatic compounds (Nyanhongo *et al.* 2002).

LiP (E.C:1.11.1.14) is a heme protein with high oxidation potential that oxidizes phenolic and nonphenolic substrates. MnP (E.C:1.11.1.13) is also a heme protein that oxidizes phenolic substrates but it is considered unable to oxidize non-phenolic substrates, although it can depolymerize synthetic or natural lignin *in vitro* (Hatakka *et al.* 2001). Laccase (benzenediol:oxygen oxidoreductase; EC. 1.10.3.2), belongs to a family of multicopper oxidases that has a wide range of reducing substrates like polyphenols, methoxy-substituted phenols and non-phenolic compounds like aromatic amines. Laccase oxidizes model lignin compounds if appropriate primary substrates such as 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), violuric acid or 1 hydroxybenzotriazole are present (Soares *et al.* 2001). Under such conditions laccase can oxidize substrates that are restrictive to LiP production, such as veratryl alcohol or polycyclic aromatic hydrocarbons. Thus, organisms able to produce all the three lignolytic enzymes are interesting in view of their potential importance in processes such as bioremediation, biobleaching of pulp paper, and degradation and detoxification of recalcitrant substances.

Trametes trogii is a wood-inhabiting mushroom which is distributed worldwide. It is characterized by having whitish to pale brownish pore surface, and tough basidiocarps attached to wood which lack stipe. This mushroom has been shown to be a good producer of laccases and other lignolytic enzymes including LiP and MnP (Levin *et al.* 2001; Deveci *et al.* 2004; Mechichi *et al.* 2005). It has also been shown to be an efficient tool for the degradation of several organic pollutants including nitrobenzene and anthracene (Levin *et al.* 2003), Polychlorinated biphenyls (PCBs) mixture (Aroclor 1150) and industrial Polycyclic aromatic hydrocarbons (PAHs) mixture (10% v/v of PAHs, principal components hexaethylbenzene, naphthalene, 1-methyl naphthalene, acenaphthylene, anthracene, fluorine and phenanthrene) (Haglund *et al.* 2002). The ability to degrade such environmental pollutants is correlated with the production of extracellular LiP, MnP and laccases.

Production of these enzymes is affected by many fermentation factors such as medium composition, carbon and nitrogen ratio, pH, incubation temperature, aeration rate and phenolic and aromatic compounds related to lignin or lignin derivatives such as xylidine, veratryl alcohol, ferulic acid or guaiacol (Gianfreda *et al.* 1999, Arora and Gill, 2001). Some white rot fungi such as *P. chrysosporium* have been found to be N-regulated while other strains are N-deregulated, for example strains of the genus *Bjerkandera* (Mester and Field, 1997; Nakamura *et al.* 1999). The nature and amount of carbon sources has also been shown to regulate production of lignolytic enzymes. For example, glucose and cellobiose were found to be the most effective carbon sources which were also utilized well by the *Trametes pubescens* (Galhaup *et al.* 2002). Copper as micronutrient has a key role as metal activator and induces both laccase transcription and production, and serves as a cofactor in the catalytic center of laccase enzyme (Palmieri *et al.* 2000).

One of the limitations of these enzymes to biotechnological use is the lack of capacity to produce them in high amounts. Masalu (2004) studied some Tanzanian white rot fungi on the basis of enzymes activity profiles and ability to degrade dyes and lignocellulosic materials. This study established different lignolytic production patterns by various white-rot basidiomycetes under non-optimized culture conditions. Mtui and Nakamura (2007) further reported activities of lignolytic enzymes from marine white-rot fungus, *Phlebia chrysocreas* isolated from the sea coast. Recent studies have also reported lignolytic activities from facultative and obligate marine fungi isolated from mangrove forests and in the Indian Ocean waters, respectively (Mtui and Masalu, 2008; Mtui and Nakamura 2008). In all these studies, no any attempt was made to optimize crude production of these enzymes. However, such studies have been conducted elsewhere in the world for some basidiomycetes, and the obtained results suggest that each species of basidiomycetes has specific optimum culturing conditions for optimum lignolytic enzymes production (Mester and Field, 1997; Gianfreda *et al.* 1999, Nakamura *et al.* 1999, Galhaup *et al.* 2002, Revankar and Lele 2006). In this study, therefore, crude production of lignolytic enzymes by *Trametes trogii*, a lignolytic fungus isolated from coastal Tanzania was improved by optimizing culture conditions and nutritional requirements using one-factor-at-a-time approach which involved changing the independent variable while fixing others at a certain levels. The effect of various concentrations of Cu^{2+} and aromatic inducers (2,5-xylidine, veratryl alcohol and ferulic acid) were also evaluated.

MATERIALS AND METHODS

Collection, screening and cultivation of the fungus

Trametes trogii (Berk.) was collected from decayed 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) and mycelia were repeatedly transferred onto new plates until cultures were pure. Rhemazol Brilliant blue R (RBBR) dye, 2,2'-azino-bis (3-ethylbenzthiazoline)-6-sulfonate (ABTS) and guaiacol were added in the solid medium to screen for lignolytic activities. The modified Kirk's medium (Dhouib *et al.* 2005) was used in the submerged culture fermentation.

Optimization of culture conditions in the submerged culture fermentation

The modified Kirk's media (adapted from Dhouib *et al.* 2005) and the modified Asther *et al.* (1988) media were used throughout the optimization strategies for laccase and peroxidases production, respectively. The pH of these culture media was set at optimal and these culture media were autoclaved at 121 °C and 1 atmospheric pressure for 20 minutes before inoculating with 10 actively growing fungal mycelia disks, which had been grown 7 days earlier on 3 % malt extract agar (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. For lignin and manganese peroxidases activities determination, 250 ml Erlenmeyer flasks were used and kept without agitation. All flasks were incubated at optimal temperature obtained after optimization for each enzyme. Mycelial liquid cultures were collected daily (after every 24-hours) into eppendorf tubes, centrifuged using eppendorf centrifuge (Humburg, Germany) at 10,000 rpm for 10 minutes, and the supernatant analyzed for enzyme activities using UV-visible spectrophotometer (Thermospectronic, Great Britain).

Incubation temperature and pH

Incubations were carried out at different temperatures ranging from 20-35 °C at 5 °C intervals while medium pH was varied from 3.5-6.5 at 0.5 intervals.

