

## Effect of nutrient nitrogen on laccase production, its isozyme pattern and effluent decolorization by the fungus NIOCC #2a, isolated from mangrove wood

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Carbon and nitrogen sources in the growth medium play an important role in the production of lignin-degrading enzymes in the white-rot basidiomycetous fungi. The role of nutrient nitrogen sources in growth media on production of lignin-degrading enzymes namely laccase, lignin peroxidase and manganese peroxidase as well as on the decolorization of industrial effluents like black liquor, molasses spent wash and textile mill effluents was studied using the basidiomycetous fungus NIOCC #2a isolated from mangrove wood. The results indicated that the type of nitrogen source used, not only influences the amount and type of lignin-degrading enzymes produced but also has an effect on the decolorization of these effluents. The amount of extracellular peroxidases increased by several fold in the presence of effluents whereas in their absence they were of negligible quantity. Some of the effluents had an inhibitory effect on laccase production. The effect of nitrogen sources in the absence as well as presence of the effluents, on the expression of laccase isoenzymes was studied by non-denaturing SDS-PAGE. It was noticed that a few new isozymes of laccase were induced in the presence of industrial effluents. Decolorization of these effluents by the concentrated culture filtrate obtained from media containing different nitrogen sources further proved the importance of the type of nitrogen source in decolorization of colored industrial effluents.

[**Key words:** Laccase, isoenzymes, lignin-degrading marine fungus, nutrients, fungi, effluents]

### 1. Introduction

Laccase (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) is a copper oxidase capable of oxidizing phenols and aromatic amines by reducing molecular oxygen to water. It is predominantly present in fungi and higher plants. Laccase is an important enzyme in the lignin-degrading enzyme complex in ligninolytic fungi, the other being lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP). Laccase has a wide range of substrate specificity and is thus used in the degradation of several of xenobiotics including synthetic dyes and industrial effluents<sup>1</sup>. Ligninolytic enzymes of white-rot basidiomycetous fungi have been extensively studied for the degradation of recalcitrant compounds and colored effluents from textile and dye-making industries<sup>2</sup>. Laccases have gained importance recently due to a number of diverse applications such as delignification of lignocellulosics and cross-linking of polysaccharides,

food technological uses, personal and medical care applications, biosensors and analytical applications<sup>3</sup>.

Effective decolorization of bleach plant effluent from paper mill, molasses spent wash from alcohol distillery and synthetic dyes by the basidiomycetous fungus *Flavodon flavus* isolated from decaying seagrass in a lagoon of the Lakshadweep island Kavaratti was reported<sup>4</sup>. This fungus produced all the three lignin-degrading enzymes namely LiP, MnP and laccase, the highest titre being that of MnP<sup>5</sup>. Another white-rot basidiomycetous fungus, NIOCC # 2a, isolated from mangrove wood produced laccase as the major lignin-degrading enzyme and effectively decolorized the colored effluents from paper mill, alcohol distillery and textile mill<sup>6</sup>.

The secretion of extracellular lignin-degrading enzymes by white-rot fungi depends on nutrient source, either carbon or nitrogen<sup>7,8</sup>. The lignin-degrading enzymes are produced during secondary metabolism under conditions of limited nitrogen<sup>9,10</sup>. Industrial effluents varying in their nitrogen content and source may sometimes inhibit the activity of fungal growth or their enzymes. Thus for effective

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bioremediation of such colored effluents by white-rot fungi, it is imperative to study these interactions. Differential regulation of ligninolytic enzyme-encoding genes in response to culture conditions has been documented<sup>11</sup>. Effect of inducers of specific laccase isozymes has been demonstrated in some of the basidiomycetes<sup>12,13</sup>.

Therefore, the major objective of this study was to investigate the effects of various nitrogen sources incorporated in the growth medium on laccase production and decolorization of industrial effluents by the basidiomycetous fungus NIOCC # 2a. Decolorization of these effluents by the culture filtrate (*in vitro*) containing lignin-degrading enzymes obtained from media with different N sources were also compared. Further, we describe the effect of various nitrogen sources and effluents incorporated in the growth medium on the production of laccase isozymes.

