

INTERNATIONAL JOURNAL OF PURE & APPLIED BIOSCIENCE

***Coprinus cinereus* Biodegradation of Colored Wastewater with Cibacron Blue**

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**ABSTRACT**

*Water pollution due to release of industrial wastewater has already become a serious problem in almost every industry using dyes to color its products. In the present study an enzymatic decolorization of Cibacron blue by a fungal peroxidase from *Coprinus cinereus* was investigated. Results show that immobilized cells in calcium alginate are able to decompose this dye in a short time (1 hour) and with a high efficiency. Moreover it also determines the most suitable and optimum conditions of *Coprinus cinereus* culture that allow a maximum decolorization.*

**Keywords:** *Coprinus cinereus*, Decolorization, Cibacron Blue, Biodegradation.

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**INTRODUCTION**

Wastewater from textile, paper and printing industries and dye houses is characterized by high chemical and biological oxygen demands (COD and BOD), suspended solids and intense color due to the extensive use of synthetic dyes<sup>1</sup>. Direct discharge of these effluents into municipal wastewater, plants and/or environment may cause the formation of toxic carcinogenic breakdown products<sup>2</sup>. Thus certain metal complex dyes adversely affect water resources, soil fertility, aquatic organisms and ecosystem integrity<sup>3</sup>. Unfortunately conventional biological wastewater treatment systems are not efficient for decolorization.

*Coprinus cinereus* is a multicellular basidiomycete, it's a source of peroxidase for industrial purposes; in fact many studies reported that several *Coprinus* species, such as *C. cinereus* UAMH 4103 found to be one of the best peroxidase producing<sup>4</sup>.

Peroxidases (EC 1.11.1.7) such as peroxidase, lignin peroxidase and manganese peroxidase are known to be useful for the removal of polycyclic aromatic hydrocarbons<sup>5</sup>, for the pulp bleaching<sup>6,7</sup> for the decolorization of colored wastewater<sup>8-11</sup> and as an enzyme for detergents<sup>12</sup>.

Dyes are colored substances that have an affinity to the substrate to which they are being applied. They are extensively used in textile, printing and dyeing industries. Many dyes are believed to be toxic, carcinogenic<sup>13</sup>.

In order to find a cheaper and easily available alternative fungi enzyme source for the decolorization of various reactive, acid, direct and basic dyestuffs; the present study aimed to examine the potential use of *Coprinus cinereus* to decolorize colored wastewater with: orange G, azorubine, trypan blue, indigo carmine and cibacron Blue; then the most decolorized dye was noted. In addition a partial purification of the enzyme was investigated. Therefore, on the basis of the advantages that immobilization techniques offer, we also aimed to eliminate the toxic dye "Cibacron blue" from synthetic water by using *Coprinus cinereus* entrapped in calcium alginate gel.

## MATERIAL AND METHODS

### Microorganism and culture media

*Coprinus cinereus* used in this study was isolated from dye-contaminated sludge collected from an industrial area in Casablanca city (Morocco). It was identified based on the visual observation of isolates grown on potato dextrose agar (PDA : Topley House, Bury, England) plates, micro-morphological studies in slide culture (Riddell 1950) at room temperature, and the taxonomic keys described by De Hoog *et al.* (2000)<sup>14</sup> as well as the compendium of soil fungi<sup>15</sup>.

Three medias were used in this study: PDA were used for routine transfer and fungal culture, minimum medium (MM) containing: 0.6 g K<sub>2</sub>HPO<sub>4</sub>, 0.1 g MgSO<sub>4</sub>, 0.6 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 g NaCl, 20 mg CaCl<sub>2</sub>, 1.1 mg MnSO<sub>4</sub>, 0.2 mg ZnSO<sub>4</sub>, 0.2 mg CuSO<sub>4</sub> and 0.14 mg FeSO<sub>4</sub>, plus 1 % of glucose and 15 g/l agar (pH was adjusted to 7 with HCl 1 M) used to monitor the decolorization on solid medium, nutrient media (NB) was used as liquid media.

