BIOELECTROCHEMICAL DEGRADATION OF PHENOLIC COMPOUNDS FROM REFINARY WASTE-WATERS USING CHLOROPEROXIDASE FROM *Caldariomyces fumago* AND ELECTROGENERATED HYDROGEN PEROXIDE

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Resumo – Cloroperoxidase (CPO) de *Caldariomyces fumago* catalisa a oxidação de vários compostos fenólicos, comumente encontrados em águas residuais de refinaria, na presença de peróxido de hidrogênio. Embora a disponibilidade do H_2O_2 controle as velocidades de reação e o tipo e a qualidade da oxidação, esta enzima pode ser inativada por altas concentrações de H_2O_2 afetando a eficiência da reação. Assim, a continua electrogeração de peróxido (EG) concilia a efetiva disponibilidade do H_2O_2 com a estabilidade da enzimatica.
4-clorofenol (4-CP) foi inicialmente selecionado como alvo da oxidação enzimática em reações em batelada com adição direta de H_2O_2 (SD) ou eletrogeração (EG) a fim de determinar as melhores condições a serem aplicadas posteriormente em matrizes complexas. Ambos dos sistemas usaram concentrações de CPO (6.0 UI/mL), de 4-CP (0.5 mM) em tampão fosfato de sódio e potássio 100 mM pH 6.0. Concentrações equivalentes de H_2O_2 foram usadas nos ensaio de SD de acordo com as concentrações atingidas nos experimentos de EG. Os experimentos de EG foram realizados numa célula bicompartimentada com eletrodo de trabalho de malha de cobre eletrolítico, fio de platina como eletrodo auxiliar e solução de calomelano saturado como referencia. Três potencias de -220, -420 e -620 mV foram avaliados na produção de H_2O_2. A concentração de fenol residual, a atividade enzimática residual (estabilidade) e a formação de precipitados (remoção) foram estudados em cada caso.

Palavras-Chave: Cloroperoxidase, degradação de compostos fenólicos, bioeletroquímica, peróxido de hidrogênio

Abstract Chloroperoxidase (CPO) from *Caldariomyces fumago* catalyses the oxidation of several phenolic compounds, common in refinery waste waters, in the presence of hydrogen peroxide. Although H_2O_2 availability drives the reactions rates, yields and the substrates oxidation degree, these enzymes could be inactivated by threshold a peroxide concentration which hinders the reaction effectiveness. The continuous electrogeneration (EG) and *in-situ* supplementation of the peroxide offers the possibility of conciliate effective peroxide availability to undisruptive concentrations in the reaction mixtures.
4-chlorophenol (4-CP) was selected as target for enzymatic oxidation in batch reactions using direct supplementation (DS) of H_2O_2 or electrogeneration (EG) in a way to establish the best removal conditions applied in further experiments with complex matrixes. Both systems used concentrations of 6 UI/mL of CPO, 0.5 mM of 4-CP in sodium-potassium phosphate 100 mM pH 6.0 buffer. Equivalent peroxide concentrations were added in DS experiments depending on the H_2O_2 concentrations obtaining in EG ones. EG employed a bicompartmented cell with a Cu electrochemical web as work electrode, Pt wire as auxiliary electrode and calomel saturated solution as reference electrode. Three different potentials were evaluated -220, -420 and -620 mV. Residual phenol concentration, residual enzyme activity (stability) and precipitate formation (removal) were studied in each case.

Keywords: Chloroperoxidase, phenolic compounds degradation, bioelectrochemistry, hydrogen peroxide.

1. Introduction

As simple as it may seem, the treatment of contaminated waters by dyes, phenolic compounds or polyaromatic hydrocarbons is as diverse and complicated as the operations from which it comes. Nowdays environment, where merely transferring contaminants from one medium to another is no longer acceptable, it is no surprise that biomolecules with oxidizing activity and powerful oxidizer as hydrogen peroxide should be so widely used. This is hydrogen peroxide can acts isolated or in combinations as co-substrate for enzyme mediated reactions, this molecule is a powerful yet versatile oxidant that is both safe and effective. The hydrogen peroxide applications in conventional biological treatment comprehend its use as a pretreatment degrading toxic, refractory or bio-inhibitory organics, rendering them more amenable to biodegradation. In conjunction with microorganisms, provides a supplemental source of dissolved oxygen in-situ (penetrating both soil columns and bioflocs, eliminating the sludge bulking phenomenon). As a polishing step using enzymes, especially peroxidases, destroys trace levels of organics pollutants that could pass through biotreatment. Nevertheless, its quantification can be hidden for its inherent instability, the limit concentrations of some colorimetric methods and false concentrations due side reactions.

