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PHENOL REMOVAL THROUGH COMBINED BIOLOGICAL AND ENZYMATIC TREATMENTS

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Abstract - This work studies the use of biological and combined biological/enzymatic treatments in phenol degradation. The systems studied were conventional batch aerobic biological followed or preceded by enzymatic treatment. Tyrosinase extracted from the mushroom *Agaricus bispora* was employed.
Biological treatment efficiently degraded effluents containing up to 420 $mg.L^{-1}$ of phenol, removing 97% of the COD and 99% of the phenol in 48-hour batches. Alterations in phenol concentration intake reduced treatment efficiency significantly. Enzymatic polishing of biotreated effluent removed up to 75% of the remaining phenol in a four-hour reaction with 46 $U.mL^{-1}$ of tyrosinase and 50 $mg.L^{-1}$ of chitosan (used as coagulant). Enzymatic pretreatment with 20 $U.mL^{-1}$ of tyrosinase reduced the phenol concentration by 25 % after 2 hours of reaction, although initial COD increased up to 58%. The subsequent biological treatment of that enzymatic pretreated effluent reduced COD to 151 $mgO_2.L^{-1}$ and phenol concentration to 1 $mg.L^{-1}$ in 24-hours batches.
Keywords: tyrosinase, phenol removal, biological treatment.

INTRODUCTION

Phenol is a major pollutant present in the wastewaters from several industrial activities: coal mining, petrol refining, pharmaceutical production, founding and steel and iron manufacture, and the tanning and finishing of leather (U.S.EPA, 1980). Commonly used conventional treatments (biological, chemical oxidation and adsorption) often fail to generate final effluents with the required discharge quality at affordable costs. Considering that in Brazil legal discharge limit for phenol is 0.5 $mg.L^{-1}$, the importance of developing treatment technologies that achieve this limit must be emphasized (CONAMA, 1991).

In the last two decades, several researchers have studied the use of enzymes in wastewater treatment (Karam and Nicel, 1997). Enzymes have many potential advantages over conventional biological treatment and/or chemical oxidation: the lack of an acclimatization period, the absence of problems related to charge shocks or toxic effects, and no generation of unexpected products due to their high specificity.

Tyrosinase (also known as polyphenoloxidase; EC 1.14.18.1) is involved in the phenomenon of browning of fruits and is present in mushrooms, apples and potatos. This enzyme catalyzes phenol oxidation with molecular oxygen through two distinct reactions: the ortho-hydroxylation of phenol producing cathecols and its dehydrogenation producing quinones. Quinones are extremely

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unstable in aqueous solutions, reacting nonenzymatically to produce brownish insoluble polymers (Duckworth and Coleman, 1970). These polymers can be removed from solution through many process such as adsorption, precipitation and flotation.

Chitosan, a polycationic polymer produced by chitin deacetilation, is commonly used as adsorbent or coagulant. Wada et al. (1995) and Gangidoust et al. (1996) showed in their research that it is more effective to use chitosan in an acetic solution as coagulant agent than to use its flakes as adsorbent. In this way smaller quantities of the product are required in order to remove suspended materials.

The present work addressed the efficiency of enzymatic treatment using tyrosinase (either for pretreatment or polishing with chitosan as a coagulant) in phenol removal together to conventional aerobic biological treatment.

MATERIALS AND METHODS

All reagents used were of analytical grade. Chitosan was from Aldrich; 4-aminoantipirin (4-AAP), from Sigma; l-tyrosine; $K_3Fe(CN)_6$ and acetone, from Vetec; and phenol, K_2HPO_4, urea and acetic acid, from Reagen. The mushrooms used (*Agaricus bispora*) were bought at a local market.

Tyrosinase Extraction

Mushrooms were triturated with acetone and then filtrated; the resulting cake was frozen. Afterwards, the frozen cake was resuspended in distilled water and filtrated again to obtain enzymatic extract containing tyrosinase (Atlow et al., 1984). The activity of extracted tyrosinase was from 2,000 to 8,800 $U.mL^{-1}$, depending on the lot of mushrooms used.

