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BIODEGRADATION OF REACTIVE ORANGE 16 BY *PHANEROCHAETE CHRYSOSPORIUM* FUNGUS: APPLICATION IN A FLUIDIZED BED BIOREACTOR

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ABSTRACT

Application of a fluidized bed bioreactor working for treatment of colored wastewaters has been studied using *Phanerochaete chrysosporium* fungus immobilized in calcium alginate biogel beads. The working volume of the bioreactor was 1 L; experiments were performed at room temperature and pH of culture medium was initially adjusted to 4. Manganese Peroxidase activity, glucose and ammonium concentrations have been assayed daily along the 7 operating days. Azo dye Reactive Orange 16 was added to the bioreactor after 7 days of incubation and decolorization was assayed by spectrophotometer for 1 h intervals. Maximum Manganese peroxidase activity of 96±8 U/L was obtained on day 7, and 70±3 % decolorization was achieved after 6 h of dye addition. The results were compared to free cell cultures from previous studies and the role of agitation and immobilization of cells in increasing of the efficiency of decolorization was discussed. The mechanism and morphology of the immobilization of cells in ca-alginate beads were studied and the relationship between glucose and ammonium consumption and ligninolytic activity of fungi were discussed.

Key words: Fluidized bed bioreactor; Azo dye; Decolorization; *P. chrysosporium*; Calcium alginate

INTRODUCTION

With the increasing usage of the wide variety of Synthetic dyes in textile dyeing, paper printing, leather dyeing, color photography and products industries, pollution from the effluents has become increasingly alarming. Color is noticeable at a dye concentration higher than 1 mg/L and an average concentration of 300 mg/L has been reported in effluents from textile manufacturing processes (O'Neill *et al.,* 1999; Gonçalves *et al.,* 2000). Over 7×10^5 ton and approximately 10,000 different dyes and pigments are produced annually worldwide, about 10% of which may be found in wastewater (Deveci et al., 2004).

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Color interferes with penetration of sunlight into waters, retards photosynthesis, inhibits the growth of aquatic biota and interferes with gas solubility in water bodies (Banat *et al.,* 1996). In addition, many dyes are believed to be toxic, carcinogenic or to be prepared from known carcinogens such as benzidine or other aromatic compounds that might be formed as a result of microbial metabolism (Novotny et al., 2006; Kariminiaae-Hamedaani *et al.,* 2007). The current existing techniques for the removal of dyes from dye containing wastewater have serious restrictions such as high cost, formation of hazardous by-products or intensive energy requirements (Stolz, 2001).

By far, the single class of microorganisms most efficient in breaking down synthetic dyes is the white-rot fungi. They constitute a diverse ecophysiological group comprising mostly basidiomycetes and to a lesser extent litter-decomposing fungi capable of extensive aerobic lignin depolymerization and mineralization. This property is based on their capacity to produce non-specific extracellular ligninolytic enzymes that are also capable of degrading a wide range of xenobiotics. The main extracellular enzymes participating in lignin degradation are lignin peroxidase (ligninase, LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13) and Cu-containing laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2). Earlier studies showed that peroxidase activity of *Phanerochaete* . *chrysosporium* is inhibited by agitation. Thus, systems which minimize intensive shear both to the organism and the enzyme and also provide a growth support for the controlled growth of the organism, are most likely to be successful in the consistent cultivation of *P. chrysosporium* and the production of lignin peroxidases (Novotny et al., 2006; Zahmatkesh *et al.* 2008 a). Immobilization of fungal cells can provide a suitable low-shear environment for ligninolytic system of fungi (Novotny *et al.,* 2006; Zahmatkesh *et al.* 2008 a, b).
The objective of the present work was to develop a system that permits production of ligninolytic enzymes by *P. chrysosporium* in laboratory scale. Therefore, a fluidized bed bioreactor was employed to produce ligninolytic enzymes due to its simplicity, reliability and low cost (Kiese *et al.* 1980). Moreover, it provides a low shear environment (Bonnarme & Jeffries, 1990;Moreira *et al.* 1996) in comparison with common stirred tank bioreactors and does not require a mechanical stirrer with which the risk of contamination and energy demand is considerably lessened (Träger *et al.* 1989). The role of agitation and immobilization on the azo dye decolorization and enzyme activity were also investigated.