The use of hydrogen peroxide in biocatalysis is limited at high concentrations (above 0.5 mM) that could damage the enzyme structure causing loss of enzyme activity. One solution for this problem is the use of low but controlled peroxide supplementation necessary for the maintenance of effective oxidation levels. Thus, the electrogeneration of hydrogen peroxide become into an alternative for this peroxide supplementation. The electroenzymatic approach provides a significantly lower and easily controllable hydrogen peroxide formation rate than any other so far. This leads to a higher reaction stability of the enzyme. The total turnover number could be increased by one order of magnitude.
Often chemistry and biotechnology are complementary; sometimes they are even competitive as in the production of fine chemicals. In view of atom- and energy-efficiency the combination of both fields is becoming more and more important. Electroenzymatic synthesis utilizes electrons as cheap reagents. The advantages of enzymes as selective catalysts are combined here in reaction cascades with the waste-free electrochemical *in situ* generation or regeneration of reagents. By means of electrochemical catalysis reactants are either synthesized *in situ* or regenerated. The use of bioelectrochemistry relies on the combination of enzyme and substrate, leading to products witch will be detected by a suitable methodology.

2. Results and Discussion

2.1. The Hydrogen Peroxide Electrogeneration (EG)

The electrochemical production peroxide it is based on the dissolved oxygen reduction present in the reaction mixture. This oxygen reduction produces, as final product, water, with the consumption of 4 electrons. However, depending on the material utilized as work electrode, this reaction could occur in two steps producing the stabilization of the peroxide in a determined range of polarization.

In order to avoid a possible anodic oxidation of the substrate during the *in-situ* generation of H_2O_2, a thermal controlled two chamber cell was used, with a glass frit that splits reaction mixture into two different cathodic and anodic chambers (Figure 1). A platinum wire was used as auxiliary electrode (AE) and a calomel saturated solution (SCE) as reference electrode (RE). As work electrode a copper electrolytic web of 27 cm^2, was used. The electrochemical measurements were made using a potenciostate/galvanostate from the Omnimetra (US). Prior to turn-on the system, the cathodic chamer with 100 mM sodium-potassioum phosphate buffer pH 6.0, was saturated with pure O_2 during an hour and then air stripping was stopped to guarante the air-dragg effect over the phenol solution.

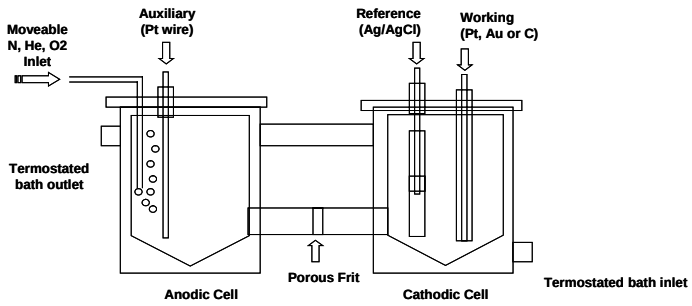

Figure 1. Diagram of the electrochemical cell used for in-situ hydrogen peroxide generation and bioelectrochemical degradation of phenolic compounds.

To evaluate the H_2O_2 generation, samples form the cathodic chamber were taken along 4 hours and the H_2O_2 was quantified using a spectrophotometric enzyme-mediated method. Thus, it was possible to confirm as shows figure 2, that with the increase of the cathodic over tension until -620 mV_{ECS}, the highest H_2O_2 generation was achieved. With even more negative potentials was observed the reduction on H_2O_2 generation due to its reduction into water. Control experiments were also carried out. In all cases, reaction conditions were maintained according to the described above, except by the following substitutions: enzyme by water distilled and oxygen by nitrogen. Beyond that, two others control experiments were carried out aiming at verify the influence of the oxidizing power of the H_2O_2 and by dissolved O_2 itself. In this way, equivalent H_2O_2 concentrations to the eletrogenerated ones and O_2 were added separately to the reactor in the absence of polarization or enzyme.