## 2. Material and Methods

The fungal culture NIOCC #2a used in this study was isolated from decaying mangrove wood from Choroa island in Goa, India (73°55'E and 15°30'N). The fungus was maintained on malt extract agar slants prepared with half-strength seawater. The culture has been deposited at the Microbial Type Culture Collection (MTCC) Chandigarh, India, under the accession number MTCC 5159 under the Budapest treaty for patent culture deposition<sup>14</sup>. Based on partial large subunit rRNA (D2) gene (~350 bps) alignment with GenBank database, it was shown to have 99% homology to an unidentified basidiomycete species AY187277 (MIDI LABS Inc, Newark, USA). Since the telomorph stage of the fungus has not yet been identified, its further identification is presently not possible. From the ITS sequence, it also showed homology to an unidentified basidiomycete. The sequencing data of ITS and D2 region are deposited in the GenBank under the accession No. AY 939879 and AY 939878 respectively.

### 2.1 Quantitative estimation of lignin-degrading enzymes

A seven day-old fungal culture grown in malt extract broth was washed with sterile water, mechanically homogenized using glass beads under sterile conditions and the resulting mycelial suspension was used at 10% (v/v) concentration for inoculating low nitrogen medium containing different nitrogen sources at a final concentration of 0.1%

nitrogen. The low nitrogen (LN) medium contained a final concentration of 2.5% fructose (1% carbon), 0.01% thiamine, 7% trace elements, 5% basal salts in a total volume of one liter with distilled water. The pH was adjusted to 7 with citrate phosphate buffer. Trace elements contained 1.5 g nitrile triacetate in 500 ml distilled water, the pH was adjusted to 6.5 with 1 N KOH, to which the following components were added; 3 g MgSO<sub>4</sub>, 0.5 g MnSO<sub>4</sub>, 0.08 g CdCl<sub>2</sub>.6H<sub>2</sub>O, 0.1 g FeSO<sub>4</sub>.7H<sub>2</sub>O, 0.1 g ZnSO<sub>4</sub>.7H<sub>2</sub>O, 0.01 g AlK(SO<sub>4</sub>)<sub>2</sub>.12H<sub>2</sub>O, 0.01 g H<sub>3</sub>BO<sub>3</sub>, 0.01 g Na<sub>2</sub>MoO<sub>4</sub>.2H<sub>2</sub>O in a total volume of one liter. Basal salts contained 40 g KH<sub>2</sub>PO<sub>4</sub>, 10 g MgSO<sub>4</sub>, 2 g CaCl<sub>2</sub> in a total volume of one liter. The type of nitrogen sources however varied namely, KNO<sub>3</sub>, glutamic acid, glycine, beef extract and corn steep liquor. The cultures were raised in 250 ml capacity Erlenmeyer flasks, containing low nitrogen media varying only in the type of the nitrogen source utilized, under stationary conditions. The cultures were oxygenated every third day with pure oxygen for 1 min using tygon tubing and Pasteur pipettes, under sterile conditions. Cupric sulphate at a final concentration of 2 mM was added to the 4-day old cultures. The effect of various colored industrial effluents on the production of lignin-degrading enzymes as well as the ability of the fungus to decolorize these effluents *in vivo*, was studied by adding different effluents namely, textile mill effluent A and B (TEA and TEB), molasses spent wash (MSW) from alcohol distillery and black liquor (BL) from a paper and pulp mill, to the 6-day old fungus grown in each nitrogen source. The controls for the experiment were the cultures grown in varying nitrogen sources without any added effluent. The effluents were added to a final concentration of 10% were added to 6-day old culture. Textile effluent A (from Atul Ltd., Gujarat) contained mainly azodye-20 and had a pH of 8.9 with 0.34% carbonate. The textile effluent B from the same source had a pH of 2.5 with 21,700 color units. The black liquor obtained from Seshasayee Paper Mills, Erode, Tamil Nadu, was from a bagasse and wood chip-based newsprint manufacturing unit. As per the data provided by the mill, the effluent had COD of 416 mg l<sup>-1</sup> and BOD of 190 mg l<sup>-1</sup>. Raw untreated molasses spent wash obtained from Rhea Distilleries Ltd., Goa was reported to have a pH of 4.3, BOD of 42,000 mg l<sup>-1</sup> and COD of 80,000 mg l<sup>-1</sup>.

