PHENOL OXIDATION OF PETROL REFINERY WASTEWATER CATALYZED BY LACCASE

María-Carolina Vargas*[1] and Nubia E. Ramírez*[1]

[1]Ecopetrol - Instituto Colombiano del Petróleo, A.A. 4185 Bucaramanga, Santander, Colombia
e-mail: mcvargas@ecopetrol.com.co e-mail: nramirez@ecopetrol.com.co

(Received 23 May 2002; Accepted 6 November 2002)

Laccase has been obtained through two different production systems, the first using *Pleurotus ostreatus* in solid state fermentation, the second one using *Trametes versicolor* in submerged culture. Different substrates (by products from yeast, flour and beverage industries) have been evaluated in both systems. Maximum laccase yield with *Pleurotus ostreatus* (25 U/ml) was obtained in a wheat bran medium. The maximum enzyme concentration level using *Trametes versicolor* (25 U/ml) was achieved in a submerged system containing 10% vinasse, 4,5% wheat bran and 0,2% molasses per liter of waste. Culture filtrate extracted from *Pleurotus ostreatus* was used to remove phenol from wastewater.

The enzymatic treatment is effective over a wide pH and temperature range. The laccase treatment has been successfully used to dephenolize industrial petrol refinery wastewater. The advantage of laccase dephenolization is that this enzyme uses molecular oxygen as an oxidant.

La lacasa se ha obtenido a través de dos sistemas de producción diferentes, el primero usando *Pleurotus ostreatus* en fermentación en estado sólido, el segundo usando *Trametes versicolor* en cultivo sumergido. Se han evaluado diferentes sustratos (derivados de la levadura, harina e industrias de la bebida) en ambos sistemas. La mayor cantidad de la lacasa producida con *Pleurotus ostreatus* (25 U/ml) se obtuvo en un medio de salvado de trigo. El máximo nivel de concentración de la enzima usando *Trametes versicolor* (25 U/ml) se logró en un sistema sumergido con 10% vinaza, 4,5% salvado de trigo y 0,2% de melaza por litro. El filtrado del cultivo extraído del *Pleurotus ostreatus* se usó para remover el fenol de las aguas residuales.

El tratamiento enzimático es eficaz para un amplio rango de pH y de temperatura. El tratamiento con la lacasa se ha utilizado para desfenolizar el agua residual de las refinerías de la industria del petróleo. La ventaja de la desfenolización lacasa es que esta enzima usa el oxígeno molecular como un oxidante.

Keywords: *Phenol, laccase, wastewater, industrial substrate, solid state fermentation.*

* *To whom correspondence may be addressed*

INTRODUCTION

Phenols are present in the wastewaters of a great number of industries, including coal conversion, petroleum refining, resins and plastics, dyes, textiles, mining and pulp and paper. All phenols are toxic; furthermore, phenol and most of its derivatives are considered to be hazardous pollutants.

Conventional methods for dephenolization of industrial wastewaters include solvent extraction, microbial degradation, adsorption on activated carbon and chemical oxidation. These methods although effective and useful, suffer from serious drawbacks such high costs, low phenol removal efficiency, formation of hazardous by-products, and applicability to only limited phenol concentration range (Aitken, 1993). Therefore, alternative technologies are highly desirable. This led to the development of an enzymatic approach to treat phenol containing aqueous effluents. Enzymes are known to be highly selective catalysts and therefore have been proposed as a means of removing targeted substances from wastewaters. Several researches have demonstrated previously that oxidation of phenolic poIlutants can be catalyzed by different enzymes (Aitken, 1993) including horseradish peroxidase, chloroperoxidase, from the fungus *Caldaryomyces fumago,* laccase from several microbial sources, lignin peroxidase from the white rot fungus *Phanerochaete chrysosporium* and polyphenol oxidase.