Tyrosinase Activity

One activity unit was defined as the amount of enzyme that increased absorbance 0.001 (λ = 280 nm) per minute, in a 3 mL reaction medium containing L-tyrosine (0.5 mM) in potassium phosphate buffer (0.05M) pH 6.5 and a temperature of 25°C.

Polishing of Phenolic Solutions

Phenol removal catalyzed by tyrosinase was initially tested in experiments using buffer solutions (potassium phosphate 0.05 M, pH 6.5) containing 20 $mg.L^{-1}$ of phenol. Reactions were performed at room temperature in 200 mL aerated and magnetically stirred reactors. The working volume was 50 or 100 mL, and samples of 2 to 3 mL were taken periodically. Reactions were stopped by adding 0.1mL of H_3PO_4 8.5% w/v. Tyrosinase concentrations tested were of 20, 30, 46 and 100 $U.mL^{-1}$ and chitosan concentrations were 0; 25; 75 e 100 $mg.L^{-1}$. Phenol concentration was analyzed at the beginning and after 20 hours reaction.

Chitosan solution (0.5% w/v) was prepared by dissolving it in acetic acid 0.5% v/v.

Enzymatic Pretreatment

Enzymatic pretreatment was conducted in aerated and magnetically stirred beakers for two hours using 250 mL of effluent and 20 $U.mL^{-1}$ of enzymatic extract. Initial pH was 6. Occasionally, initial pH was lower than 6 and was adjusted to 6.0-6.5 with NaOH 0.1 N.

After enzymatic pr-treatment, the effluent was fed to biological reactor which operated under a batch time of 24 hours.

Biological Treatment

The bioreactor used was a 600 mL beaker, aerated with porous material and mechanically stirred.

The sludge used had been collected at the Wastewater Treatment Station of Ilha do Governador (ETIG-Cedae). Sludge was acclimatized in 24 hours batches, in which the ratio between synthetic effluent and municipal wastewater was gradually increased until it reached 100% synthetic wastewater containing 200 $mg.L^{-1}$ of phenol. Phenol content was further increased, and when it reached 360 $mg.L^{-1}$ (75^{th} acclimatization day) batch time was increased to 48 hours. Acclimatization continued until the phenol concentration in the feeding medium was 420 $mg.L^{-1}$. After the 119^{th} day of acclimatization, 25mL of tap water were added to distilled water and used as diluent for the feed medium (250 mL of total volume). It was a source of micronutrients.

Synthetic wastewater was conveniently diluted from a concentrated parentsolution containing phenol, urea and phosphate (COD:N:P 100:5:1), according to desired phenol concentration. The pH of the feeding medium was set to 7 with NaOH 1.0 N or H_2SO_4 5% (v/v).

Enzymatic Polishing of Biotreated Effluent

Biotreated effluents in which phenol concentration was above target levels (0.5 $mg.L^{-1}$) were enzymatically treated.

Reactions were conducted in a 200 mL aerated beaker, magnetically stirred at room temperature (T=25°C). The working volume used was 50 mL. Chitosan and tyrosinase concentrations were 50 $mg.L^{-1}$ and 46 $U.mL^{-1}$, respectively. Periodically, samples were taken and reaction was stopped by adding 0.1mL of H_3PO_4 8.5% w/v.

A blank reaction corresponding to each experiment was carried out using the same biotreated effluent under equal stirring and aeration conditions, but without chitosan and tyrosinase.

Analytical Methods

Phenol analysis was done according to the direct colorimetric method in Standard Methods (APHA, 1992).

Color was determined spectrophotometrically at λ = 400 nm after filtrate samples through Whatman nº 4 filter paper (Wada et al., 1995).

Chemical oxygen demand (COD) was determined in Hach equipment by the closed reflux colorimetric method (APHA, 1992).

Total suspended solids (TSS) and volatile suspended solids (VSS) concentrations of sludge were determined according to the standard proceedings established in Standard Methods (APHA, 1992).