The cultures were allowed to grow for another 6 days after the addition of the effluents. The biomass

of each of the 12-day old cultures was obtained by filtering the contents through oven-dried, pre-weighed Whatman No. 1 filter paper discs. The dry weight of the fungus was determined as the difference in weight after drying the filter papers at 60°C until a constant weight.

The lignin-degrading enzymes, lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase as well as the amount of decolorization that had occurred in the filtrate obtained from these cultures were estimated. The activity of LiP was determined<sup>15</sup> by measuring the rate of oxidation of veratryl alcohol to veratryldehyde in the presence of H<sub>2</sub>O<sub>2</sub>. Manganese peroxidase was determined<sup>16</sup> by measuring the rate of oxidation of Mn<sup>+2</sup> to Mn<sup>+3</sup> in the presence of H<sub>2</sub>O<sub>2</sub>. Laccase activity was assayed<sup>17</sup> using ABTS (2,2'-azino-bis-3-ethyl benzothiazoline-6-sulphonic acid) (Sigma, USA) substrate at pH 3.0. The enzyme units were expressed as units per liter of culture filtrate (U l<sup>-1</sup>).

## 2.2 In vivo decolorization of effluents

Decolorization of the effluents on day 12 was determined by monitoring the absorbance at their absorbance maxima. The absorbance obtained immediately upon addition of the effluent was considered to be 100%. The extent of decolorization was recorded as percentile residual color or percentile decolorization. Decolorization of TEA and TEB were monitored at their absorbance maxima of 505 and 667 nm respectively and MSW and BL were monitored at 475 and 317 nm respectively. All experiments were carried out in triplicates and the average values are presented.

## 2.3 In vitro decolorization of effluents

The enzyme source for the decolorization of effluents, *in vitro* was the five-fold concentrated culture filtrate obtained from the culture grown in various nitrogen sources without the added effluent. Thus, five different enzyme sources corresponding to the different nitrogen sources were used for the *in vitro* decolorization of each of the effluents. The concentration of the culture filtrates was by ultra filtration using Centricon tubes with a 10 kDa cut-off membrane (Millipore, USA) at 5,000 rpm in the cold (4°C). Each of the concentrated culture filtrates with 10 U of laccase activity was incubated at 30°C at a final concentration of 1000 color units of the effluents. Color units of the various effluents were

determined by measuring its absorbance maximum in a UV/Visible spectrophotometer (Shimadzu, Japan). One color unit is defined as the amount of colored material in 1 ml giving an optical density of 1.0 in a path length of 1.0 cm at its absorbance maximum<sup>18</sup>. Decolorization of various effluents *in vitro* was monitored at 6, 12, 24, 36 and 48 hours after addition of the enzyme to the effluent. Decolorization achieved was calculated with reference to the zero hour reading.

## 2.4 Effect of nitrogen source on the laccase isozyme pattern

The culture filtrates from the 12 day-old cultures grown in different nitrogen sources as well as those supplemented with effluent were concentrated by ultra filtration with nanosep centrifuge tubes (Pall Gellman, USA) having 3 kDa cut-off membrane at 5,000 rpm (4°C). After estimating the laccase activity in the concentrates, non-denaturing SDS-PAGE using 12% resolving and 7% stacking gel was carried out with a constant voltage of 60 volts at 6°C. One unit of laccase from each sample was loaded in each well. The gels were observed for laccase activity by staining them with 2.5% guaiacol (SD-Fine Chemicals Ltd., Mumbai). The molecular weights of the laccase isozymes were determined by silver-staining of the corresponding gels<sup>19</sup>.