Carbon and nitrogen sources

Glucose, cellulose and glycerol were used as a source of carbon 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^{-1}) were added in the culture medium. Various amounts of glycerol (2, 4, 6, 8 and 10 g $l^{-1)}$ were added in the culture medium to examine its effect on lignin peroxidase and manganese peroxidase activities. Various concentrations of cellulose (2, 4, 6, 8, 10, 15 and 20 g l^{-1}) were added in the culture medium separately, to test their effects on lignin peroxidase, manganese peroxidase and laccase activities. Ammonium tartrate was used as a source of nitrogen and was added in the culture media at 2.7, 5.4, 10.9, 16.3, and 21.7, 24.4 and 27.1 mM concentrations. 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 LiP by veratryl alcohol addition

Veratryl alcohol (3,4-dimethoxybenzyl alcohol) was added to the culture media to final concentrations of 0.2, 0.4, 0.8, 1.0, 2.0 3.0 and 4.0 mM during culture media preparation. The control flask contained culture media without veratryl alcohol.

Induction of MnP by Mn^{2+} addition

To determine the effect of Mn^{2+} on MnP production, $MnSO_4 .H_2O$ was added in 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 cultures contained no $MnSO_4 .H_2O$.

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 , to final concentrations of 0.1, 0.5, 1.0, 2.0, 3.0 and 4.0 mM. 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 to the culture media was made during culture media preparation, before sterilization; such that the final concentrations to the culture media were 0. 01, 0.05, 0.1, 0.5, 1.0, 1.5 and 2.0 mM of ferulic acid. In the control experiment, no ferulic acid was added to the control flasks.

Enzyme activity assays

The reaction mixture for lignin peroxidase determination contained 20 mM succinate buffer (pH 3.0), 1 mM veratryl alcohol, 600 µl of mycelial culture filtrate and 5 mM H_2O_2 (Sugiura *et al.* 2003). The increase in absorbance was followed spectrophotometrically at 310 nm (extinction coefficient, ε_{310} = 9300 $M^{-1}cm^{-1}$) due to oxidation of veratryl alcohol to veratraldehyde (3,4-dimethoxybenzaldehyde). Manganese peroxidase activity was determined by monitoring the oxidation of guaiacol (2-methoxyphenol) as the substrate at 465 nm with extinction coefficient, ε_{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 µl of mycelial culture filtrate, and 1 mM H_2O_2. The laccase activity was determined via 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 -100 µl culture supernatant. Oxidation of ABTS was monitored spectrophotometrically by determining the increase in absorbance at 420 nm, (A_{420nm}) with a molar extinction coefficient, ε_{420} = 36000 $M^{-1}cm^{-1}$. One unit (U) of enzyme activity was defined as the amount of enzyme oxidizing 1 µmole of substrate per minute under assay conditions.

RESULTS AND DISCUSSION

Initial screening for LiP, MnP and Lac activities

In the initial screening, complete (100%) RBBR decolorization and ABTS oxidation was observed after six and five days of incubation, respectively. 72 % guaiacol was oxidized after 7 days of incubation. Oxidation of ABTS and guaiacol was confirmed by the formation of green and reddish-brown halo zone around the microbial growth, respectively while colorless halo zone was for RBBR decolorization (Figure 1b, c). The fungal filtrate exhibited maximum LiP, MnP and Lac activities of 0.001, 0.005 and 2.3 U/ml, respectively, in the submerged culture fermentations under non-optimized conditions. Dye decolorization and halo formation as a result of oxidation of colored compounds is due to lignolytic enzymes production (Machado *et al*. 2005). 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 (Masalu, 2004).

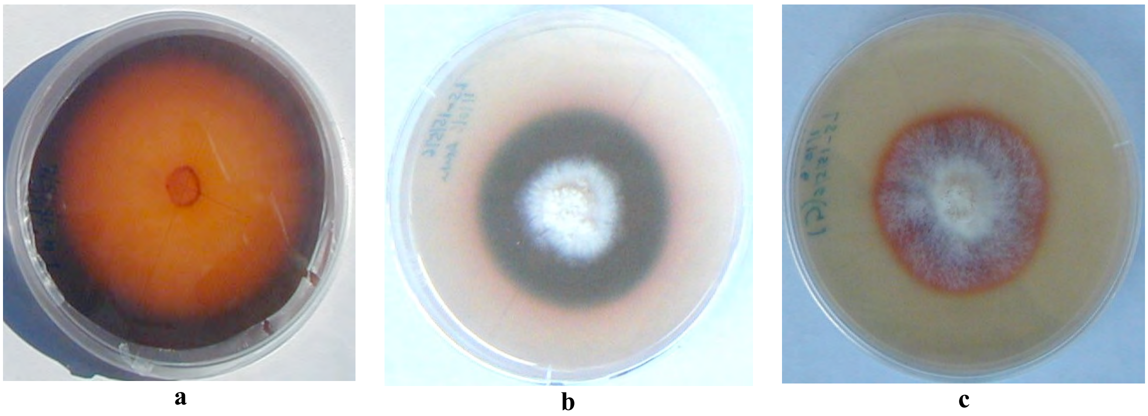


Figure 1: Halo formations as a result of (a) RBBR decolorization (b) ABTS oxidation and (c) guaiacol oxidation by extracellular enzymes from *Trametes trogii* grown on the malt extract agar (MEA) medium.

