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## RESEARCH ARTICLE

# DISCOLORATION OF DYES BY *HEXAGONIA APIARIA* FUNGUS ISOLATED IN GABON AND SCREENING OF ENZYMES ON SOLID CULTURE MEDIUM

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**ABSTRACT**

The white-rot fungus *Hexagonia apiaria* isolated in wood parks in Gabon showed a high capacity to degrade synthetic dyes used in the textile industry. The results of this study show that this fungus has an optimum growth temperature between 30°C and 40°C. Optimum physicochemical parameters of discoloration on solid culture media are 35°C and 0.3g/L. On solid culture medium, the fungus produces different enzymes (cellulase, laccase, phenoloxidase, and peroxidases) in the presence of different substrates. At the concentration of 0.3g/L after 6 days, the discoloration rates are of 0.83, 0.76 and 0.78 (cm/day) respectively for Orange G, Reactive blue 4 and Congo red. The study reveals the opportunity of using this yet poorly known fungus for discoloration of synthetic dyes.



## INTRODUCTION

Dyes are chemicals which are consumed by a large number of chemical industries like textile, printing, paper, food and cosmetics (Pavko, 2011). Approximately 10,000 different dyes and pigments are used industrially and over 0.7 million tons of synthetic dyes are produced annually in the world India, the former USSR, Eastern Europe, China, South Korea and Taiwan consume approximately 600 thousand tons of dyes per annum (Ishikawa *et al*., 2000). The use of dyestuff has increased these last years because of the tremendous increase of industrialization and consumer demand for colour. The dyes disposed of during the coloration process are dissolved or suspended in wastewater. The coloration of industrial effluent is the most obvious indicator of water pollution. In addition, most dyes are resistant to breakdown, with the potential for persistence and accumulation in the environment (Talarposhti, 2001). However, most dyes are toxic, carcinogenic and mutagenic (Pinheiro *et al*., 2004). To remove dye from industrial effluent, physical and chemical methods such as adsorption, chemical precipitation, flocculation, chemical oxidation and reduction and electrochemical treatment are generally used (Pavko, 2011).

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Biotechnological methods using fungi, bacteria and algae in dye degradation has been well appreciated in general (Libra *et al*., 2003; Rigas and Drista, 2006; Pandey *et al*., 2007;Vaithanomsat *et al*., 2010; Atteke *et al*., 2013). Different fungi species such as the white rot fungi (*Pycnoporus sanguineus, Trametes versicolor, Phanerocheate chrysosporium* etc.) have been found to discolour dyes by using enzymatic systems, mainly lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac) (Libra *et al*., 2003; Kumaran and Dharani 2011; Atteke *et al*., 2013). In the present work, we have investigated the discoloration abilities of *Hexagonia apiaria,* a white-rot fungus isolated in Gabon, on textile dyes Congo red, Orange G and Reactive blue 4. In addition, this study reports simple screening methods for enzymes activities.

## MATERIALS AND METHODS

### Microorganisms

*Hexagonia apiaria* MUCL 51323 fungus was collected from wood parks in Gabon and identified using morphologic, macro/microscopic observations and identification keys by two mycologists Dr Cony Decock and Ms Yombiyeni Prudence (Université catholique de Louvain, Belgium). They conditioned and replicated the fungus in Petri dishes containing malt-agar

at 25°C. The fungus *Phanerochaete chrysosporium* MARD78 was obtained from the Interactions Arbres-microorganismes laboratory fungus collection and was used as control for discoloration and enzymatic activities.

**Dyes and chemicals**

The three dyes, Congo Red (RC), Reactive Blue 4 (RB4) and Orange G (OG) were obtained from Sigma-Aldrich (Sigma-Aldrich Chimie Sarl, Saint Quentin Fallavier, France); agar powder and malt extract were obtained from Acros Organics (Fisher Scientific SAS, Strasbourg, France); ABTS (acid, 2, 2-azino-bis (3-ethylbenzothiazoline)-6-sulfonic acid), Carboxy Methyl-Cellulose (CMC), guaiacol and cresol-p were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Methanol and gallic acid were obtained from Carlo Erba Réactifs (Carlo Erba Reactifs-SDS, Val de Reuil, France). Glucose was obtained from Prolabo (VWR International S.A.S, Strasbourg, France). All minerals to culture media were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA).