Atlow *et al.,* (1983) observed in their horseradish peroxidase system that compounds having high removal efficiencies assisted in the removal of more recalcitrant substances. This is likely due to the radicals produced from one phenolic compound reacting with the more recalcitrant compound. To investigate whether this phenomenon occurred in the laccase system, reaction mixtures were prepared with 2,4 dichlorophenol (readily removed) and phenol (recalcitrant). The results showed an improvement in the removal efficiency of 5% when phenol and 2,4 dichlorophenol were incubated with an enzyme extract compared with phenol alone.

The purpose of this work is to present a concise study of the laccase originating from *Pleurotus ostreatus* and *Trametes versicolor*. Specifically, their production, oxidation procedure and conditions for phenol removal in spiked matrix and refinery wastewater sample were investigated.

MATERIALS AND METHODS

Microorganisms and Culture Conditions

Trametes versicolor ATCC 20869 and *Pleurotus ostreatus* (isolated from Chapas, México) were maintained on malt agar plates at 4°C. They were used to inoculate 200 ml of Malta broth in 1 liter Erlenmeyer flasks. Ten to twelve day old pellets incubated at 20°C and 200 rpm were used as inoculum.

Enzyme production

Two simultaneous studies were conducted for enzyme production. The first one was done in submerged state using *Trametes versicolor*. Different products from beverage, flour and yeast industry, with different ratios among them, were studied as potential substrates. Table 1 shows substrate and mixtures composition evaluated for enzyme production. The second study was performed using *Pleurotus ostreatus* in solid state fermentation system. The medium contained 250 g of sterilized wheat bran, 250 ml buffer phosphate pH 6,5 and vinasse 10% as an inducer. The fungus was cultivated in flasks at 22°C. After 20 days, the enzyme was extracted from the culture medium as a paste that was washed with a phosphate buffer (pH 6,3) at 1:1 v/w ratio.

The suspension was filtered through cheesecloth and the supernatant obtained from both *Pleurotus ostreatus* and *Trametes versicolor* was centrifuged at 4°C 10 000 rpm for 15 minutes for solids separation. Cultures filtrates were concentrated by membrane ultrafiltration (MWCO = 50 000). Samples were taken aseptically, and protein concentration and laccase activity defined as the amount of ABTS oxidized per unit of time, were determined (Kadhim *et al.,* 1999; Bollag and Leonowicz, 1984).

Chemicals

Phenols and all other chemicals used in this study were obtained commercially of analytical grade; wheat bran and vinasse were obtained from the flour and yeast industry, respectively. Two different samples of phenol containing wastewater were examined in this work.

Table 1. Media composition for laccase production using *Trametes versicolor*

Medium identification	Composition
A	10% Vinasse + 4,5% Wheat bran
B	10% Vinasse + 4,5% Wheat bran + 0,2 % Molasses
C	10% Vinasse + 4,5% Wheat bran + 0,5 % Liquor
D	10% Vinasse + 5% Liquor
E	10% Vinasse + 1% Liquor
F	10% Vinasse + 4,5% Wheat bran + 10 % Yeast
G	10% Vinasse + 10% Yeast

One is sour water, petrol refinery process water that has come into contact with the process streams (nafta). The other one is from the wastewater treatment plant. A Colombian petroleum refinery kindly provided both.

Phenol quantification

The disappearance of phenols was monitored by high performance liquid chromatography (HPLC) using a HP 1090 series II with a diode array detector and the software of HP ChemStation version A.06.03. A reverse phase column, Supelcosil LC_{18} (5 µm, 4,6 mm i.d. x 25 cm), was used and the mobile phase consisted of acetonitrile in acetate buffer (50 mM, pH 4,6) at a gradient from 40 to 100% acetonitrile in 30 min. After column equilibration, 20 µl of reaction solution was injected. Compounds were detected by their absorbance at 270 nm. The absorption spectra of reaction solution were determined using the same spectrophotometer.