Dye concentration was obtained by measuring the absorbance at 590 nm using a Jenway 6405 UV/Visible spectrophotometer.

### Decolorizing screening

5 colorants at 50 mg/l were tested in both solid and liquid medium in order to determine which colorant is efficacy decolorated with *Coprinus cinereus*.

### pH and Temperature effect

Medium was adjusted at different pH: 4, 5, 6, 7, 8 and 9 in order to evaluate the effect of pH on decolorizing cibacron blue.

The waterbath was equilibrated at 25°C, 30°C, 37°C, 40°C et 45°C during 28 hours of incubation to determine the optimum temperature of decolorization.

### Enzyme assay

Nutrient media was prepared in 250 ml baffled Erlenmeyer flasks and autoclaved (120°C, 20 min). Before inoculation of *Coprinus cinereus*, the culture was supplemented with dye solution to a final concentration of 50 mg/l, and then placed in a rotary shaker (150tr /min) at 37°C until decolorization. The culture broth was filtered trough amicon stirred cells for the concentration of proteins, then was centrifuged at 15 000 g for 20 min.

The peroxidase activity was measured as followed: the reaction mixture contained 25 mM sodium citrate buffer (pH 3.4), 0.2 mM H<sub>2</sub>O<sub>2</sub> and 120 µM of Cibacron Blue. The reaction was started by adding 100 µl of concentrated extract<sup>16</sup>.

### Protein assay:

Protein content was measured according to the Bradford procedure (Bradford, 1976) by using bovine serum albumin (BSA) as standard<sup>17</sup>. Protein reagent was added to protein solutions. The absorbance was measured at 595 nm after 10-15 mn of incubation in the dark.

Enzyme activity expression:

The specific activity of each enzyme was calculated using the following formula:

$$AS = (\Delta Abs/mn \times 1000) / (\epsilon \times [P] \times Ve)$$

$\Delta Abs/mn$ : absorbance variation/minute

$\epsilon$  (extinction coefficient) :

$$\epsilon (H_2O_2) = 40 M^{-1}.cm^{-1}$$

[P]: protein concentration

Ve: assay volume

### Cell Immobilization: Entrapment in calcium-alginate gel

100 mL of sterile sodium alginate solution (2% W/V) was mixed until homogeneous, with 10 g of a fresh mycelium culture obtained by centrifugation at 15000 g for 10 min at + 4°C. The mixture was extruded through a needle (1 mm) into 150 mM CaCl<sub>2</sub>, forming white beads of 3.0 mm (diameters). Then the beads were allowed to harden in CaCl<sub>2</sub> solution at room temperature for 30 and were washed with 50 mM tris-HCl buffer (pH 7)<sup>18</sup>.

### **Decolorization with immobilized cells in fluidized bed bioreactor**

The fluidized bed bioreactors are composed of 500 mL conical flasks containing the immobilized mycelium in calcium alginate suspended in 100 mL of liquid medium with 50 mg/L of Cibacron blue. The bioreactors were placed in a rotary shaker and the fluidization is assured by a high stirring of 100 rpm. Decolorization rate was followed according time.

### **Denaturing polyacrylamide gel electrophoresis**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) on one-dimensional 12 % polyacrylamide slab gels containing 0.1 % SDS [19]. Gels were run on a miniature vertical slab gel unit. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 at 0.025 % (w/v) in methanol/acetic acid/water (4:1:5, v/v/v) for 30 min at room temperature. Destained was done in methanol/acetic acid/water (4:1:5, v/v/v). The apparent subunit molecular weight was determined by measuring relative mobilities and comparing with the pre-stained SDS-PAGE molecular weight standards.

## **RESULTS AND DISCUSSION**

### **Isolation of CB decolorizing fungus**

Preliminary selection of CB decolorizing fungi was based on the decolorization of CB on MM plates. Four fungal isolates, which decolorized this anthraquinone dye (CB), were isolated from dye-contaminated sludge. Among these fungi, one fungal strain, which was identified as *Coprinus cinereus*, with higher CB decolorization potential in MM plates was selected for further studies.