Optimization of lignolytic enzymes production

Effect of incubation temperature

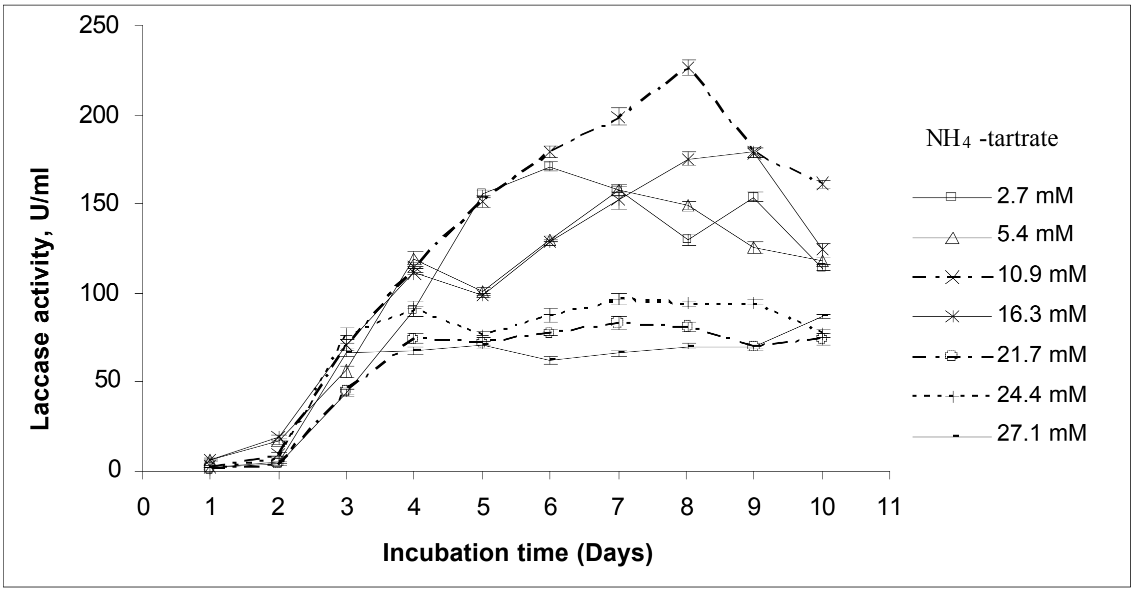
Temperature optima for maximum production of lignolytic enzymes were determined to be 25 ºC for laccase on day 6 with activity of 0.36 U/ml. MnP production reached maximum at 30 ºC on day 3 with activity of 0.019 U/ml. LiP production reached maximum at 20 ºC on day 4 with activity of 0.0009 U/ml. Very little lignolytic activities were observed at temperatures above 25 ºC probably due to cell death. The same trend has been demonstrated by Zadrazil *et al*. (1999) when *Pleurotus sp* and *Dichomitus squalens* were cultivated at temperatures higher than 30 ºC. Similar results have been reported by Nakamura *et al*. (1999) whereby maximum lignolytic activity from cultures of *Bjerkandera adusta* were attained at 30 ºC, but above 37 ºC there was no activity observed.

Effect of initial medium pH

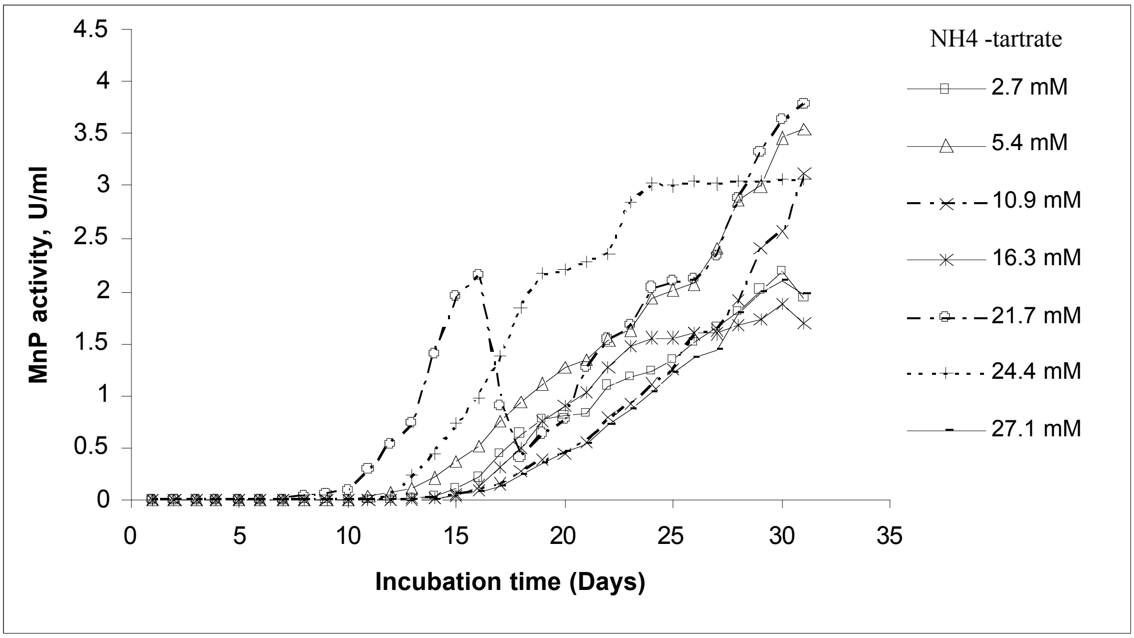
Maximum LiP, MnP and laccase production were 0.03, 0.26 and 9.3 U/ml, respectively and were achieved on day 10, 12 and 9 in a culture medium of pH 5.5, 6.5 and 5.0, respectively at their determined optimum temperatures. Activities in the most acidic medium (pH 3.5) were little compared to slightly acidic 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 nutrient nitrogen on Lignolytic enzymes production

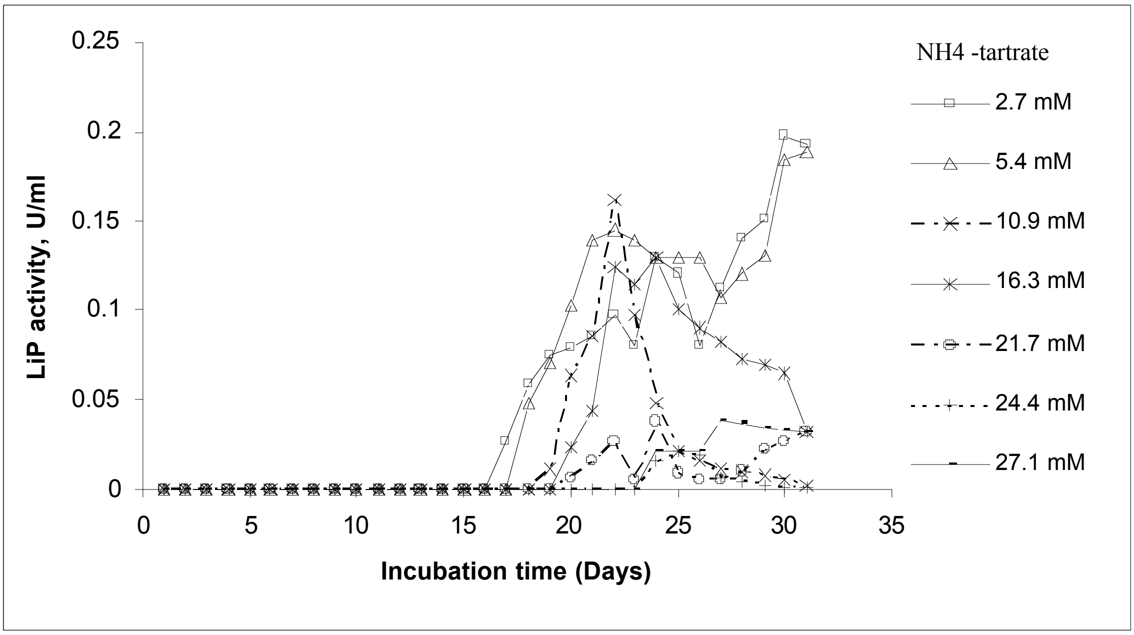
Results are shown in Figure 2a-c. The highest laccase produced was 227 ± 4 U/ml in day 8, and was observed in culture medium with 10.9 mM ammonium tartrate categorized as medium-N culture (Figure 2a). Such observed laccase activity was improved 33 times as compared to that obtained in he optimum pH of 5.0. The high nitrogen (27.1 mM) content medium gave the least laccase production. 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. 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).