## 3. Results and Discussion

### 3.1 Growth of NIOCC # 2a in media with different N sources and effluents

Fungal growth was best in the presence of glutamic acid as the nitrogen (N) source. When supplemented with black liquor (BL), the fungus showed enhanced growth in all the nitrogen sources except glutamic acid. Fungal biomass was more in KNO<sub>3</sub> and beef extract (BE) supplemented with molasses spent wash (MSW) than in other N sources. No significant difference was observed between glutamic acid and the same supplemented with MSW. On the other hand, growth was inhibited in corn steep liquor (CSL) and glycine supplemented with MSW. The fungus showed enhanced growth in all of the TEA-supplemented media (Table 1) whereas, growth was inhibited in media containing TEB except in the presence of glycine.

### 3.2 Effect of N source and effluent on the production of lignin-degrading enzymes

Among several of the carbon sources tested, maximum laccase activity was detected when fructose

was used as the carbon source. Among all the nitrogen sources, glutamic acid supported maximum laccase production (Table 1). This trend was maintained even in the presence of TEA and MSW. On the other hand, TEB and BL supported maximum laccase production when glycine was the N source (Table 1). Glutamic acid and glycine being amino acids not only serve as a nitrogen source but also as a readily available source of carbon. This carbon in addition to the carbon supplied by fructose probably aided in biomass build-up which in turn positively affected laccase production. Among, the two amino acids, glutamic acid supplies more carbon than glycine for a constant amount of nitrogen. This is

reflected in the difference in the amount of biomass obtained (Table 1). Irrespective of the nitrogen source used, laccase production was inhibited when supplemented with BL and TEA.

Production of manganese peroxidase (MnP) was enhanced by several fold in the presence of BL in all of the N sources (Table 1). To a certain extent, TEA and MSW also enhanced the production of MnP in some of the nitrogen sources. Similarly, production of LiP was enhanced by several fold in the presence of BL and to a certain extent in the presence of MSW, followed by TEA and TEB (Table 1). Fungal biomass and lignin-degrading enzyme production did not show any correlation.

Table 1—Effect of nitrogen source and effluents on the production of biomass and lignin-degrading enzymes by NIOCC # 2a.

Nitrogen Source	Effluents				
	Control (without effluent)	TEA <sup>#</sup>	TEB <sup>#</sup>	MSW <sup>#</sup>	BL <sup>#</sup>
Biomass (mg/ 20 ml)					
KNO <sub>3</sub>	106.5	141.6	80.4	149.7	118.2
Glycine	81.2	104.8	111.5	53.9	154.2
Glutamic acid	144.7	150.5	96.5	142	106.2
Beef extract	77.4	89.3	74.9	190.4	138.5
Corn steep liquor	95.4	99.1	94.6	61.7	146.1
Laccase Activity (U L <sup>-1</sup> )					
KNO <sub>3</sub>	16,687	2,224	10,385	6,652	2,622
Glycine	34,659	3,702	49,628	5,644	12,210
Glutamic acid	47,567	9,756	20,471	26,552	2,287
Beef extract	10,346	1,979	8,254	8,320	1,445
Corn steep liquor	12,973	620	12,172	2,553	2,058
MnP Activity (U L <sup>-1</sup> )					
KNO <sub>3</sub>	0	353	0	0	2421
Glycine	12	508	30	0	1621
Glutamic acid	0	0	38	394	1679
Beef extract	25	493	0	450	2372
Corn steep liquor	46	1760	222	604	2178
LiP Activity (U L <sup>-1</sup> )					
KNO <sub>3</sub>	26	266	150	85	913
Glycine	2	343	65	98	1114
Glutamic acid	17	75	103	180	2591
Beef extract	25	473	141	0	671
Corn steep liquor	26	29	0	75	0
Percentile Decolorization					
KNO <sub>3</sub>	—	64	78	30	0
Glycine	—	56	88	61	5
Glutamic acid	—	64	70	36	1
Beef extract	—	28	77	49	0
Corn steep liquor	—	53	92	71	0

# TEA =Textile effluent A; TEB =Textile effluent B; MSW =Molasses spent wash;  
BL = Black Liquor

Mansur *et al.*,<sup>20</sup> showed that fructose induced 100-fold increase in laccase production in the basidiomycetous fungus CECT 20197. Stajic *et al.*,<sup>21</sup> demonstrated the effect of inorganic and organic nitrogen sources on laccase production in different species of *Pleurotus*. Our results showed that well defined organic N sources such as glutamic acid and glycine were better than beef extract and corn steep liquor for laccase production. Elisashvili *et al.*,<sup>22</sup> observed highest laccase activity in the medium with ammonium sulfate as the N source in *Cerrena unicolor* IBB 62.