### **Decolorizing screening**

The decolorization of 5 dyes samples during the fungal culture of the isolated strain was investigated. Dyes were at a concentration of 50 mg/L. *Coprinus cinereus* showed a higher ability to decolorize and degrade medium with 50 mg/L of cibacron blue than the others dyes as it's shown in figure 1 and 2, 100 % of decolorization was archived at 72 hours.

### **Temperature, pH and concentration effects**

Cibacron blue concentrations were varied between 25 and 150 mg/L. Percentage of decolorization reached the maximum level after 72 h of incubation when dye concentration was less than 50 mg/L. While it decreased with increasing dye concentrations above 50 mg/l that may be due to substrate inhibition at high dye concentrations (figure 3). This may suggest that dye concentration should not exceed 50 mg/L for higher percentage decolorization.

The slowest and lowest decolorization was obtained with pH 9, only 20 % of initial dye was removed, while the process decolorization was higher and faster at pH 7 up to 100 % of initial dye was removed; as it's shown in figure 4.

According to figure 5, the highest decolorization of Cibacron blue was obtained at 37°C followed by 30°C which suggest that the optimum temperature of *Coprinus cinereus* is 37°C.

### **Decolorization with immobilized cells in fluidized bed bioreactor**

At various times concentrations of Cibacron blue were measured spectrophotometrically at 590 nm for immobilised cells with *Coprinus cinereus*.

Figure 6 shows a fast decrease of Cibacron blue concentration after only 1 hour until decolorization was mostly complete. This might be due to the hydrodynamic behavior and mechanical bead properties of calcium alginate and make that polymer, a matrix of choice for the utilization in a fluidized bed bioreactor<sup>20,21</sup>.

### **Partial purification of peroxidase**

After a centrifugation at 15000 g for 30 min, an ultrafiltration trough amicon stirred cells (10 Kda) was investigated to obtain 42.1 mg/ml of proteins. Electrophoretic gel (figure 7) showed two bands were clearly observed that we correlated to peroxidase. The molecular weight of the enzyme was found to be approximately 69 Kda and 61.5 Kda (figure 8). In order to confirm the activity of the enzyme we test the fraction by using native PAGE gel in presence of Cibacron blue and as it shown in figure 9, degradation was observed. This may suggest that this enzyme is implicated in the degradation of Cibacron blue.

Fig. 1: Percentage of decolorization of 5 dyes in liquid medium by *Coprinus cinereus*