Measurement of protein and laccase activity

Extracellular Protein concentration in the extracellular extract was determined using Bio-Rad reagent (Bradford, 1976). Laccase was able to oxidize ABTS [2,2'-Azino-di-(3-ethylbenzothialozin-6-sulfonic acid)]. The assay mixture contained 0,5 mM ABTS and 40 mM citrate buffer, pH 5,6. Oxidation of ABTS was followed by an absorbance increase at 420 nm (ε_{420} = 36 m M^{-1} cm^{-1}) at a temperature of 25°C. Enzyme activity was expressed in international units.

Enzyme stability with respect to pH and temperature

For determination of temperature stability, samples of crude laccase, from *Pleurotus ostreatus* solid fermentation, were pre-incubated for 24 h in a range of temperatures from 10°C to 70°C. Samples were taken every two hours, brought to assay temperature and tested for remaining laccase activity. The pH stability was determined by incubating samples in acetate buffer 0,2 M (pH 4,5 and 6); phosphate buffer 0,1 M (pH 7 and 8); carbonate buffer 0,1 M (pH 9,19 and 11) for 96 hours at room temperature. Samples were tested at assay pH for remaining activity.

Laccase-catalyzed oxidation of Phenols

Typical experiments were performed in 100 ml flasks. Each flask contained 25 ml of the total reaction medium, including a predetermined volume of laccase concentrate (~ 25 U/ml) to achieve the desirable enzyme concentration, a specific volume of phenol spiked solution, and the remaining volume of citrate phosphate buffer solution (50 mM, pH 5,0). The reaction was conducted at room temperature and 100 rpm.

At regular intervals, aliquots were taken and acidified with 20% HCl solution until achieving a pH 2 to stop the reaction. The solution was assayed for phenol quantification after centrifugation to remove the precipitate. Two more experiments were simultaneously conducted: buffer+phenol without enzyme for evaporation and monitoring photoxidation effects, and buffer+phenol+enzyme replicate.

EXPERIMENTAL STRATEGY

Substrate study for laccase production

Submerged and solid state fermentation were evaluated using different substrate composition with

Pleurotus ostreatus and *Trametes versicolor*, as described above.

Study on spiked phenol matrix

Three differents phenol concentrations were selected to explore phenol oxidation performance: 25, 100 and 400 mg/l, they were chosen based on preliminary industrial wastewater characterization. Crude laccase broth from solid state fermentation was used.

Petrol refinery wastewater enzymatic oxidation

Two petrol refinery samples were tested, sour water and wastewater. Oxidation was conducted without pH adjustment with wastewater sample. The enzyme concentration was 5 U/ml . The enzyme was added in the following order: 1,5 ml at time zero, and 0,5 ml at 1, 2 and 4 hours of reaction time. Crude Laccase broth from solid state fermentation was used.

RESULTS AND DISCUSSION

Laccase production by *Pleurostus ostreatus* and *Trametes versicolor* fermentation

Wheat bran, vinasse, molasses, liquor and inactive yeast were selected as a low cost and readily available substrate for the grow of *Trametes versicolor*, see Table 1.

Table 2. Enzymatic phenol oxidation. Effluent of wastewater treatment plant at Colombian refinery, 16,1 mg/l of phenol, pH 4,6 , laccase 5 U/ml

Time, hours	Average phenol concentration, mg/l	Average removal, %
0	16,01	
1	11,00	31,67
2	8,32	48,32
4	5,19	67,76
6	3,23	79,93
12	0,22	>98,63
24	ND	>98,63
48	ND	>98,63

N.D.: not detectable

Table 3. Enzymatic phenol oxidation. Sour water from a petrol refinary 248,36 mg/L of phenol, pH 7,3 , laccase 5 U/ml

Time, hours	Average phenol concentration, mg/l	Average removal, %
0	248,35	
1	210,6	15,20
2	188,15	24,24
4	160,19	35,49
6	121,80	50,95
12	37,89	84,74
24	0,49	99,8
48	0,39	99,84

During submerged state fermentation samples were taken at 5, 12 , 15, 20 and 25 days. Figure 1 shows laccase activity behavior along time for the different substrates and mixtures evaluated. Enzyme production shows the higher slope from 5 to 12 days. Medium B gave the highest enzyme activity, 25 U/ml on the 20th day followed by media C and F with laccase activities of 18,2 and 16,4 U/ml , respectively. Fermentation times longer than 20 days resulted in a decrease in enzyme activity for all experiments.