MATERIALS AND METHODS

Fungal strain

Phanerochaete chrysosporium RP78 was maintained through periodic (monthly) transfer on potato dextrose agar (PDA). Cultures were stored at -20°C and -70°C on potato dextrose broth (PDB) containing 15% and 30% glycerol as cryoprotective compound, respectively.

Immobilization

The spores of *P. chrysosporium* grown into the stationary phase in PDA plates were collected and suspended in PDB. Spore suspension of 8 mL (approximately 10^7 cells) was added to 100 mL of 3% sodium alginate. The mixture was gently stirred at room temperature to produce a uniform suspension and then dropped into 100 mL of 30% calcium chloride solution. Nozzle with 4 mm diameter was used to form beads of 4 mm size. The beads so obtained were stored in calcium chloride solution at 4 °C for 2 h to complete gel formation. The insoluble and stable immobilized *P. chrysosporium* alginate beads thus obtained were further used for the decolorization studies in the bioreactor.

Equipment and operational conditions

The experiments were carried out in a fluidized bed bioreactor (New Brunswick, USA) with total working volume of 1.5 L. Eight baffles were added to the bioreactor to conduct the flow and help the stirring of the medium. Bioreactor was filled with liquid medium and agitated by air flow. Solid support consisting immobilized fungal spores were added to this medium and the bioreactor was working in three phase mode. Temperature was maintained at 30°C by circulation of temperature controlled water. Air was supplied to the bioreactor in a continuous way at 3 L/min and pH was allowed to vary freely.
Liquid N-limited culture was prepared as described by Tien & Kirk (Tien *et al.*, 1988) with addition of Tween 80 (0.1% v/v). The bioreactor vessel was filled with 1 L of above mentioned medium and then the immobilized spores (calcium alginate beads) were added. Cultivation was carried out for 7 days and MnP activity, glucose and ammonium concentrations were detected daily. In day 7 after 156 h of cultivation, 40 mg/L of dye Reactive Orange 16 (RO16), was added to the bioreactor and biodecolorization was determined in 1 h interval for 7 h. Samples were collected once a day, centrifuged (8000×*g*, 7min) and analyzed in duplicate. The figures were presented as mean values with standard deviation.

Analytical determinations

Glucose concentration in the medium was measured spectrophotometrically by enzymatic glucose reagents (Chem Enzyme Co.) using glucose oxidase as the enzyme and D-glucose as standard.

Ammonium concentration was assayed spectrophotometrically by enzymatic ammonium reagents (Chem Enzyme Co.) using glutamate dehydrogenase enzyme.

MnP activity was assayed as described by Paszczynski et al. (Paszczynski *et al.,* 1988). The method is based on monitoring the enzymes oxidation of Mn(2) to Mn(3). The reaction mixture contained enzyme, 0.1 M sodium tartrate (pH 5.0), 0.1 mM H_2O_2, and 0.1 mM $MnSO_4$. The product, Mn(3) showed a characteristic absorbance (A) at 238 nm (ε=6500). Reactions were initiated by adding of H_2O_2, and the reference cuvette