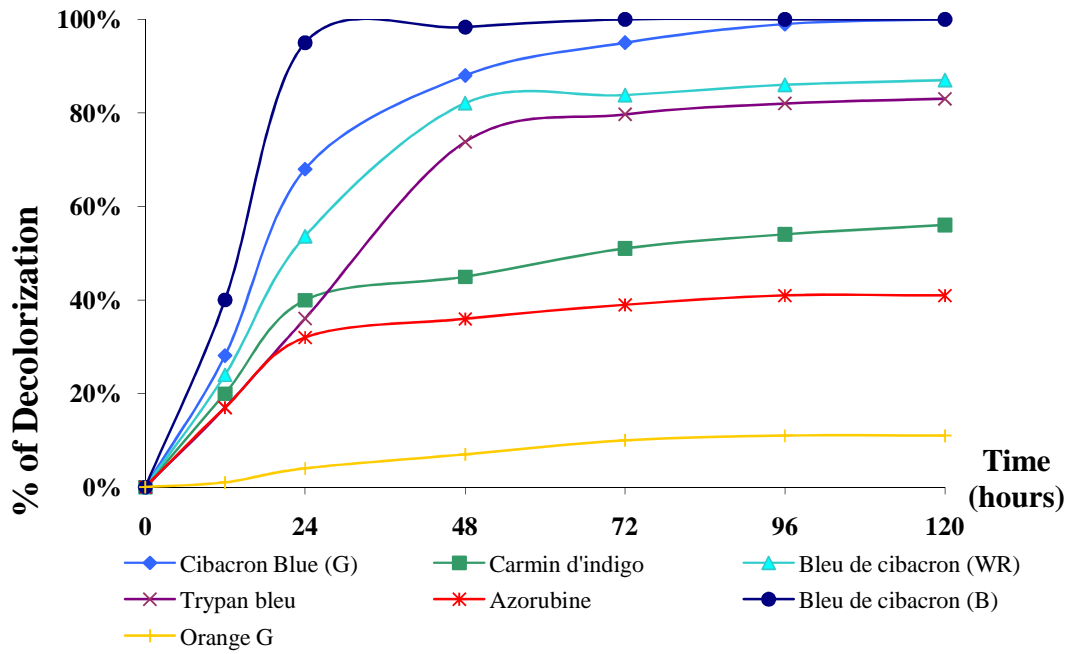


Fig.2: Decolorization of Cibacron Blue (50 mg/L) with *Coprinus cinereus* in