Similarly, Bollag and Leonowicz (1984) reported a laccase activity of 14 U/ml with *T. versicolor* after 40 days of fermentation using rich medium and 2,5 xylidine as an inducer. They achieved an activity of 5 U/ml with *P. ostreatus* using the same medium. Hunolstein *et al.,* (1986) obtained an activity of 100 U/ml with no inducer addition with a mutant strain of *T. versicolor*. Eggert *et al.,* (1996) achieved 10 U/ml using *T. versicolor* with xylidine as an inducer. The present work achieved a laccase activity of 25 U/ml after 20

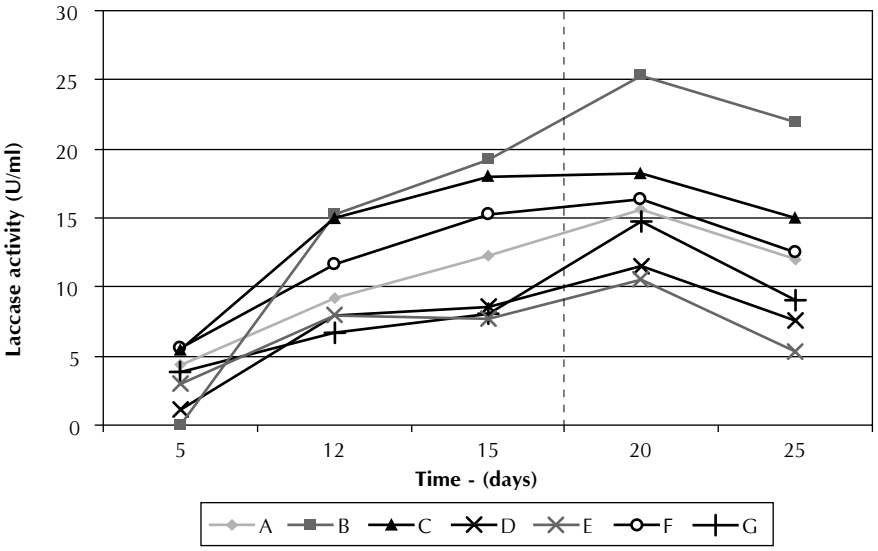

Figure 1. Laccase activity as function of different substrate composition. Letters A to G refer to media as described in Table 1

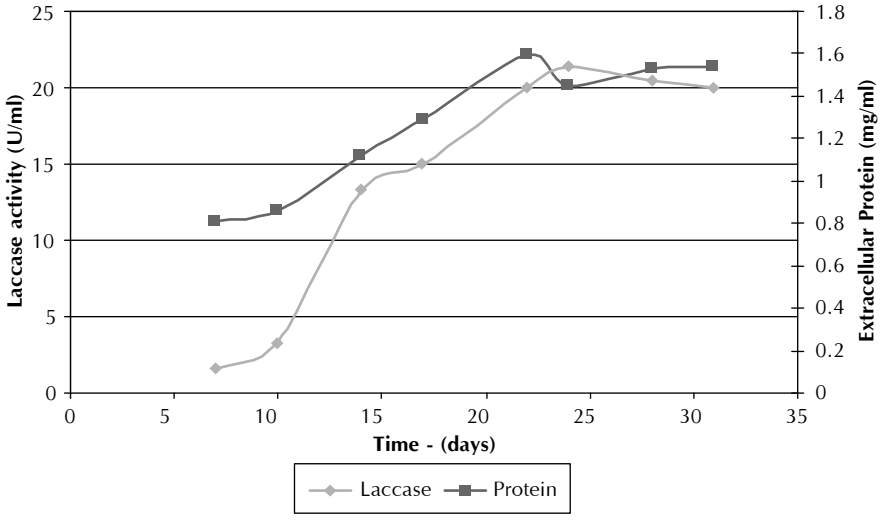

Figure 2. Time course of a *Pleurotus ostreatus* solid-state fermentation. The ABTS was used to measure the residual activity after different pre-incubation times