### 3.3 Effect of N source on (in vivo) decolorization of colored effluents

Among the effluents, BL was least decolorized, irrespective of the N source used, whereas TEB was decolorized equally well in all the media (Table 1). Decolorization of TEA and MSW was achieved to a moderate extent in media with different N sources. As the volume and not the color units of the effluent added (10% v/v) was kept constant, decolorization of the individual pollutants varied vastly. The black liquor at the same volume gave intense color with much higher color units than the other effluents. Textile effluent B was less turbid than the other effluents and had a pH of 2.5, the pH at which most of the lignin-degrading enzymes show their optimum activity<sup>23</sup>. Textile mill effluent A with a pH of 8.9 showed less decolorization since the lignin-degrading enzymes show<sup>23</sup> negligible activity at alkaline pH.

Although MnP and LiP production was enhanced in the presence of black liquor, this did not result in its decolorization. On the other hand, mutants of *Phanerochaete chrysosporium*, a well-known lignin-degrading white-rot fungus that lacked the ability to produce LiP but produced MnP, showed about 80% decolorization of bleach plant effluent, suggesting that MnPs play an important role in decolorization of bleach plant effluent<sup>24</sup>. Black liquor enhanced the growth of the fungus, whereas, TEB inhibited its growth. In spite of this, TEB was decolorized up to 70-90%, whereas, black liquor was decolorized only up to 5%. Maximum laccase was produced in the presence of TEB and similarly TEB was decolorized to the maximum extent (Table 1) suggesting that laccases play a key role in decolorization of textile effluents. The important role of laccases in textile dye decolorization has been reported<sup>25</sup>.

### 3.4 Decolorization of the effluents in vitro

All the effluents were decolorized to the maximum by 48 hours *in vitro*. The process might have continued, but the experiment was terminated at 48 h in the present study (Fig. 1). Enzymes from media containing only KNO<sub>3</sub> as N source performed continuous decolorization of TEA (Fig. 1A). On the other hand, enzymes from all the N sources, decolorized TEB continuously (Fig. 1B) without any repolymerization. A similar phenomenon was observed in the case of MSW where continuous decolorization occurred only when enzyme from beef