Volumetric sludge index (VSI) was defined as the volume occupied by 1 g of active sludge (dry weight) after settling for 30 minutes, accordingly to Standard Methods (APHA, 1992)

RESULTS AND DISCUSSION

The effectiveness of using crude tyrosinase extracted from mushrooms together with chitosan solution for enzymatic polishing of phenolic effluents was first studied in experiments using phenol in buffer solutions. Reaction time was 20 hours in order to guarantee complete phenol reaction and precipitation of brownish products. Although enzymatic reaction is relatively fast, polymer precipitation and/or adsorption is much slower and is noticeable only after 7 hours reaction. Figure 1 shows the phenol removal achieved by oxidation catalyzed by tyrosinase along with precipitation with acetic chitosan solution. The best results were achieved using 50 $mg.L^{-1}$ of chitosan. Higher chitosan concentrations were deleterious to process, probably due to interference with enzymatic activity or to complexing with the enzyme. Color removal in those experiments is shown in Figure 2. Chitosan solution contributed to reducing color in the phenolic solution, which was enzymatically treated. The best results were achieved ($Abs_{400\ nm}$ less than 0.2) by using at least 50 $mg.L^{-1}$ chitosan with up to 46 $U.mL^{-1}$ of tyrosinase. The enzyme solution was dark and higher concentrations of tyrosinase increased color. Excess chitosan also negatively affected color as it increased turbidy.

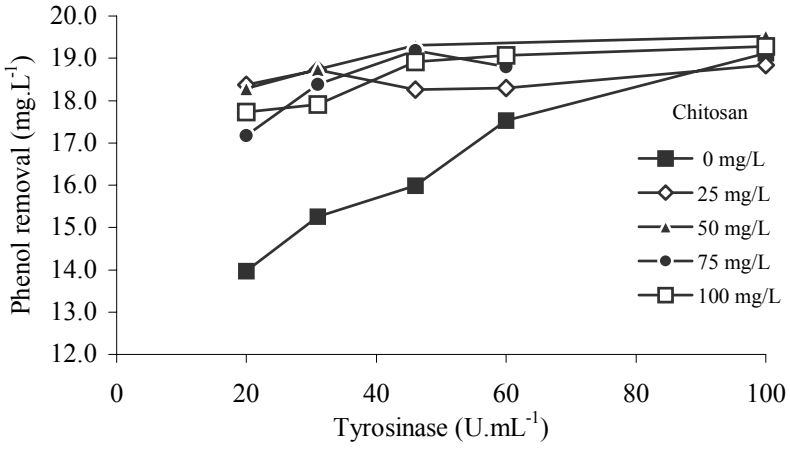

Figure 1: Phenol removal by crude tyrosinase together with acetic chitosan solution (reaction time of 20 hours).

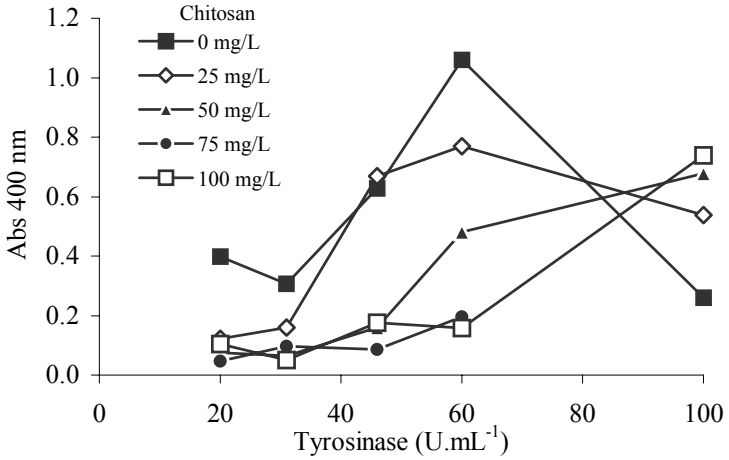

Figure 2: Evaluation of color in reactional media with buffered phenol solution, crude tyrosinase and acetic chitosan solution (reaction time of 20 hours).