days of fermentation operating in a submerged state with *Trametes versicolor* ATCC 20869 and industrial by-products.

Laccase production using *Pleurotus ostreatus* is shown in Figure 2. The culture was allowed to grow until the laccase level had reached a peak and then declined. The pH of the medium initially was 6,3 and dropped over the course of 31 days to pH 5,8. Laccase production increased gradually, reaching a peak after 22 days of incubation (25 U/ml). Protein production appeared to coincide with peak laccase concentration (1,6 mg/ml) after a gradual increase. Laccase and protein concentrations on the 31st day were 21 U/ml and 1530 mg/l, respectively. Finally, after incubation for 31 days at 22°C, the culture had grown sufficiently to be harvested for enzymes. Laccase activity from the wheat bran culture was 21 U/ml ± 2 U/ml.

Based on similar laccase production level in both submerged and solid state systems, this paper focus the following stability and oxidation studies on the laccase obtained from solid state fermentation by *P. ostreatus*.

Enzyme stability with respect to pH and temperature

Laccase had an optimum temperature of 50°C (Figure 3). Laccase was stable at temperatures ranging from 10°C to 50°C for 24 h monitoring time. As shown in Figure 3, laccase was sensitive to heat, and it lost 40% of its activity after 24 h exposure to 70°C. However, laccase retained 85% of its activity after being pre-incubated at 60°C, depicting some temperature stability. No loss of activity was detected when laccase samples were stored at lower temperatures (22 or 4°C) for up to a month (data not shown). Most of the laccase samples were stable in a pH range from 7 to 9. Whereas at pH 10 (Figure 4), laccase became partially inactivated. Incubation at pH 4 caused a marked and irreversible inactivation of laccase.

Phenol removal by crude laccase preparation

Based on preliminary experiments (data not shown), when 5 U/ml of laccase was added to 15 ml of phenol solution (25 - 400 mg/l), the solution gradually turned black. Upon subsequent stirring, a black precipitate formed gradually. After one hour of stirring (first sampling), the precipitate was separated by centrifugation and the supernatant assayed for phenol. No appreciable reduction in phenol concentration was observed in the controls having the same phenol concentrations but no laccase.

Effect of Phenol Concentration

It was important to establish how the efficiency of the laccase catalyzed phenol removal depends on various parameters, such as pH, aeration, substrate con-