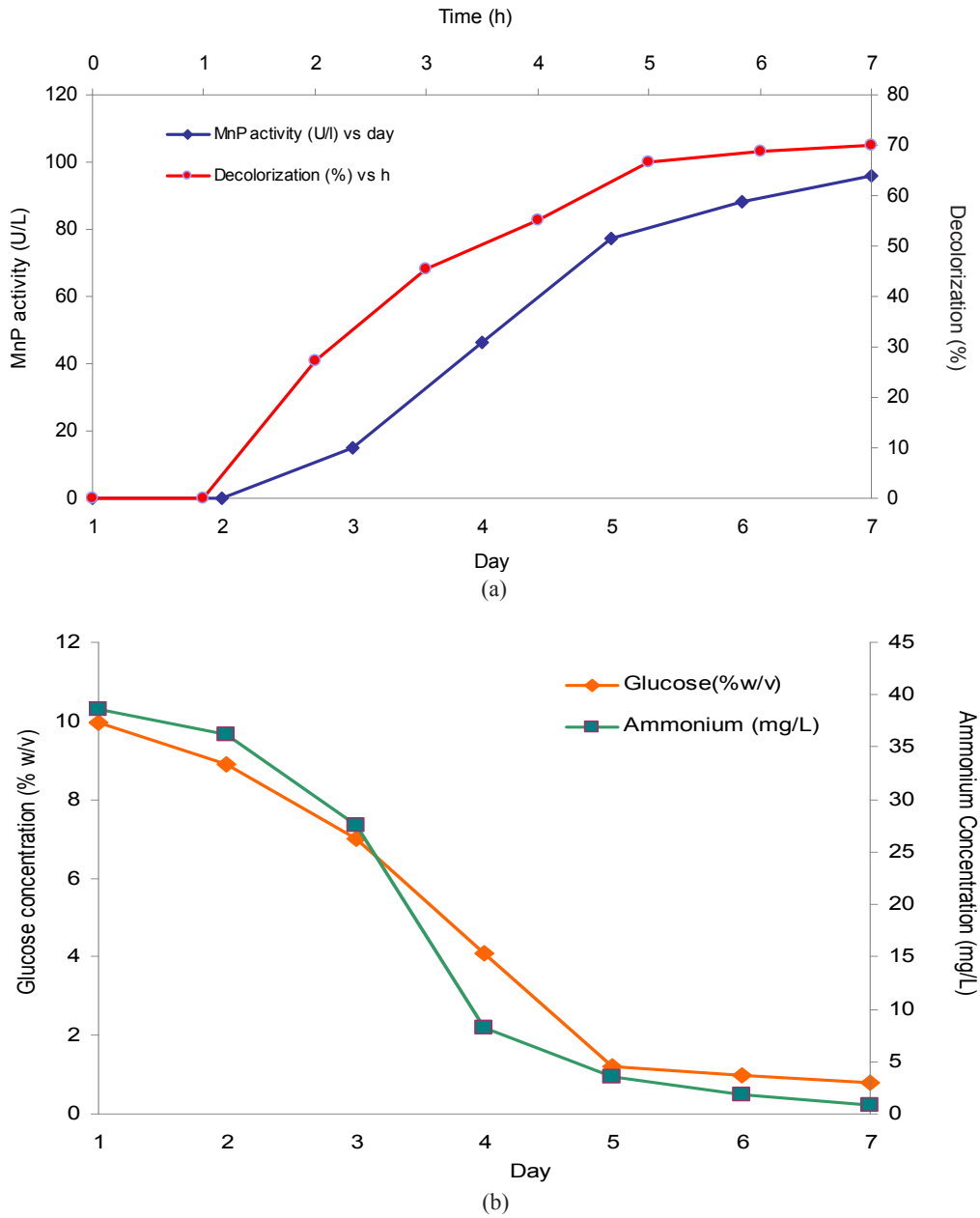

Fig. 1: (A) Glucose and ammonium concentrations during 7 days of cultivation in bioreactor and (B) MnP activity (U/L versus day) during 7 days of cultivation and biodecolorization percentage (versus h) during 7 h after dye addition

contained all components except Mn(2). Increase in $A_{238\ nm}$ was monitored during the first 5-30 s of reaction. One unit of peroxidases oxidizes 1µmol of Mn(2)/min.

Dye disappearance was determined spectrophotometrically by monitoring the absorbance at 494 nm which is the maximum wavelength of RO16.

RESULTS

In this study, special attention has been paid to the influence of the shear stress of the system on enzyme productivity. These data are of great interest in order to optimize the reaction conditions in subsequent applications of the obtained enzymes.

The ammonium and glucose consumption and MnP activity during 7 days of cultivation of immobilized *P. chrysosporium* on calcium alginate beads in the fluidized bed bioreactor are shown in Fig. 1.

MnP activity first appeared after 72 h as 15±4 U/L and peaked on day 7 as 96±8 U/L. As it is shown, the ammonium consumption rate increased from day 3 to day 4 and it is exactly when the MnP activity begins to increase with high rate. Ligninolytic enzymes of *P. chrysosporium* are produced by secondary metabolism which induced by limitation of nitrogen or sulfur concentration in the cultivation medium. It can be seen that when the nitrogen is very low in the medium (day 7), the MnP activity is high. Glucose concentration had less effect on ligninolytic system of *P. chrysosporium* as it can be seen from Fig. 1 and has been studied and reported before.