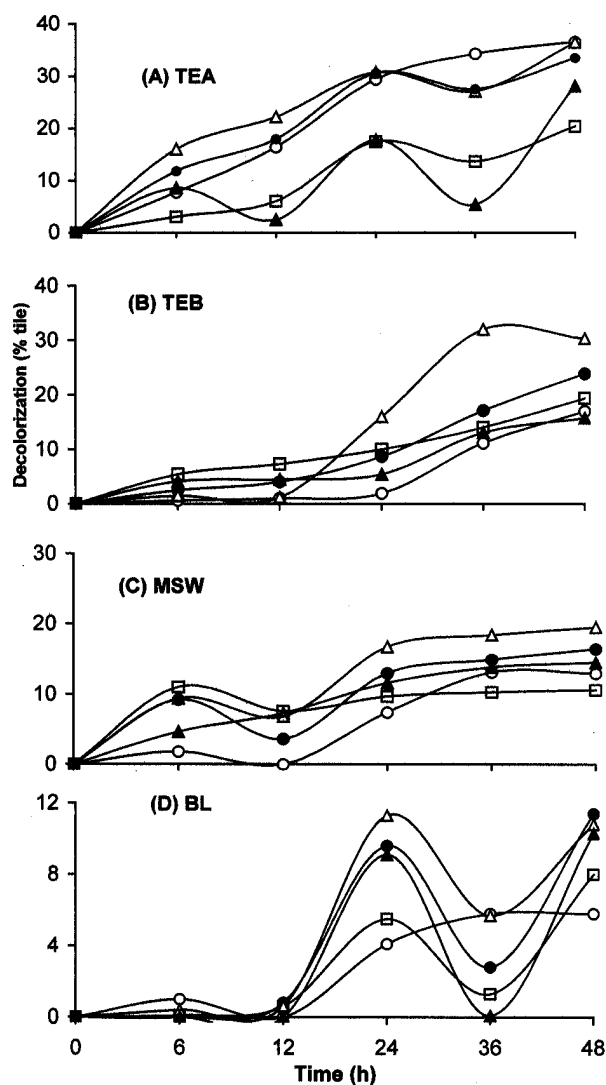


Fig. 1—*In vitro* decolorization of effluents with 1000 color units each, using 10 U of laccase obtained from different nitrogen sources namely, KNO<sub>3</sub>, glycine, glutamic acid, beef extract and corn steep liquor. Decolorization was monitored from 0 to 48 h. KNO<sub>3</sub>= ○ ; Glycine ● ; Glutamic acid △ ; Beef extract ▲ ; Corn steep liquor □.

extract as N source was used (Fig. 1C). Continuous depolymerization of black liquor occurred, when the enzyme source from KNO<sub>3</sub> was used (Fig. 1D). In the presence of enzymes obtained from media containing other N sources, an initial decolorization of BL was followed by increase in color, which is probably due to its repolymerization (Fig. 1D). The decolorization was not the effect of pH, as there was no change in pH at the end of 48 h in any of the reaction mixtures. These results led us to hypothesize that the N source in the medium regulated the production of lignin-degrading enzymes and this in turn affected its decolorization ability. However, no direct correlation between enzyme units and percentage decolorization was observed in any of the N sources. Therefore, it appears that besides the lignin-degrading enzymes and the source of N, the composition of the effluents plays an equally important role in decolorization.

### 3.5 Influence of N source on the isozyme pattern of laccase

To study the production of different isoenzymes of laccase as a function of the type of nutrient nitrogen in the presence as well as absence of effluents, samples containing one unit of laccase were analyzed by non-denaturing SDS-PAGE. The results showed that the nitrogen source not only affects laccase production quantitatively but also the type of laccase

isozymes produced (Figs. 2-4). One of the reasons for differential decolorization of various effluents in the presence of constant laccase units may be attributed to differential isozyme patterns and amount of laccase produced under different N sources. The isozymes may have altered substrate affinity which may affect its decolorization potential. In case of the enzyme source from media without effluent supplementation, The 47 kDa laccase was present irrespective of the N source used in the medium and those supplemented with the effluents except black liquor (Fig. 2). Incidentally, black liquor (BL) showed the lowest decolorization. A faint band of 27 kDa was observed in KNO<sub>3</sub> alone. A 55 kDa laccase was induced in the presence of glycine, beef extract and corn steep liquor, whereas a 25 kDa laccase was induced only in the medium containing glutamic acid as the N source. The 47 kDa laccase was present in all the N sources supplemented with various effluents except BL (Fig. 2). The 55 kDa laccase was present in media containing glycine, beef extract and corn steep liquor as N source (Fig. 2). It was induced only in medium containing KNO<sub>3</sub> supplemented with MSW, whereas it was inhibited in glycine and corn steep liquor (Figs. 2, 3). Only one laccase of 47 kDa was detected in medium with glycine as N source supplemented with TEB (Figs. 2, 4) where maximum laccase