(a)



(b)



(c)

Figure 2(a-c): Lignolytic enzyme activities in *Trametes trogii* under different concentrations of nitrogen during the submerged culture fermentation (a) laccase at pH 5.0, 25 ºC (b) manganese peroxidase at pH 6.5, 30 ºC (c) lignin peroxidase at pH 5.5. 20 ºC

Maximum MnP (3.8 ± 0.3 U/ml) was obtained on day 30 in the medium containing 21.7 mM ammonium tartrate. The activity observed was about 12 fold higher than that obtained in the trial to optimize initial medium pH. But 24.4 mM ammonium tartrate containing medium seemed to be the best concentration because at that amount a relatively high MnP was produced in a shorter time from day 11 to day 24 as compared to other concentrations used. However, on the 25th day there was no further increase of MnP (Figure 2b).

While previous studies (Nakamura *et al.* 1999) show that it is better to use N-limited conditions, this study shows that 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 the 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*, and *Bjerkandera adusta* is triggered in response to N limitation (Nakamura *et al.* 1999), many white rot fungi produce higher MnP enzymes in N-sufficient media as shown by Tekere *et al.* (2001) who found highest MnP production in the high N containing medium.

LiP production (0.15 ± 0.009 U/ml) reached maximum on day 22 in the medium with 5.4 mM ammonium tartrate but thereafter declined gradually until day 27, where it increased again and reached maximum after 30 days of incubation (Figure 2c). The reason why LiP production declined gradually at some incubation periods could be attributed to enzyme degradation by proteases. Medium containing 2.7 mM N had LiP production close to that of 5.4 mM N but this overshoot on day 30. This suggests that the production of LiP by *T. trogii* is enhanced in a nitrogen-limited culture, which is in agreement with previous reports that lignin degrading enzymes of other white-rot fungi such as *P. chrysosporium*, and *B. adusta* are synthesized in response to nitrogen starvation (Nakamura *et al.* 1999).

Lignolytic enzymes production under different carbon concentrations

Three different carbon sources with different concentrations were used; glucose (5 – 40 g/l), glycerol (2 -10 g/l) and cellulose (2-20 g/l). The optimum concentration of glucose and cellulose required for maximum laccase production were determined to be 30 g/l and 8 g/l respectively (Figure 3a and 3b). The highest amount of laccase produced under the above concentrations of the two carbon sources were 386 ± 6 U/ml for glucose and 183 ± 2 U/ml for cellulose on day 14 and day 8, respectively. The trend in laccase production using glucose as carbon source increased with increasing glucose concentration in the culture medium up to 30 g/l, but above this production declined. The least production was observed in the culture medium containing 5 g/l glucose and 2 g/l cellulose. The laccase activities obtained under optimum glucose concentration was enhanced 1.3 times compared to that measured with the optimum nitrogen concentration.

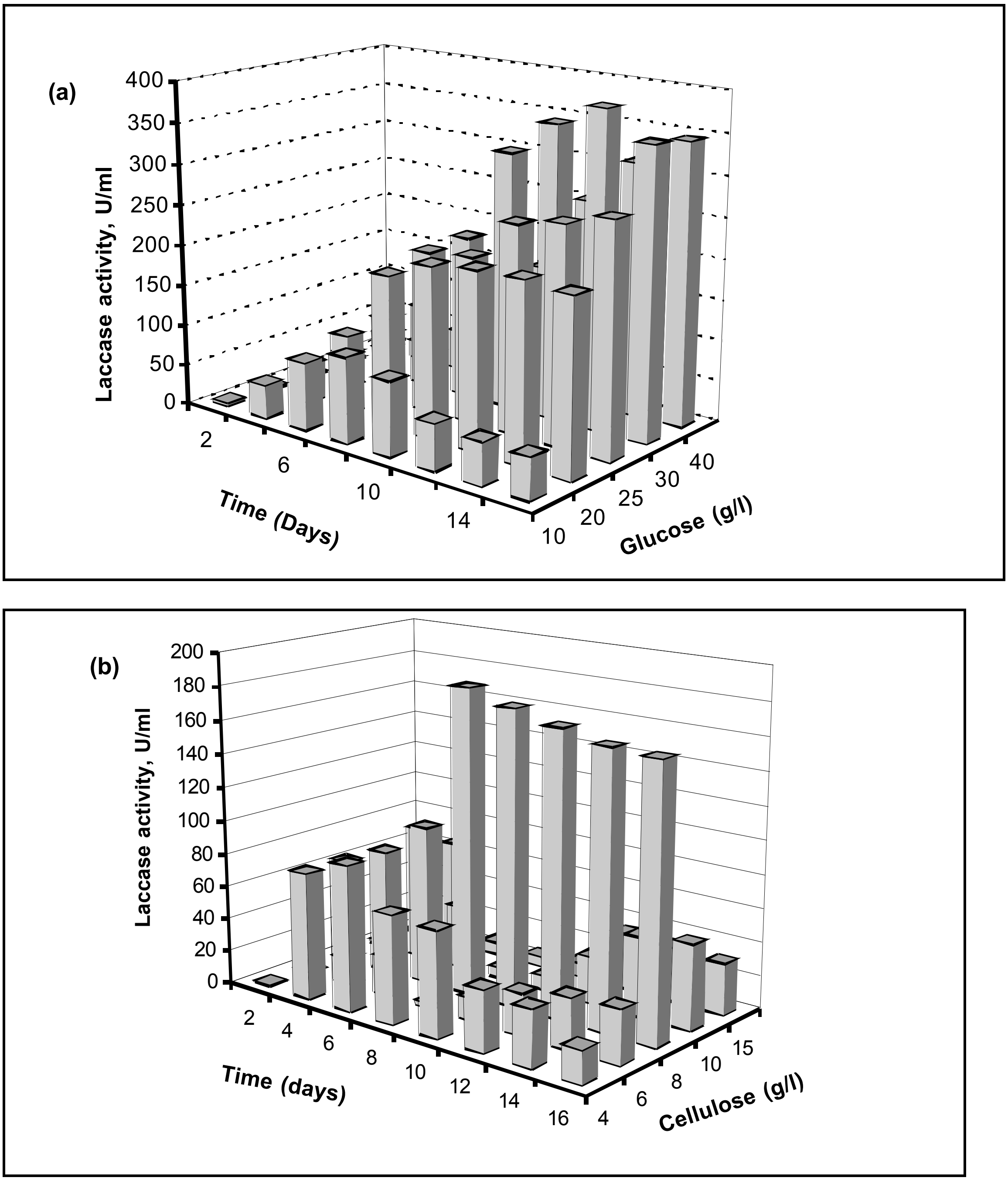
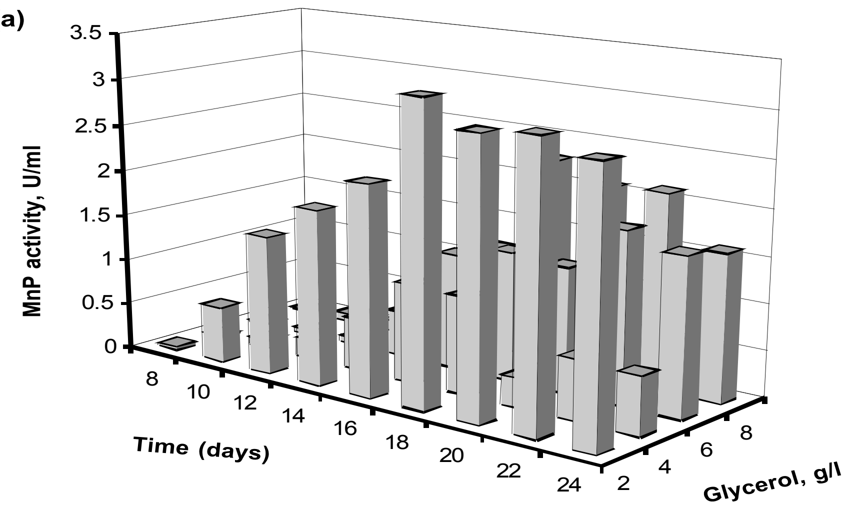