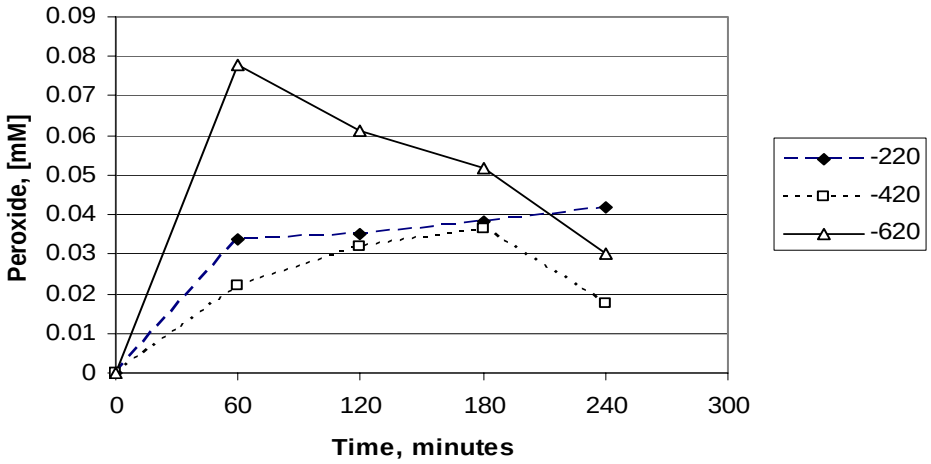

Figure 2. Curves for H2O2 generation using different potentials in function of time. Cathodic chamer with 100 mM sodium-potassioum phosphate buffer pH 6.0, was saturated with pure O_2 during an hour and then air stripping was stopped to guarante the air-dragg effect over the phenol solution.

2.2. Degradation of 4-CP

2.2.1. Batch Experiments with Hydrogen Peroxide (EG)

Although, a lower potential of -620 mV demonstrated the highest H_2O_2 generation, is possible that this condition will not indicates also the best condition for phenol degradation and removal. Due this, we decided to test two additional potentials of -220 and -420 mV. Results demonstrated that at -620 mV besides the highest H_2O_2 generation, the best removal condition was also obtained. In figure 3, it shows the effect of the potential over bioelectrochemical degradation of 4-chlorophenol using CPO. Even thought, anodic oxidation was also observed this was not even closer to the levels achieved with cathodic one, where the enzymatic oxidation occurred. As was expected, the H_2O_2 generation also drove the formation of precipitated, thus when higher H_2O_2 levels were used, the highest precipitate formation was observed.

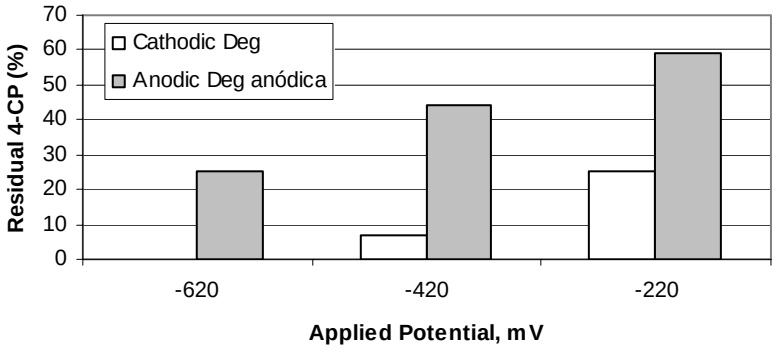

Figure 3. Bioelectrochemical degradation of 4-chlorophenol (0.5 mM) using CPO, Sodium/Potassium phosphate buffer 100 mM pH 6.0 and EG hydrogen peroxide. Comparison between three different potential applied of -220, -420 and -620 mV.

2.2.2. Peroxide Direct Supplementation (DS)

Figure 4, shows the conventional 4-CP oxidation (DS), performed using a phenolic solution of 0.5 mM in Sodium/Potassium phosphate buffer 100 mM pH 6.0, with the addition H_2O_2 in an equivalent amount of 0.1 mM, closer the one obtained with EG where best oxidation and removal levels where reached.

A rapid decrease in the 4-CP concentration was observed during the first hour using both methodologies, specially when DS was applied, the biodegradation reached almost 80% of the initial phenol concentration. Similar results were observed after 2 hours where both methodologies showed approximately 90% of phenol degradation. In both cases the maximum precipitate formation occurs during the first hour. No color loss was observed even when the reactions were extended for long time periods of 24 h (data not shown) even when no phenol was detected at this point.