A kinetic profile of enzymatic phenol removal was performed under two different sets of conditions (46 $U.mL^{-1}$ of tyrosinase and 50 $mg.L^{-1}$ of chitosan; 60 $U.mL^{-1}$ of tyrosinase and 100 $mg.L^{-1}$ of chitosan). It was verified that most of the phenol is removed during the first 5 hours (Figure 3) and a small amount of additional removal occurs thereafter, probably due to physicochemical phenomena (volatilization and adsorption). Removal of up to 98% of the phenol was achieved, producing final solutions with only 0.4 $mg.L^{-1}$ of phenols, therefore below target limits. Higher tyrosinase and chitosan concentratrions were not reflected in kinetic improvements of process.

The sludge collected at ETIG-Cedae had 1090 mg of $TSS.L^{-1}$ and 229 $mL.g^{-1}$ of VSI. It was necessary to concentrate the sludge before inoculating it in the reactor. The initial sludge concentration in the bioreactor was 2000 mg of $VSS.L^{-1}$.

Figures 4 and 5 show microbial acclimatization to effluent containing phenol. The proportion of synthetic effluent to sewage was augmented gradually, what increased COD of the feeding solution. Despite of that, COD removal efficiency also improved, reaching about 80% by the fourth week of operation. COD removal remained between 90 and 97% and phenol degradation efficiency was 99% in the first 60 days of operation, when phenol concentration in the feeding solution was up to 200 $mg.L^{-1}$.

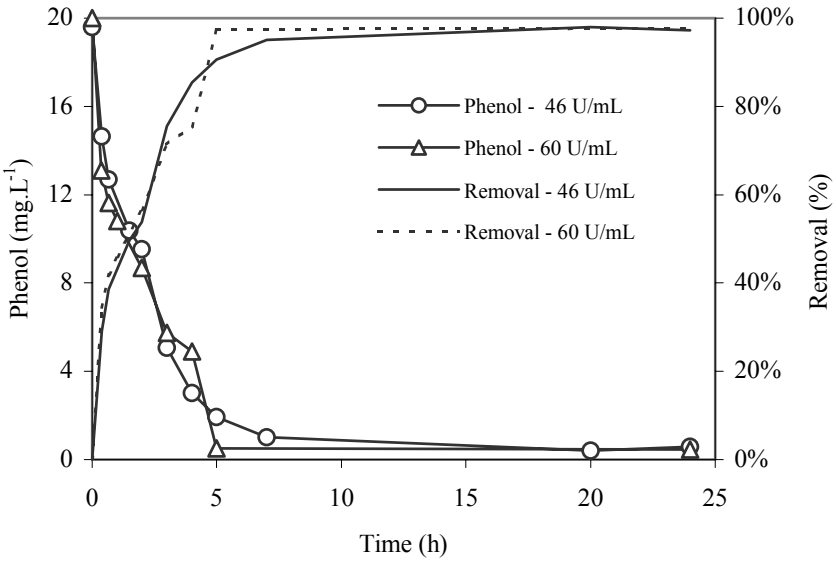

Figure 3: Kinetic profile of phenol removal using 46 $U.mL^{-1}$ of tyrosinase and 50$mg.L^{-1}$ of chitosan or 60 $U.mL^{-1}$ of tyrosinase and 100 $mg.L^{-1}$ of chitosan.