Figure 3(a- b): Time course of laccase production by *Trametes trogii* under different concentrations of glucose and (b) cellulose, in submerged culture fermentation (pH 5.0, 25 ºC)

With cellulose as carbon source, maximum laccase activity observed was 40 % and 52 % less as compared to that observed in the optimum nitrogen concentration and that obtained in the optimum amount of glucose, respectively. 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 concentration up to 30 g/l glucose and 8 g/l cellulose at which their maximum values were reached, and then declined at a concentration of 40 g/l glucose and 15 g/l cellulose, respectively. It was thus found that 30 g/l glucose and 8 g/l cellulose were the suitable carbon concentrations for maximizing the amounts of laccase produced by *Trametes trogii*.

For manganese peroxidase production, glycerol and cellulose were used as carbon sources. 2 g/l of cellulose and 2 g/l glycerol were found to be the optimum amounts required for maximum MnP production (1.6 ± 0.04 and 3 ± 0.06 U/ml, respectively) as shown in Figure 4a and 4b. Maximum production was reached on day 20 and day 18 for cellulose and glycerol containing culture media, respectively. The activities obtained at optimum cellulose and glycerol concentrations were 56 % and 12 % 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 favored by low-carbon medium although production was not significantly higher than that in optimized N medium. High carbon medium resulted into 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 (Tekere *et al.* 2001; 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, which agreed strongly with the present findings.



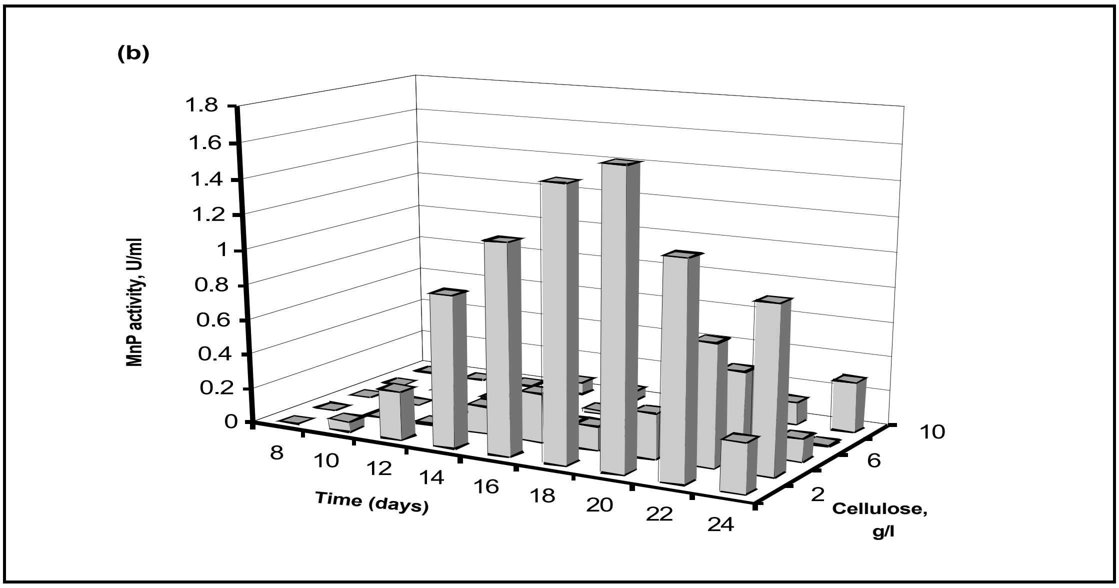
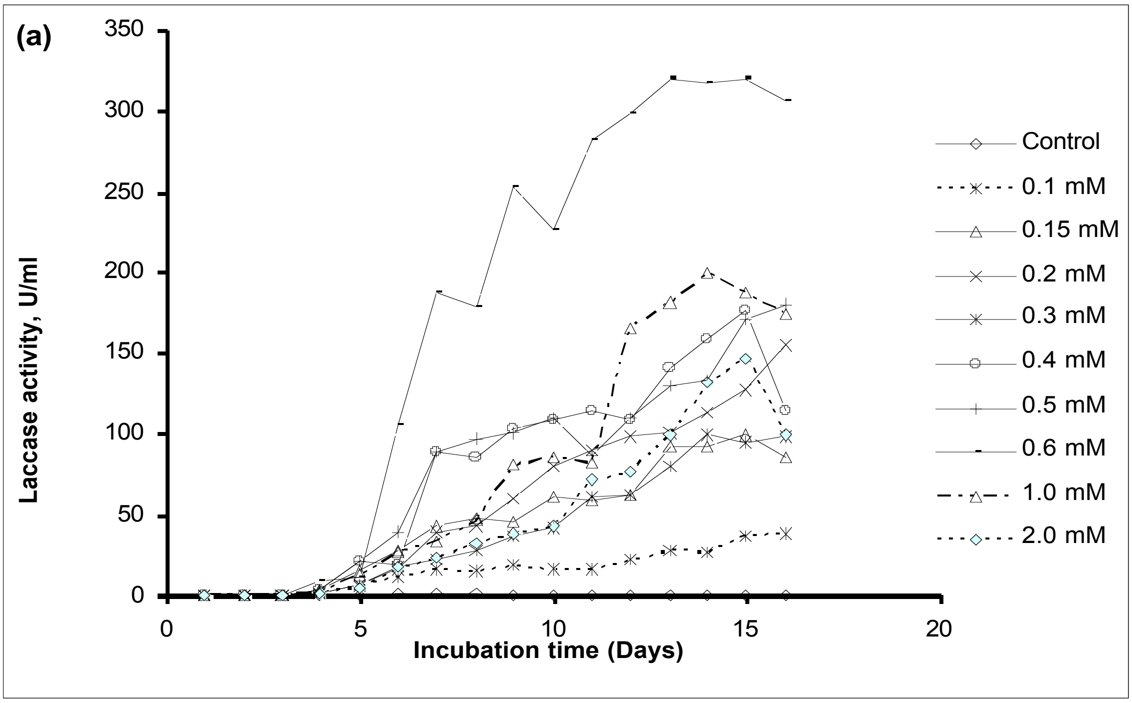