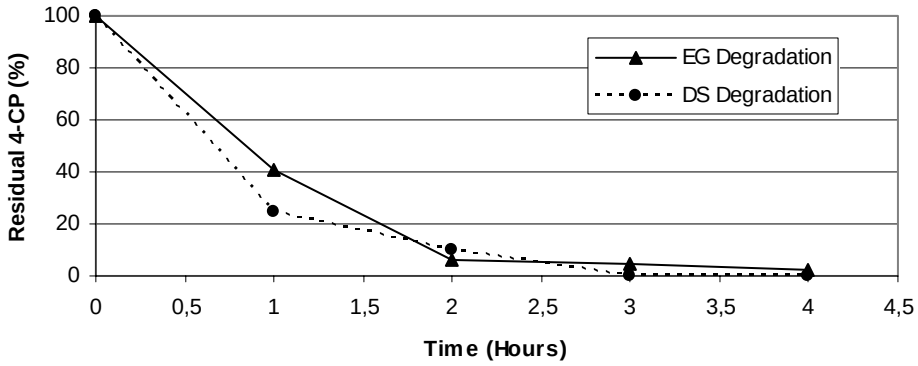

Figure 4. Biodegradation of 4-chlorophenol (0.5 mM) using CPO, Sodium/Potassium phosphate buffer 100 mM pH 6.0 and Direct supplementation of hydrogen peroxide (0.1 mM). Comparison between the *in-situ* supplementation using electrogeneration (-620 mV) and the direct supplementation of hydrogen peroxide.

2.2.3. Evaluation of Residual Peroxidase Activity

This parameter indicates the real advantages between each methodology evaluated. Despite both methodologies showed to have similar efficiencies in terms of phenol oxidation and precipitate formation, was observed that CPO loses activity more rapidly when the direct supplementation is used. Thus, decrease in activity was observed up to 60% when a solution containing CPO (6 UI/mL) plus peroxide (0.1 mM) was incubated during 4 hours. This effect is even more remarkable when both phenol oxidized products and peroxide were simultaneously present during the direct supplementation. Thus, figure 5 shows how after 4 hour of process, EG caused the lowest loss in peroxidase activity (less than 20% of initial activity), followed by the control of a solution of CPO and 0.1 mM of hydrogen peroxide (loss of 40% of initial activity) and finally, DS caused the highest loss with almost 60% of the initial activity after 4 hours.

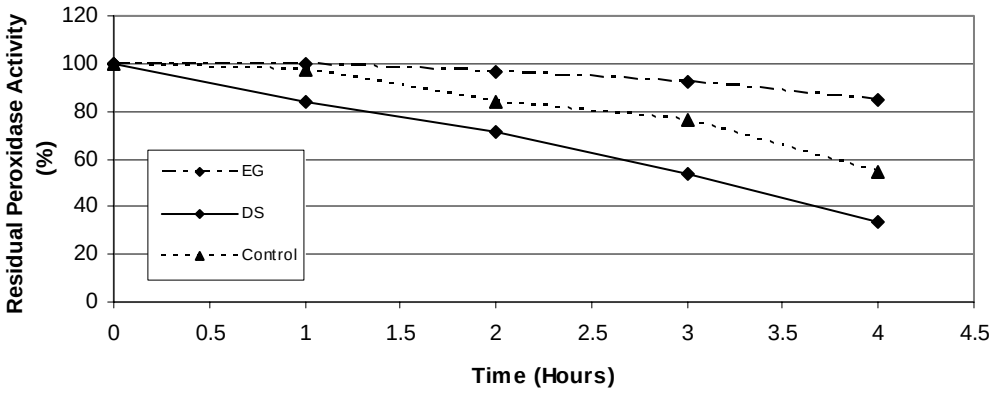

Figure 5. Comparison between the effect electro generation or direct supplementation of hydrogen peroxide over the activity of CPO.

4. Conclusions

From the results obtained to the present moment, the following points can be concluded:

- The enzymatic oxidation occurs at the cathodic chamber;
- The product profile o in this chamber depends on the applied potential
- With long reaction periods, there is an intense fall in the phenol removal suggesting the formation of products derived from the oxidation and polymerization of 4-chlorophenol.
- There is oxidation process at the anodic chamber.
- At Lower potentials up-to – 620 mV the maximum peroxide generation was achieved and coincides with the optimal conditions for 4-CP removal.
- The condition will be applied during further experiment using other chlorinated phenols and complex matrixes.

5. Acknolegments .

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6. Referências

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