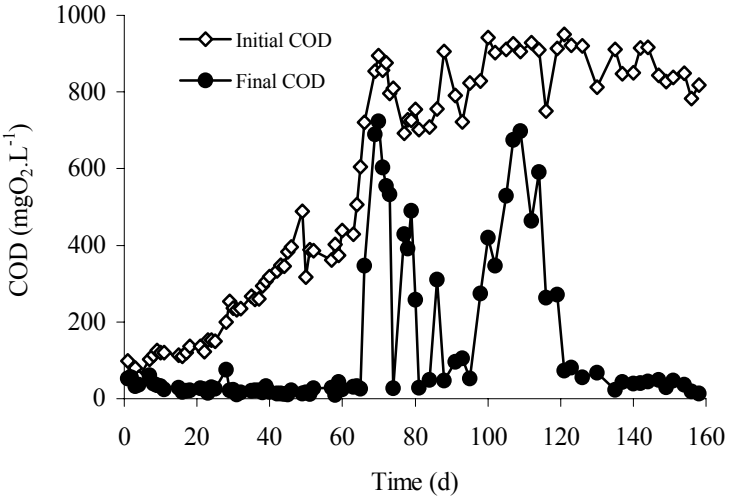

Figura 4: COD removal during microbial acclimatization.

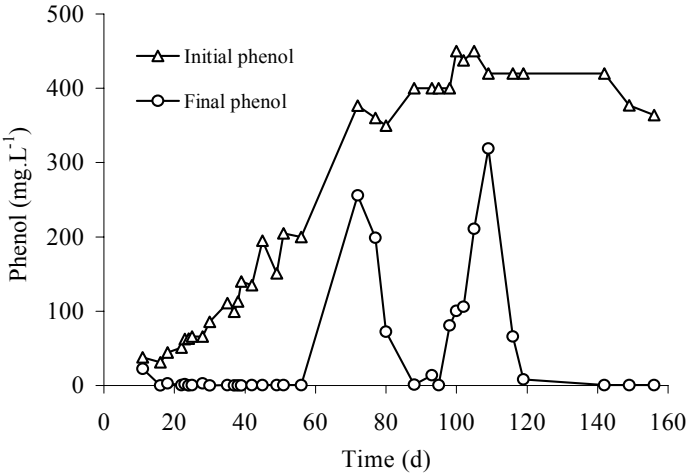

Figura 5: Phenol removal during microbial aclimatization.

An increase in phenol concentration in the feeding solution from 200 to 400 mg.L^{-1} over a short period of time dramatically reduced treatment quality. COD removal efficiency fell to only 26% and phenol removal was reduced to 32%.

In order to overcome this problem, phenol concentration in the feeding solution was reduced to 360 mg.L^{-1} until operation of the bioreactor returned to former patterns. In spite of this, final effluent quality was not improved. Therefore batch operation time was increased from 24 to 48 hours. This batch operation time was mantained until the end of acclimatization period.

A few days after the bioreactor had started operating in these conditions, COD removal efficiency was again over 90%. Then phenol concentration in the feeding solution could be once again increased to 400 mg.L^{-1}. Since this time the microorganisms were already acclimatizated to high phenol concentrations this did not caused charge shock. However, as increase in phenol concentration in the feeding solution to 450 mg.L^{-1}, carried out on the 100th acclimatization day was prejudicial to biological treatment efficiency, and it was necessary to reduce phenol concentration back down to 420 mg.L^{-1}. Even after this alteration, reactor efficiency did not recover rapidly. As this could have been caused by the lack of mineral micronutrients essential to microorganisms, tap water was included as diluent of the parentsolution.

The operational conditions established for the bioreactor were as follows: feeding solution containing 420 mg.L^{-1} of phenol (at a 1:9 ratio of tap to distilled water as diluent) and a batch time of 48 hours. Bioreactor operation was mantained in this condition for an additional 30 days, and medium values of COD and phenol removal were 95.7% and 99.9%, respectively.

Experiments of enzymatic polishing were performed with samples obtained during

acclimatization whose phenol concentration was above the legal discharge limits (0.5 $mg.L^{-1}$). Tyrosinase and chitosan concentrations used were first optimized in experiments with buffered phenol solutions (20 $mg.L^{-1}$) (see Figures 1 and 2).

Figure 6 shows the results for average phenol removal, obtained in polishing tests. It can be observed that maximum phenol removal (75%) occurred when the initial phenol concentration in the enzymatic reactor was equal to or less than 20 $mg.L^{-1}$. This result was already expected as enzyme and chitosan concentrations had been optimized for this phenol concentration.