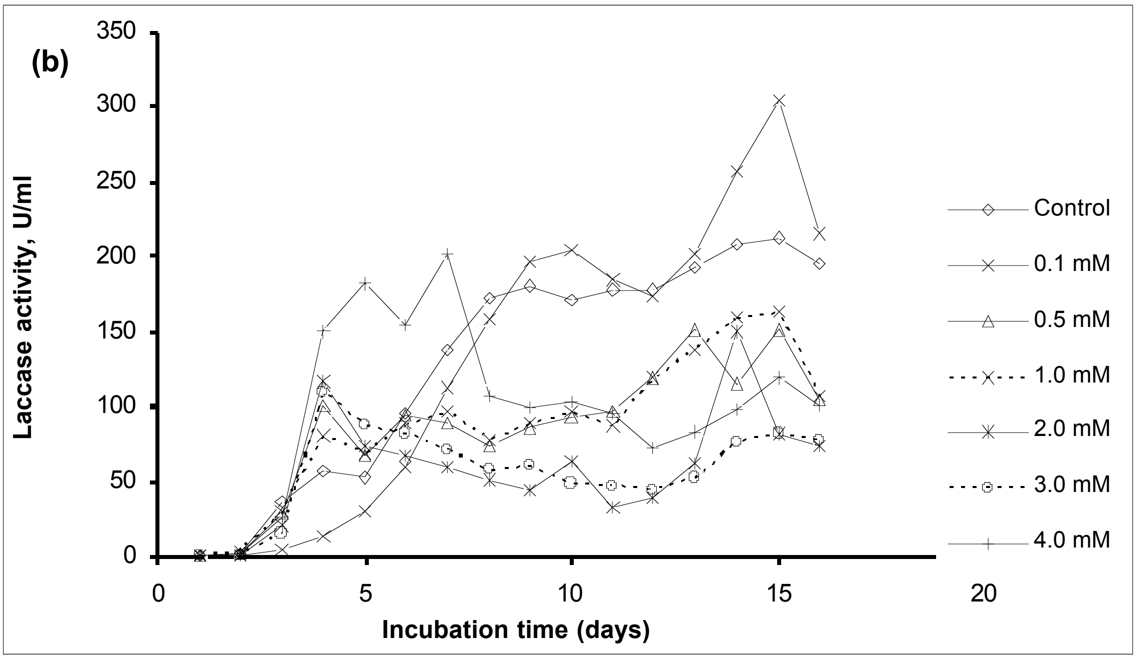
Figure 4 (a- b): Time course of MnP production under different concentrations of (a) glycerol and (b) cellulose, in submerged culture fermentation (pH 6.5, 30 ºC)

Laccase induction

Production of laccase is often enhanced by phenolic and aromatic compounds related to lignin or lignin derivatives such as guaiacol, xylidine or ferulic acid (Gianfreda *et al.* 1999). Cupric ions have been found to be strong stimulants of laccase activities too (Galhaup *et al.* 2002). In the present study, the influence of different concentrations of Cu^{2+}, 2,5-xylidine and ferulic acid on laccase production by *Trametes trogii* was also investigated.

The optimal copper concentration for the maximum laccase production in the submerged cultures was 0.6 mM (Figure 5a). The maximum laccase activity (320 ± 7 U/ml) obtained at this concentration was 366 times higher compared to Cu^{2+} free cultures. The optimal Cu^{2+} concentration observed for this isolate was lower than that (2.0 mM, added after 4 days of incubation) reported by Galhaup and Haltrich (2001) for submerged cultures of *Trametes pubescens,* but was still within the range of 2 to 600 µM 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, Pleurotus ostreatus* and *Volvariella volvacea*, (Palmieri *et al.*, 2000; Chen *et al.*, 2003). It has also been reported (Palmieri *et al.* 2000) that the induction of laccase in *Pleurotus ostreatus* occurred when the fungus is cultivated in a nutrient-rich medium supplemented with 150 µM $CuSO_4$ at the time of inoculation. Also, a Cu^{2+} dose of 1.0 mM is required for enhancement of laccase synthesis by *Trametes multicolor* in bioreactor cultures (Hess *et al.*, 2002). From this study, it was clearly seen that copper concentration above the optimum amount obtained leads to reduction in laccase activity. This may be because at high concentrations, copper acts as a potent inhibitor of fungal growth.