liquid (2a) and solid (2b) mediums

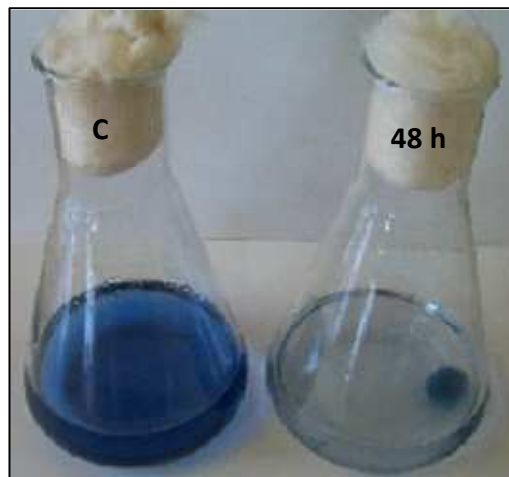
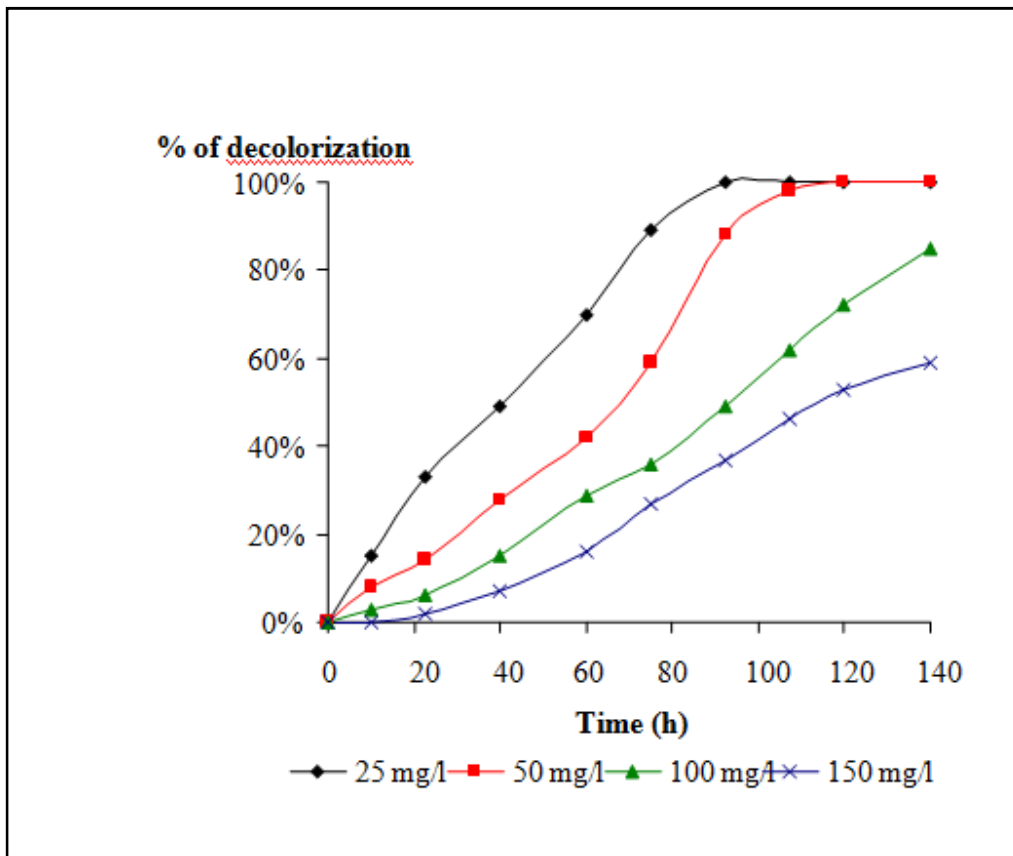