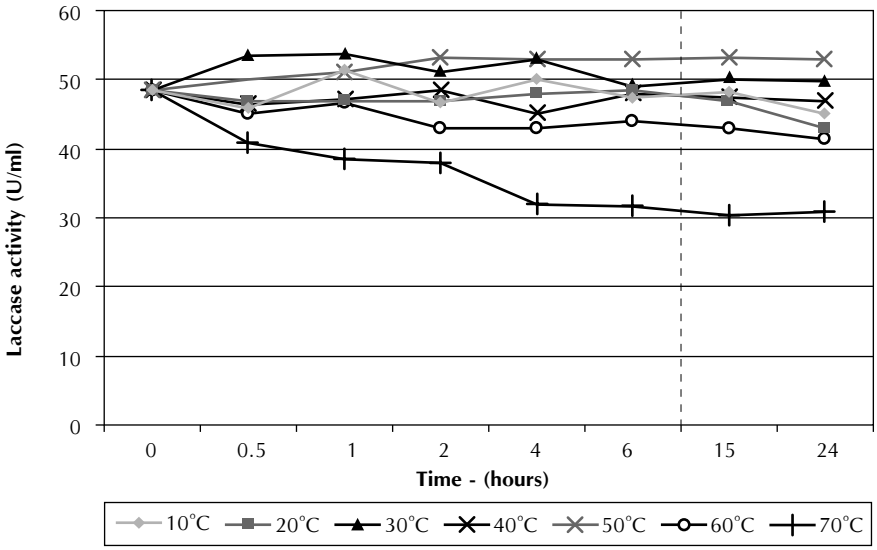

Figure 3. Stability of laccase at various temperatures. The ABTS was used to measure the residual activity after different pre-incubation times

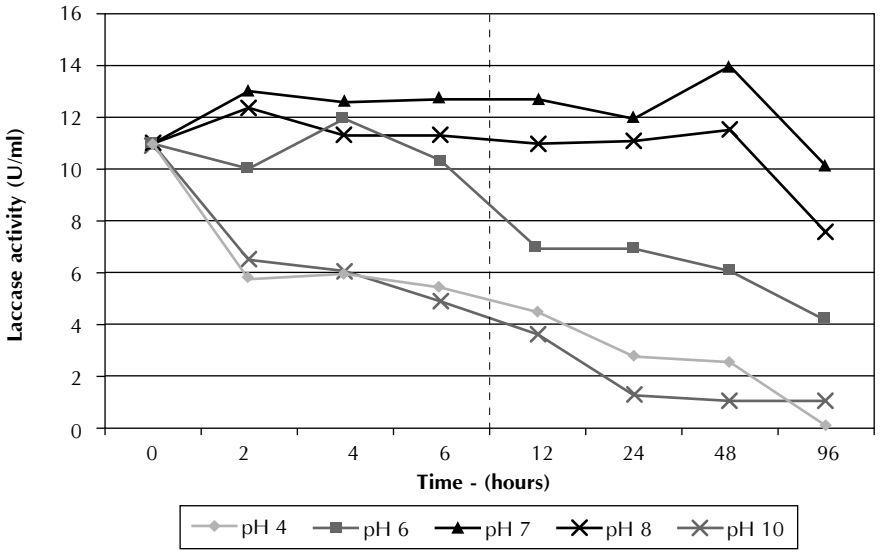

Figure 4. Stability of laccase in buffers with variable pH-values

centration, and co-substrate presence. Figure 5 shows the phenol concentration dependence of the removal efficiency, i.e., the percentage of phenol removed. It can be seen that the enzyme was effective at all of the phenol concentrations (25, 100 and 400 mg/l) evaluated. At a fixed enzyme concentration, the maximum removal percentages varied with the initial phenol concentrations. For instance, at 400 mg/l phenol removal was 16% faster than that at 25 mg/l.

Effect of Aeration

Laccases are enzymes containing copper. They utilize molecular oxygen as a terminal electron acceptor. Therefore, an air addition strategy was evaluated to