The results achieved in synthetic media (up to 98% phenol removal) during optimization of reaction conditions were better than those obtained with biotreated effluent. This was probably due to the fact that synthetic effluent is an ideal medium, without microbial metabolites and other compounds that could cause enzymatic inhibition or compete with phenol.

The highest removal efficiencies, in terms of quantity of phenol removed, were achieved when treating effluents with high initial phenol concentrations (phenol removal of 92 $mg.L^{-1}$ in the experiment with an initial phenol concentration of 146 $mg.L^{-1}$).

Enzymatic inhibition caused by biotreated effluent led to an innovative strategy: enzymatic pretreatment of effluent. The aim of pretreatment was to reduce phenol concentration in the bioreactor feeding solution, which could diminish operational batch time and improve removal efficiency.

Although biomass growth had not been quantified, the fast increase in sludge volume could be visually observed as more biodegradable substrates were introduced (proteins contained in enzymatic solution). During conventional biological treatment, only that biomass which was arrested in the effluent discharges of the bioreactor was removed. After enzymatic pretreatment started, biomass was drained daily and a maximum sludge volume of 50 mL was allowed.

Residence time for biological treatment preceded by enzymatic pretreatment was reduced from 48 hours to 24 hours, since initial phenol concentration was lower (on average 283 $mg.L^{-1}$). Figures 7 and 8 show results obtained in the biological treatment of enzymatically pretreated effluents.

Pretreatment with enzymatic solutions increased initial COD in 18 to 52 %, as a consequence of higher levels of organic matter added. Initial COD could not remain constant as quantity of enzyme was varied due to alterations in its activity. During pretreatment, 20 $U.mL^{-1}$ of tyrosinase were added, which meant adding volumes of enzymatic solution from 1.27 to 1.68 mL. The most significant alterations of COD intake occurred after the 15^{th} day of operation, when enzymatic solution was changed and average the COD in the feed changed from 1013 $mgO_2.L^{-1}$ to 1300 $mgO_2.L^{-1}$.

The enzymatically pretreated and biotreated effluent had average COD and phenol contents of 151 $mgO_2.L^{-1}$ and 1.0 $mg.L^{-1}$, respectively. These values are higher than those obtained in conventional biological treatment (average COD and phenol content at the end of batches were 35 $mgO_2.L^{-1}$ and 0.4 $mg.L^{-1}$, respectively). This result was probably a consequence of difference between biomass aclimatization periods, becaused acclimatization to synthetic wastewater exceeded 100 days in conventional biological treatment, and was much shorter when effluent was enzymatically pretreated.

Enzymatic pretreatment of phenolic effluent produced brownish products (originating in quinone polymerization) that were not degraded by subsequent biological treatment. Additional polishing steps would be necessary to remove effluent color.

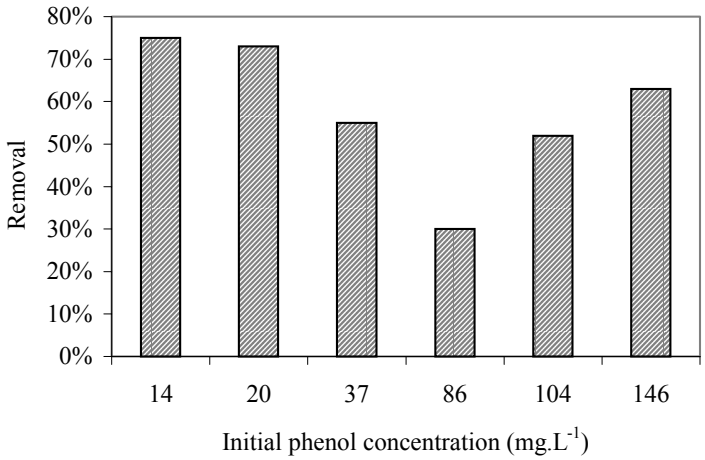

Figura 6: Phenol removal efficiency (values are the average of duplicates) after enzymatic polishing of biotreated effluent with 46 $U.mL^{-1}$ of tyrosinase and 50 $mg.L^{-1}$ of chitosan reaction time was 4 hours.