Fig 2a: culture in liquid medium



Fig 2b: culture in solid medium

**Fig.3: Variation of rate percentage decolorization with dye concentration**



**Fig.4: Variation of rate percentage decolorization with pH**

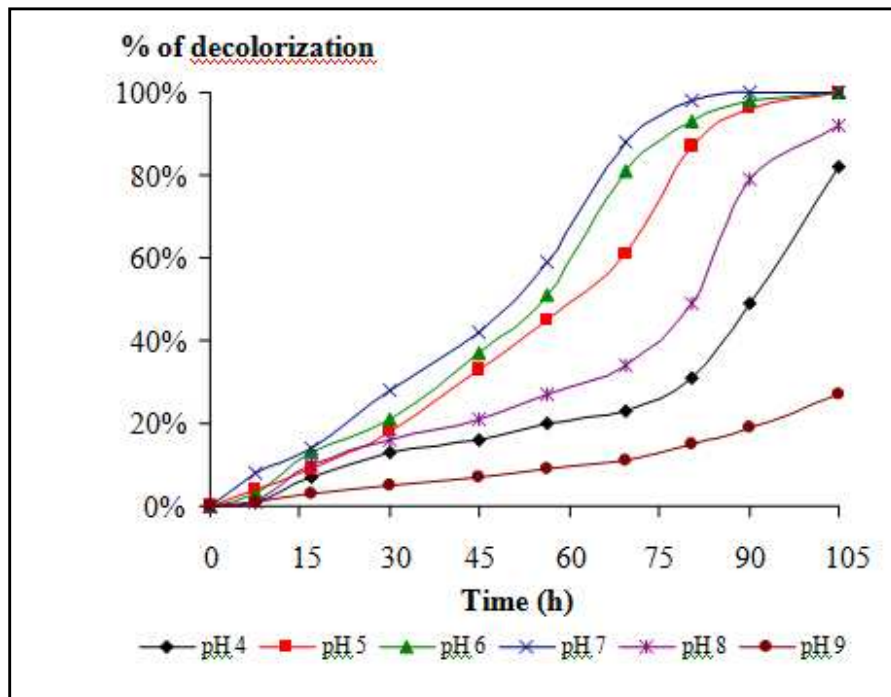