MnP activity reached about 96±8 U/L in day 7. This amount of enzyme activity is much higher (about 3 times higher) than what was attained by free cells cultivation with same medium from pervious studies (Zahmatkesh *et al.,* 2008a). This is probably due to the immobilization of cells.

DISCUSSION

The immobilization mechanism of *P. chrysosporium* into calcium alginate is entrapment. As it shown in scanning electron micrograph of calcium alginate beads which have been used in this study (Fig. 2), calcium alginate biogel has a high porous structure which can entrap fungal cells and immobilize them.

These biogel beads cannot last in stirred tank and would be damaged by the stirrer which could result in releasing the fungal cells (Kiese *et al.* 1980).

In previous studies, biodecolorization of RO16 by *P. chrysosporium* free cells have took about 10-32 h to reach about 40-70% of color removal (Zahmatkesh *et al.,* 2008a,b), but with

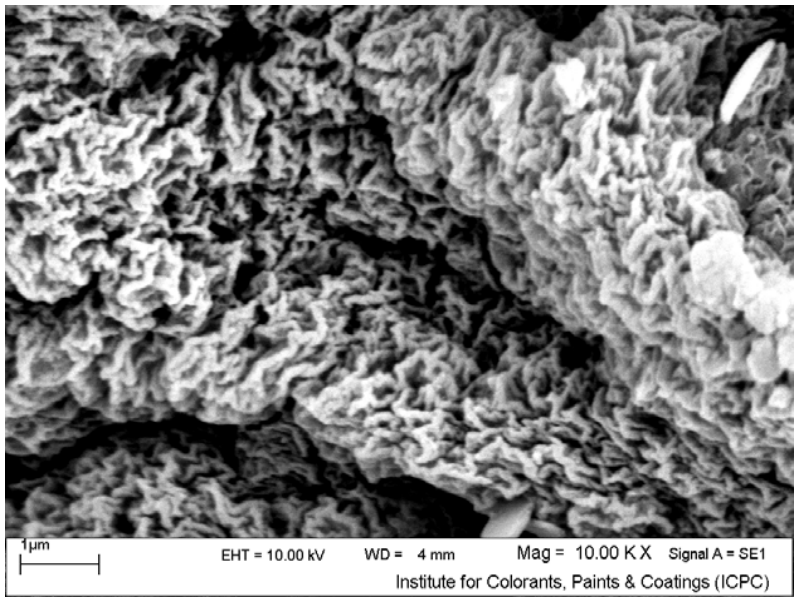

Fig. 2: Scanning electron micrograph of calcium alginate biogel bead

immobilized fungal cells in a fluidized bed bioreactor in this study, it took only 7 h to reach the biodecolorization of 70±3%.

The whole pattern of glucose, ammonium and enzyme activity and biodecolorization was similar to what was gained by immobilized spores in erlenmeyer flasks from pervious studies, which shows that the scale up of the experiments to bioreactor scale did not reduce the efficiency of the system and it can be developed to higher volume bioreactor systems.

One of the most common problems in other types of bioreactors with different supports is clogging. Clogging may be detected due to the clinging of the supports together with the fungal hyphae forming agglomerates, which hindered mass and oxygen transfer rate. But in this study, clogging was not happened. It seems that the structure of calcium alginate beads along with the fluidization of carriers in the medium did not let the clinging of supports and the limited growth of mycelia in calcium alginate beads prevented the formation of agglomerates by fungal hyphae.

calcium alginate beads proved their efficiency when applied in fluidized bed bioreactors. Several reasons may have contributed to these results: (i) they improved growth and ligninolytic activity of the fungi, the mycelium covered the entire cartridge surface and invaded its interior rapidly; (ii) they did not alter physiological characters analyzed, ligninolytic enzyme activities were high and an efficient decolorization of RO16 was achieved; (iii) calcium alginate beads showed a fast recovery after their exposure to the dye, allowing their reuse; (iv) the change of the carriers was easy and quick, allowing a continuous use of the bioreactor in the decolorization process.