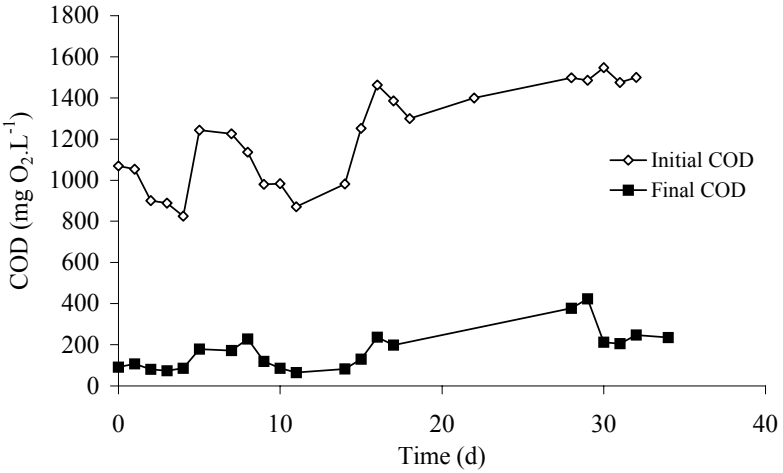

Figure 7: COD removal achieved through biological treatment of enzymatically pretreated effluent.

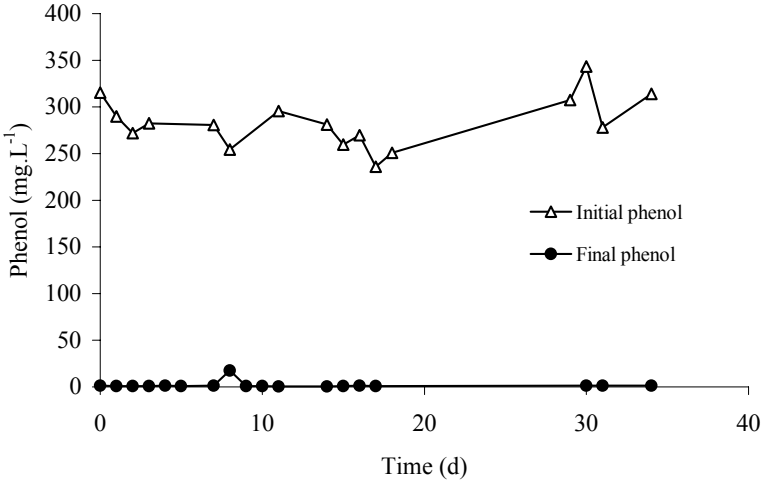

Figure 8: Phenol removal through biological treatment of enzymatically pretreated effluent .

CONCLUSION

The best results for the experiments on phenol oxidation in buffered solution were achieved using 46 U.mL^{-1} of tyrosinase and 50 mg.L^{-1} of chitosan; these experiments produced final effluent containing only 0.4 mg.L^{-1} of phenol after 20 hours of reaction.

Conventional biological treatment is a suitable process for degrading phenol and is highly efficient in removal of this pollutant. However, alterations in feeding concentration may affect biological treatment, diminishing final effluent quality. When this happens, it is necessary to adopt complementary procedures to bring effluent quality within legal discharge limits.

When the bioreactor was not operating properly, enzymatic polishing of biotreated effluent caused a considerable reduction in phenol content, althought the legal discharge limit was not achieved.

Enzymatic pretreatment may be a useful tool for reducing residence time in biological reactors and preventing stress caused by increasing pollutant content. Nevertheless, color generated by enzymatic reaction and COD content increases in bioreactor intake make this technology less interesting for wastewater treatment. Use of immobilized tyrosinase through development of new techniques that stabilizes it and remove generated quinones may encourage combined biological/enzymatic treatments to degrade phenols, specially in emergency situations.

ACKNOWLEDGEMENT

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