(a)
Laccase activity, U/ml
350
300
250
200
150
100
50
0
0
5
10
15
20
Incubation time (Days)
Control
0.1 mM
0.15 mM
0.2 mM
0.3 mM
0.4 mM
0.5 mM
0.6 mM
1.0 mM
2.0 mM


(b)
Laccase activity, U/ml
350
300
250
200
150
100
50
0
0
5
10
15
20
Incubation time (days)
Control
0.1 mM
0.5 mM
1.0 mM
2.0 mM
3.0 mM
4.0 mM

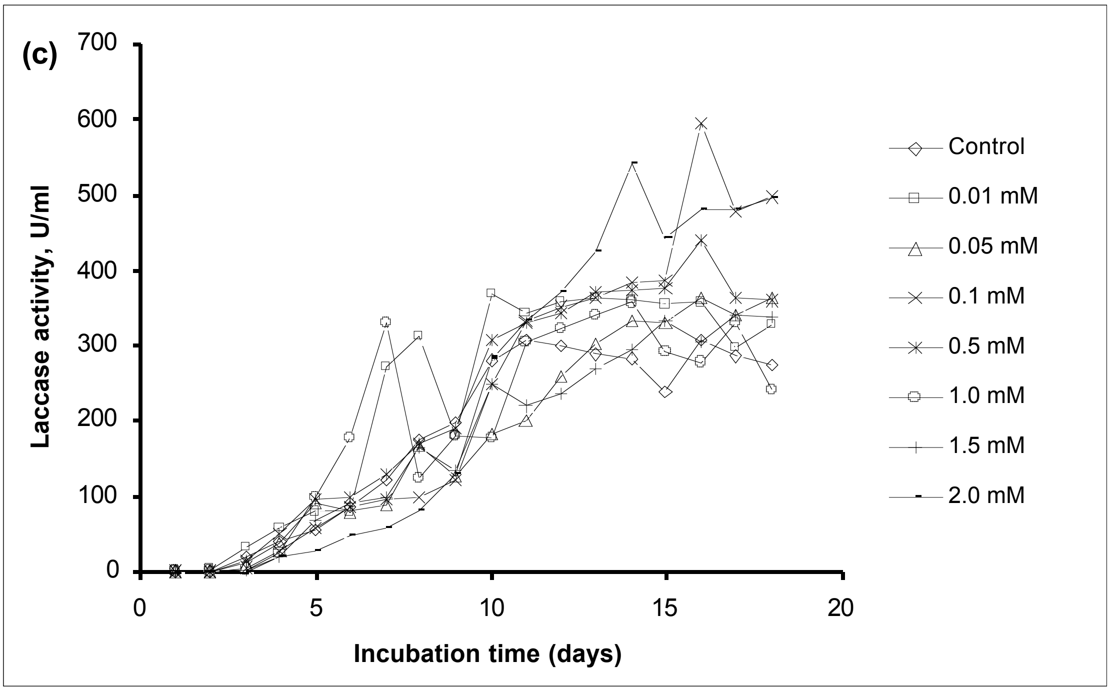


Figure 5(a-c): Laccase activities in *Trametes trogii* under different concentrations of (a) Cu^{2+} (b) xylidine and (c) ferulic acid, in the submerged culture fermentation

2,5-xylidine, the most reported laccase inducer, enhanced laccase production by a factor of 1.5 at the concentrations of 0.1 mM. Maximum laccase activity obtained at this concentration was 304 ± 2.8 U/ml (Figure 5b). Concentrations more than 0.1 mM had a detrimental effect on the organism and the laccase activities were below the control cultures. This may be because at very 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). The present results were consistent with those of other studies carried out by different research groups (Galhaup and Haltrich, 2001; Jang *et al.* 2002; Chen *et al.* 2003; Rancano *et al.* 2003), which reported that xylidine enhanced laccase production in various *Trametes* species and in *Volvariella volvacea*.

The effect of laccase production was examined by adding ferulic acid on the first day of inoculation. The acid was shown to improve production of the enzyme by *T. trogii* markedly. The laccase produced (593 ± 9.6 U/ml) was observed in the cultures with 2.0 mM ferulic acid on the 14th day of cultivation (Figure 5c). This laccase activity is 2 times higher compared to that obtained in cultures without ferulic acid, which had an activity of 307 ± 6.1 U/ml. This is consistent with 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. However, a study by Janusz *et al.* (2006) showed ferulic acid to have no effect on laccase production by *Rhizoctonia praticola* when compared to control culture.

Effects of Manganese ions (Mn^{2+}) concentration on MnP production

The highest MnP production was 4.44 ± 0.18 U/ml and this was observed in cultures containing 0.6 mM Mn^{2+} on the 23rd day of cultivation (Figure 6). This MnP activity obtained is about 5 times higher than the maximum activity (0.93 ± 0.03 U/ml) obtained in the control cultures on the 22nd cultivation day. Figure 6 clearly shows that MnP production was increasing as the concentration of the Mn^{2+} (0.05 – 0.6 mM) increased in the culture medium, but above 0.6 mM, a reduction in MnP activity was observed.

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). However, Fu *et al.* (1997) found both the amount and time of appearance of MnP being affected by the concentration of Mn^{2+} in the *P. sajor-caju* culture medium with highest enzyme levels recorded in cultures supplemented with 15 ppm Mn^{2+}. In the present study, excluding Mn^{2+} had negative effect for Mn-dependent peroxidase and the overall manganese peroxidase activity for *T. trogii* because manganese peroxidase was lowest in the medium with no Mn^{2+} added.