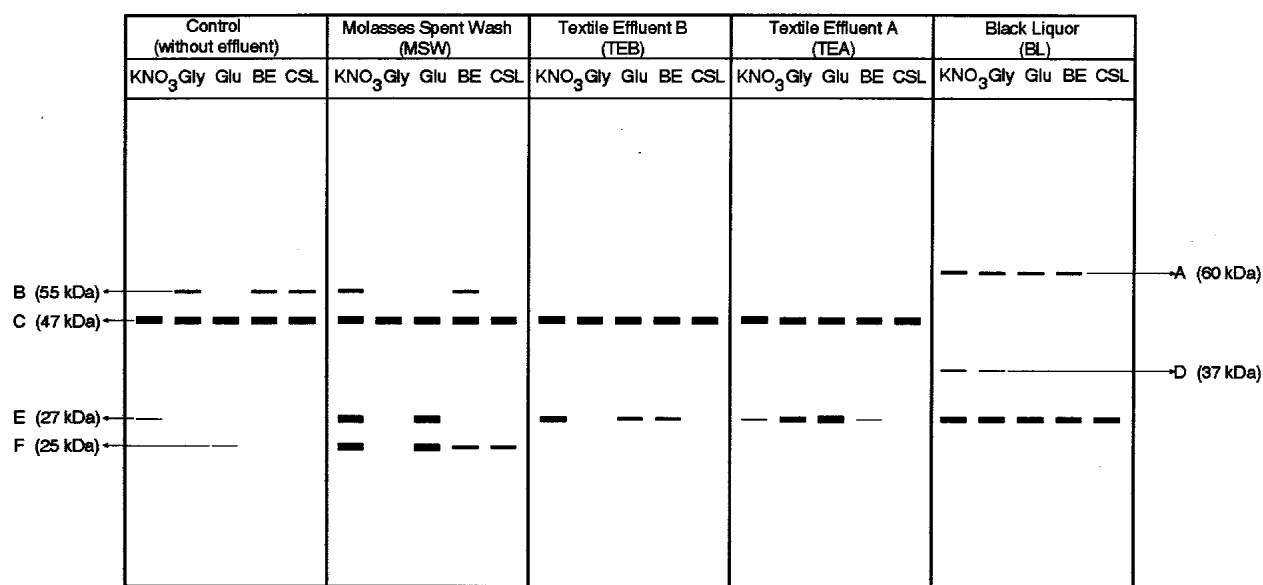


Fig. 2—Schematic representation of the laccase isozyme pattern obtained with non-denaturing PAGE using 12% resolving gel stained with 2.5% guaiacol. The figure shows isozyme pattern of laccase induced in different nitrogen sources, with and without effluent supplements. The nitrogen sources were KNO<sub>3</sub>, Gly (Glycine), Glu (Glutamic acid), BE (Beef Extract) and CSL (Corn Steep Liquor). Bands A – F represent the different laccase isozymes induced and their corresponding molecular weights are given within parenthesis. The figure is drawn to the original scale.

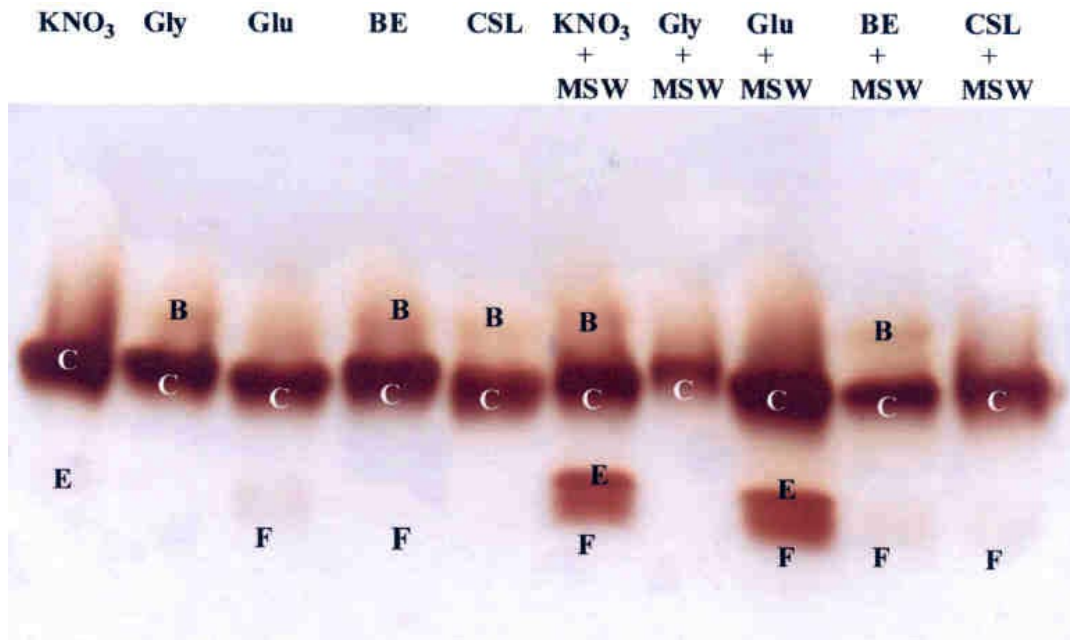


Fig. 3—Non-denaturing PAGE using 12% resolving gel stained with 2.5% guaiacol for detecting laccase isozymes induced in different nitrogen sources and these supplemented with molasses spent wash (MSW). [Band B = 55 kDa; C = 47 kDa; E = 27 kDa and F = 25 kDa].