In view of the results obtained, it can be concluded that the modified fluidized bed bioreactor with *P. chrysosporium* immobilized in calcium alginate beads is suitable for the production of ligninolytic enzymes. Moreover, the enzymatic complex secreted decolorized the azo dye RO16, verifying its ligninolytic ability. Nonetheless, more studies are necessary to optimize the conditions that allow obtaining a continous and stable production of ligninolytic enzymes.

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REFERENCES

Banat, I.M., Nigam, P., Singh, D., Marchant, R., (1996). Microbial decolorization of textile dyes containing effluents: a review. Bioresour. Technol., **58**: 217 –27.

Bonnarme, P., and Jeffries, W., (1990). Mn(II) regulation of lignin peroxidase and manganese-dependent peroxidases from lignin- degrading white rot fungi. Appl. Environ. Microbiol., **56**: 210– 217.

Deveci, T., Unyayar, A., Mazmanci, M. A., (2004). Production of Remazol Brilliant Blue R decolorizing oxygenase from the culture filtrate of Fungal iatrogii ATCC 200800. J. Mol. Catal. B: Enzyme., **30**: 25 – 32.

Gonçalves, I., Gomes, A., Brás, R., Ferra, M.I.A., Amorim, M.T.P., Porter, R.S., (2000). Biological treatment of effluent containing textile dyes. J. Soc. Dyes Color, **6**: 393 – 7.

Kariminiaae-Hamedaani, H.R., Sakurai, A., Sakakibara, M., (2007). Decolorization of synthetic dyes by an manganese peroxidase producing white rot fungus. Dyes Pigm., **72**: 157–62.

Kiese, S., Ebner, H.G., Onken, U., (1980). A simple laboratory airlift fermentor. Biotechnol. Lett., **2**: 345–350.

Moreira, M.T., Sanromán, A., Feijoo, G., Lema, J.M., (1996). Control of pellet morphology of filamenteous fungi in fluidized bed bioreactors by means of a pulsing flow. Application to *Aspergillus niger* and *Phanerochaete chrysosporium*. Enzyme Microbiol. Technol., **19**: 261–266.

Novotny, C, Dias, N., Kapanen, A. , Malachova, K., Vandrovcova, M., Itavaara, M., (2006). Comparative use of bacterial, algal and protozoan tests to study toxicity of azo and anthraquinone dyes. Chemosphere, **63**:1436 –42.

O'Neill, C., Freda, R.H., Dennis, L.H., Nidia, D.L., Helena, M.P., Wouter, D., (1999). Color in textile effluent sources, measurements, discharge contents and simulation: a review. J. Chemical Technol. Biotechnol., **74**: 1009 –18 .

Paszczynski, A., Crawford, R.L., Huynh, V.B., (1988). Manganese peroxidase of *Phanerochaete chrysosporium*: purification. Methods Enzymol., **161**: 264–70.

Stolz, A., (2001). Basic and applied aspects in the microbial degradation of azo dyes. Appl. Microbiol. Biotechnol., **56**: 69 –80.

Tien, M., and Kirk, T.K., (1988). Lignin peroxidase of *Phanerochate chrysosporium*. Methods Enzymol., **16**: 238–249.

Träger, M., Qazi, G.N., Onken, U., Chopra, C.L., (1989). Comparison of airlift and stirred reactors for fermentation with *Aspergillus niger*. J. Ferment. Bioeng., **2**: 112–116.

Zahmatkesh, M., Tabandeh, F., Ebrahimi, S., Ghasemi, F., (2008a). Study of biodecolorization activity of *Phanerochaete chrysosporium* fungi in free cells and

immobilized cells conditions and determination of kinetic constants. 12th National Congress of Chemical Engineering, Iran, Tabriz

Zahmatkesh, M., Tabandeh, F., Ebrahimi, S., (2008b). Optimization of immobilization conditions of *Phanerochaete Chrysosporium* fungus on calcium alginate beads for biodecolorization of synthetic dyes. The 2nd International Student Conference of Biotechnology, Tehran, Iran