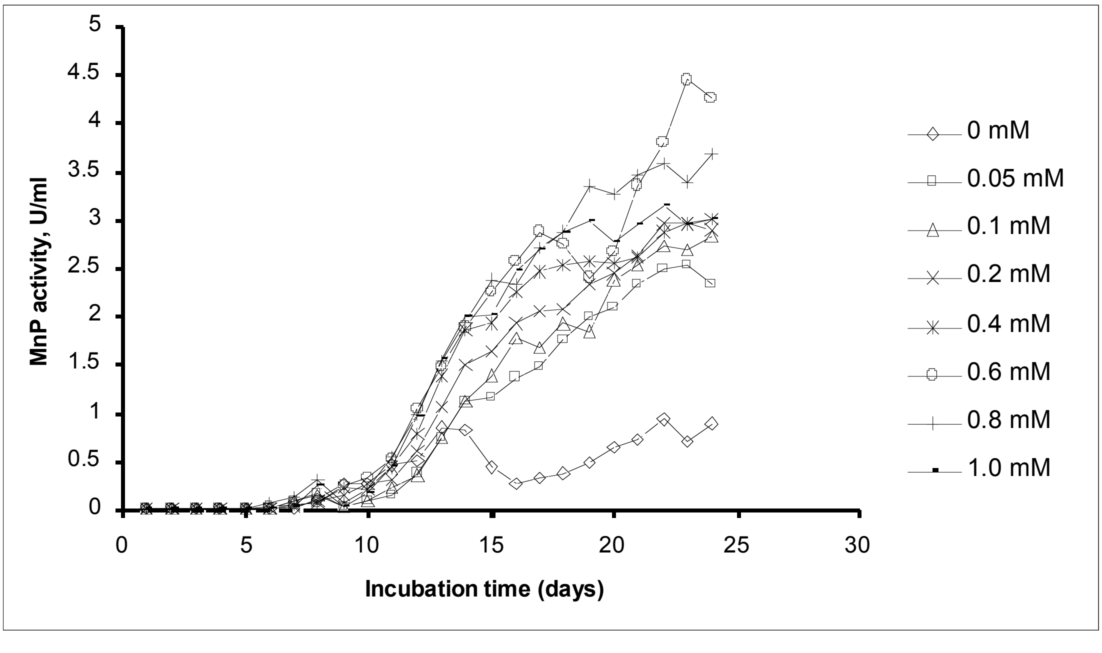


Figure 6: Effects of different concentrations of Mn^{2+} on MnP production by *T. trogii*

Effects of veratryl alcohol concentration on LiP production

The highest LiP produced (0.18 ± 0.01 U/ml) was observed in the culture medium with 1.0 mM on the 20th day of cultivation. The LiP activity obtained here is only 1.2 times higher than the maximum activity (0.15 ± 0.01 U/ml) achieved in the control cultures on the 19th day cultivation period (Figure 7). However, the LiP activity declined in all studied concentrations of veratryl alcohol after 21 days of submerged fermentation.

It has been reported that presence of veratryl alcohol increased LiP activity in *T. versicolor and P. radiata* (Gianfreda *et al*. 1999). In previous studies (Gianfreda *et al*. 1999, Tekere *et al*. 2001) veratryl alcohol was added on the day 3 to 4 when fungal growth was initiated, but in this work, it was added at the time of inoculation. This might have interfered with fungal metabolism, accounting for the observed slight increase in LiP activity. Veratryl alcohol is also produced as a secondary metabolite by some white rot fungi and its exogenous addition may further raise the concentration to levels toxic for enzyme production. Furthermore, veratryl alcohol is believed not to act as an inducer per se, but as either a stabilizer of lignin peroxidase activity or as a redox mediator for substrates that are not directly oxidized by the enzyme (Collins and Dobson 1995).

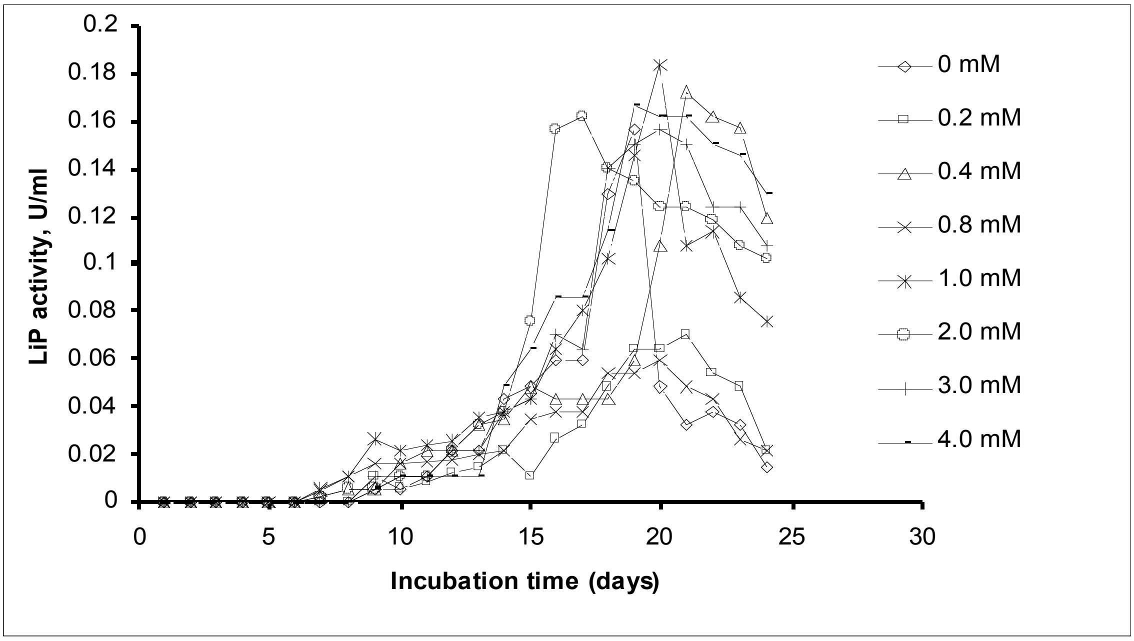


Figure 7: Effects of different concentrations of veratryl alcohol in the culture medium on LiP production by *T. trogii.*

CONCLUSION

This study attempted to optimize culturing conditions in order to improve LiP, MnP and Lac production from *Trametes trogii*. Varying the physicochemical parameters such as incubation temperature and initial medium pH improved the amounts of enzymes produced. Furthermore, altering the media composition, including the addition of inducers such as Cu^{2+}, Mn^{2+}, veratryl alcohol and ferulic acid were significant in the enhancement of enzyme yields.

When *T. trogii* was cultured in the medium with combination of all optimum factors obtained during optimization, LiP, MnP and Lac activities of 0.18 ± 0.01, 4.4 ± 0.18 and 593 ± 9.6 U/ml, respectively, were obtained. Thus, it was found that combination of all optimized operational parameters in the submerged fermentation of *T. trogii* increased LiP, MnP and Lac activities 180, 444 and 258 –fold compared to that observed in non-optimized conditions.

This work provides baseline information on growth parameters optimization for *T. trogii* under submerged fermentation using the optimal conditions. Future studies are focused towards purifying the enzymes as well as applying them in industrial and environmental biotechnology including pulp delignification and bioremediation of recalcitrant pollutants such as textile effluents and soil contaminated with crude oils and agrochemicals.

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