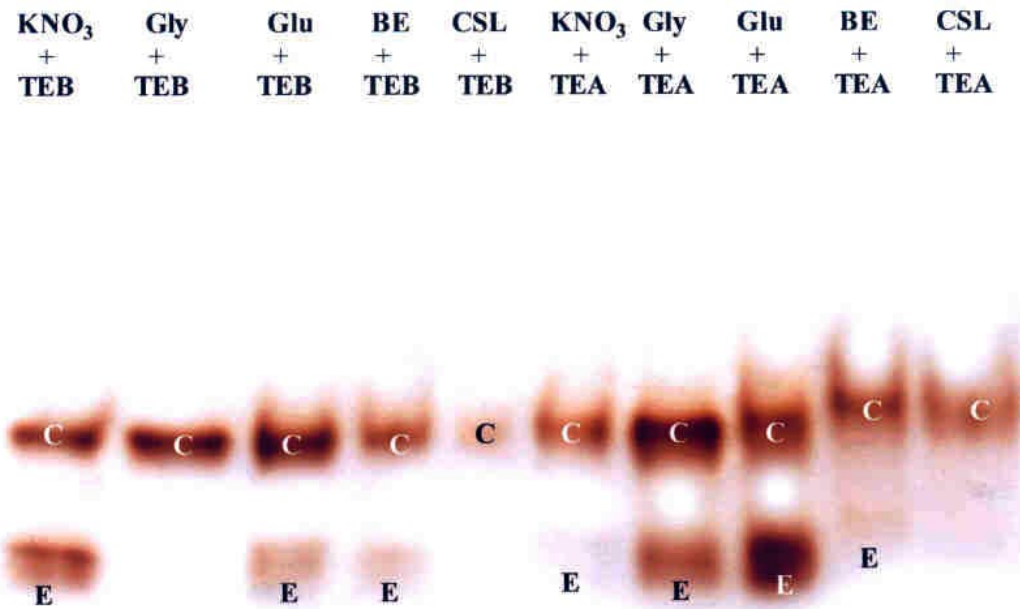


Fig. 4—Non-denaturing PAGE using 12% resolving gel stained with 2.5% guaiacol for detecting laccase isozymes induced in different nitrogen sources supplemented with TEB and TEA. [Band C = 47 kDa and E = 27 kDa].

activity was detected (Table 1). Therefore, the amount of decolorization achieved is dependant on the type of laccase isozymes induced as well as their concentration. The 27 kDa laccase was induced in several other combinations of N source and effluents (Figs. 2-4). The 25 kDa isozyme was present in all the nitrogen sources only when supplemented with MSW, except in glycine (Figs. 2, 3). A 37 kDa isozyme was specific to black liquor supplemented-KNO<sub>3</sub> and glycine media (Fig. 2). Similarly, a 60 kDa isozyme unique to BL-supplemented media was observed except when CSL was the N source (Fig. 2).

Differential regulation of laccase-encoding genes in response to culture conditions has been documented in the terrestrial fungus *Phanerochaete chrysosporium*<sup>11</sup>. Effect of copper on induction of laccase isoenzymes was demonstrated in the white-rot fungus *Pleurotus ostreatus*<sup>13</sup>. We have demonstrated here the effect of the type of N source as well as type of effluent used on the laccase isoenzyme production. Western blot analyses using specific anti-laccase isozyme-antibodies will help in confirming this activity to protein production. Genes that encode laccase isoenzymes in *P. ostreatus* have been cloned and sequenced<sup>26, 27</sup>, but very little is known about the regulation of the laccase gene expression.

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