Fig.5: Variation of rate percentage decolorization with temperature

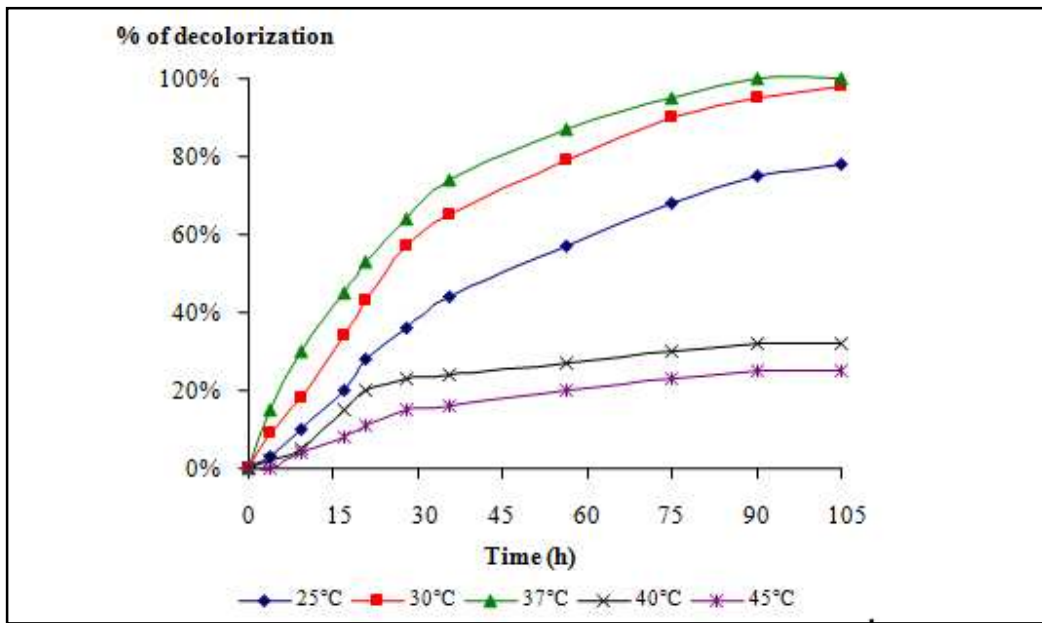
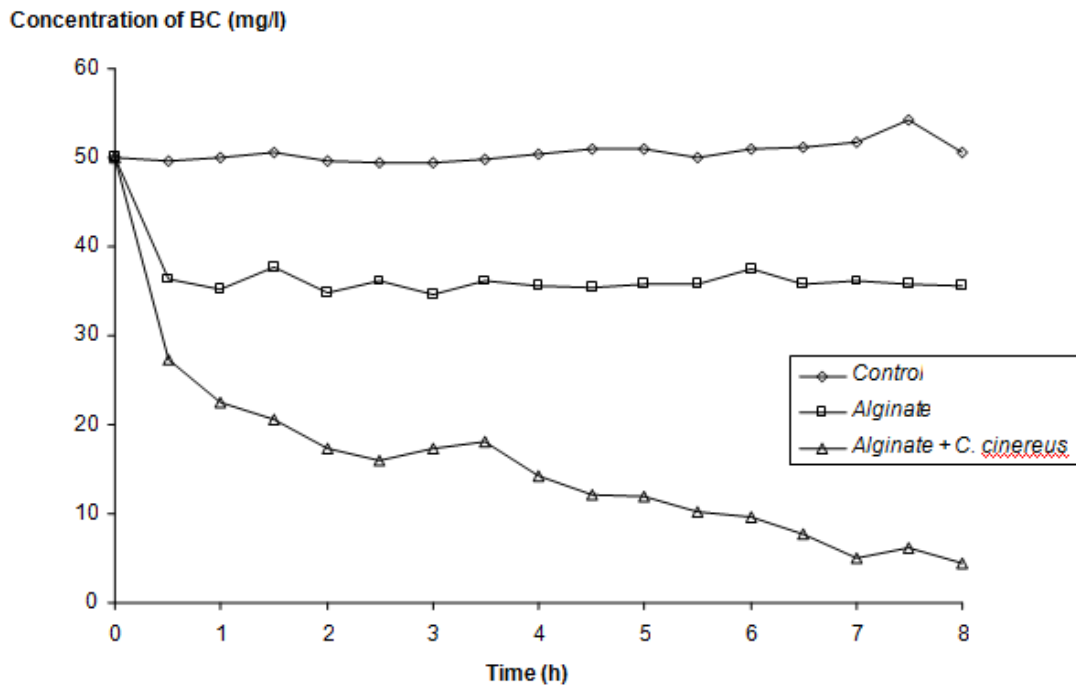
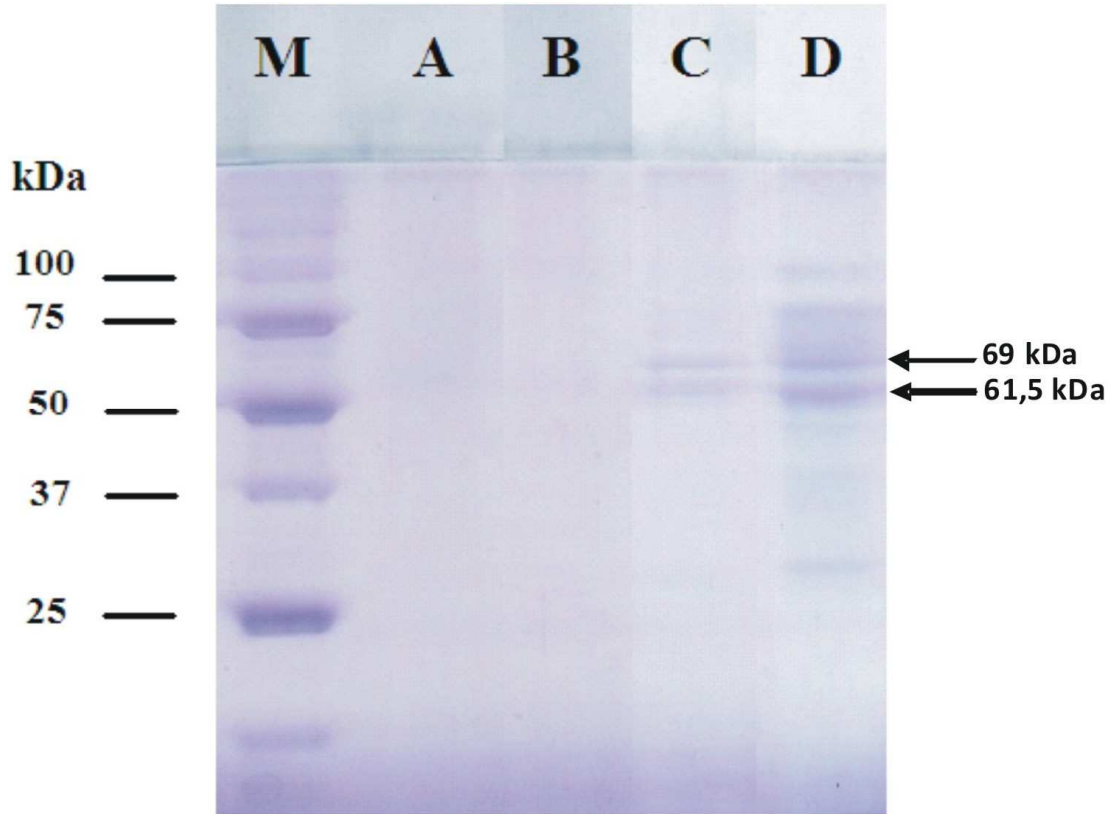