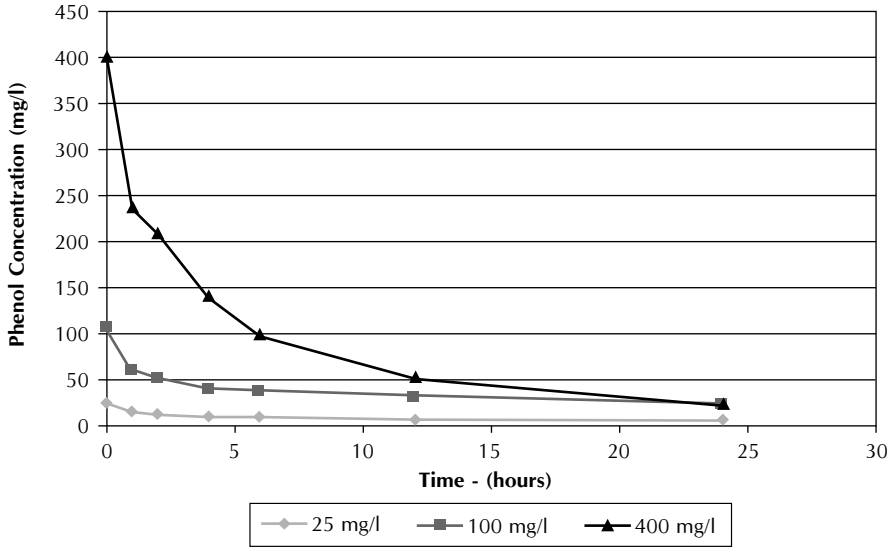

Figure 5. Effect of initial phenol concentration on phenol removal, crude laccase 5U/ml, pH 5,0 without air addition

study its effect on enzymatic oxidation. The experiment was conducted with solutions of 100 mg/l of phenol.

While all systems were stirred, only one set was under aeration. The auxiliary aeration system did not cause appreciable effects, 90% of phenol was removed after 24 h of incubation (data not shown), indicating that the oxygen supply by stirring was enough under the experimental conditions. The results from the controls without laccase showed that the phenol loss was 1,4% for the systems with aeration and 0,1% for those without.

Phenol removal in real industrial samples

Removal results for both water samples are summarized in Tables 2 and 3. The wastewater effluent was studied without pH adjustment, while the pH of sour water was adjusted to 7,3 before initiating the experiments. Both showed phenol removal efficiency higher than 98%. It should be noted that the phenol removal percentages were higher with these real samples than with the pure phenol solutions. The responsible mechanisms need to be identified. Nonetheless, the use of crude laccase preparation proved to be satisfactory for the removal of a range of phenolic compounds.

CONCLUSIONS

- Laccase could be obtained from both submerged and solid state fermentation processes using industrial by- products. *Trametes versicolor* showed the highest enzyme activity, 25 U/ml on the 20th day. achieved the highest laccase level, 25 U/ml after 22 days of incubation

- Laccase had an optimum temperature of 50°C and it was stable in a pH range from 7 to 9. Crude laccase broth was able to oxidize all the phenol concentrations evaluated. The use of crude laccase broth proved to be satisfactory for the phenol removal in both real industrial samples.

ACKNOWLEDGEMENTS

The authors express sincere appreciation to Fulvia Sánchez (Chemist MSc) for her contribution in phenol analysis. The authors also express appreciation to Ecopetrol - ICP for the financial support.

REFERENCES

Aitken, M. D., 1993. "Waste treatment applications of enzymes: opportunities and obstacles". *The Chemical Engineering Journal,* 52: B49-B58.

Atlow, S., Bonadonna-Aparo, L. and Klibanov, A. M., 1983. "Dephenolization of Industrial wastewaters catalyzed by polyphenol oxidase". *Biotechnology and Bioengineering,* 16: 599-603.

Bollag, J. M. and Leonowicz, A., 1984. "Comparative studies of extracellular fungal laccases". *Appl. and Environm. Microbiol.,* 48(4): 849-854.

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

Eggert, C., Temp, U. and Eriksson, K. E., 1996. "The ligninolytic system of the white rot fungus Pycnoporus cinnabarinus: Purification and Characterization of the laccase". *Appl. Environm. Microbiol.,* 62(4): 1151-1158.

Hunolstein, C. V., Valenti, P., Visca, P., *et al.*, 1986. "Production of Laccases A and B by a mutant strain of Trametes versicolor". *J. Gen. Appl. Microbiol.,* 32, 185-191.

Kadhim, H., Graham, C., Barrat, P., Evans, C. S. and Rastall, R. A., 1999. "Removal of phenolic compounds in water using *Coriolus versicolor* grown on wheat bran". *Enzyme and Microbial Technology,* 24: 303-307.