Fig.6: Decolorization of Cibacron blue by immobilized Coprinus cinereus

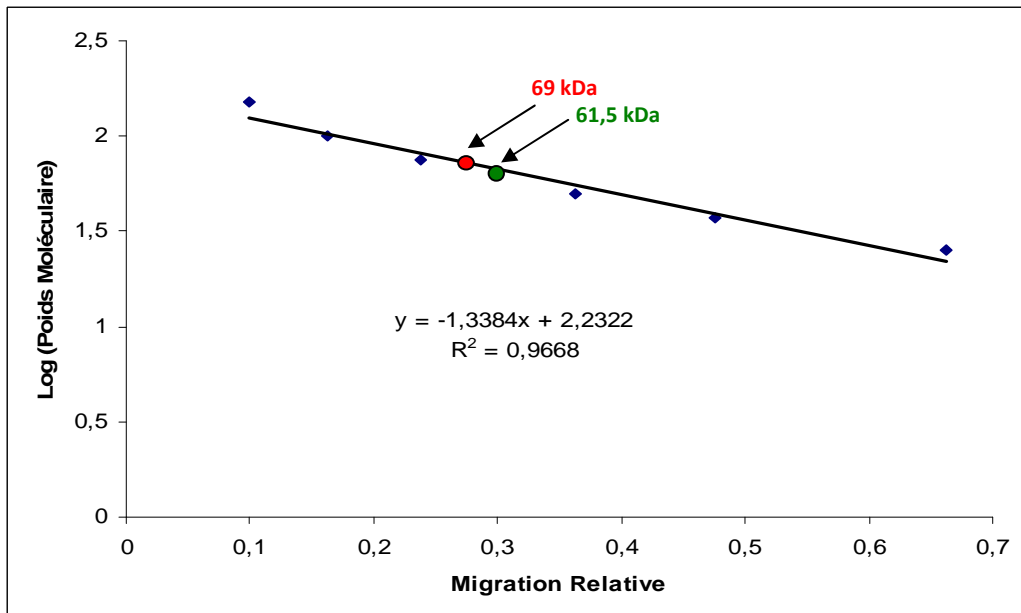


**Fig.7: Electroforetic profile of concentrated extract PAGE-SDS**

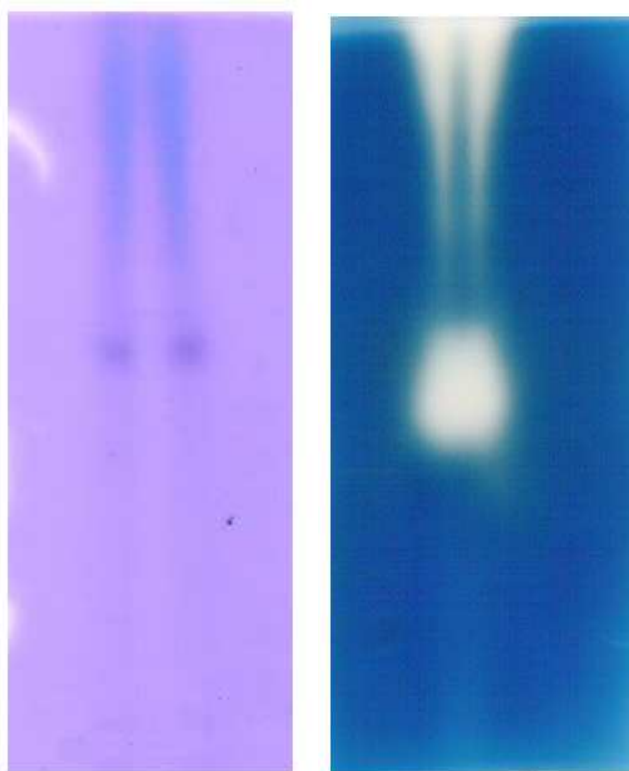


M: Precision Plus Protein Standards (Bio Rad). A et B: 2 extracts before ultra filtration  
C: concentrated extract. D: extract at 60°C

**Fig. 8: Molecular weight determination of the two subunits**



**Fig. 9: Electroforetic profile of concentrated extract on polyacrylamide gel without SDS**  
**A: Control. B: with dye (Cibacron blue)**



### CONCLUSION

It has been shown that high efficiency and fast decolorization of Cibacron blue was obtained when *Coprinus cinereus* was immobilized in calcium alginate. It shows that this technology allows specific and reproducible decolorization of the dye of high yield. Moreover the high specific decolorization rate obtained and the simplicity of the immobilization method mean that alginate would be a suitable immobilization matrix for using fungal strains to remove dyes from wastewater on industrial scale. This study also shows that the optimum conditions of a reproductive culture is 37°C and pH 7 for a complete degradation of 50 mg/L. After a partial purification, PAGE-SDS and Native PAGE gels suggest that a peroxidase is implicated in this decolorization.

### Acknowledgements

This project (PROTARS) was supported by the National Center of Scientific Research; the authors would thank them for their generous funds.

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