**Culture Conditions**

The composition of the solid growth medium used was: 0.2% glucose, 0.2% malt extract, 0.01% $MgSO_4$, 0.01% $MnCl_2$, 0.03% $NH_4NO_3$, 0.026% $KH_2PO_4$, 0.026% $Na_2HPO_4$, 0.01% $CaCl_2.2H_2O$, 0.0001% $FeC1_3.6H_2O$, 0.0001% $ZnCl_2$, 1.6% and 1.6% agar in 1 L of distilled water. The pH was adjusted to 5 using hydrochloric acid (0. 5 N) before autoclaving at 121°C for 15 min. The culture medium was transferred into 4 200-mL Erlenmeyer flasks and each reagent (enzymatic revelation) or dye, depending on its final concentration, was added. The mixture was shaken and then about 15 mL were distributed into Petri dishes (diameter 8.5 cm). All tests were conducted four times (averages are presented with the corresponding standard deviations).

**Optimal conditions of growth and discoloration**

**Optimal growth temperature**

Growth was carried out in wide range of temperatures (15°C, 22°C, 30ºC, 35ºC, 40 and 45ºC) by measuring the total diameter of growth (colony expansion) each day for one week at 75% humidity, in order to optimize the growth. A 5-mm diameter disc that contained mycelia was placed in the centre of each dish. The average growth was computed in $mm.day^{-1}$. Measurements of each colony in the Petri dish were made and all tests were conducted in quadruplet. Data are expressed as means and standard deviations.

**Discoloration at various temperatures**

Temperature plays an important role in the growth of an organism. In order to optimize the discoloration of the dyes by fungus, the process was evaluated at different temperatures 15°C, 22°C, 30ºC, 35ºC, 40°C and 45ºC on solid culture medium. The diameters of discoloration halos and growth colonies were measured. Then, different ratios and rates were determined as described by Sánchez-López *et al*. (2008). We have two controls were prepared: Petri dishes containing dye but not inoculated (to assess possible abiotic discoloration) and Petri dishes inoculated but not containing dye.

**Discoloration at various concentrations**

Different concentrations (0.05; 0.1; 0.3 and 1.5g/L) of CR, OG and RB4 were added to the culture medium at 35°C in order to evaluate their optimal concentration at discoloration. The diameters of discoloration halos and growth colonies were measured each day for a week. Each test was conducted in quadruplet. Cultural conditions and results of the discoloration were determined as described above.

**Screening for extracellular enzyme activities**

Screening methods are based on colorimetric measurements for which colour changes of reagents in the solid media are associated with specific fungal enzyme activities. Laccase activity was obtained according to the slightly modified method of Mishra *et al*. (2011) on the culture medium containing 0.01% (w/v) guaiacol. One disc of agar (1-cm diameter) containing fungus was placed at the centre of a Petri dish. After a few days, a red coloration appeared around the colony, indicating the presence of laccase. Extracellular phenoloxidase, peroxidase and laccase (ABTS) activities were evaluated by the methods described by Pointing (1999)**.** For tyrosinase activity, fungi were grown for 3 days on solid culture medium. Then wells were dug at the ends of the colony and drops of 0.11% p-cresol and 0.05% glycine were introduced. After 24 hours, appearance of a red brown colour in the wells indicated tyrosinase activity. For cellulase activity, the solid culture medium used contained 2% carboxymethyl cellulose (CMC) instead of glucose as a carbon source. The inoculated plates were incubated for 3 days at 30°C and the growth diameters of the colony were measured. 10-mL aliquots of Congo red dye (0.1 g/L) were then added to each Petri dish. After 20 min, the solution was discarded and the cultures were washed with 10 mL of 1 mol/L NaCl for 30 min. Cellulase production was indicated by the appearance of a pale halo with orange edges, indicative of areas of hydrolysis. Four independent experiments were performed for each enzyme screened.

## RESULTS

**Radial extension of *Hexagonia apiaria***

The results of the effect of temperature on the growth of *Hexagonia apiaria* fungus are represented on Figure 1.

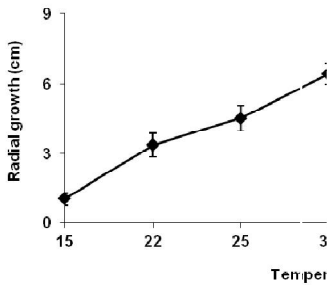



**Fig 1. Effect of temperature on growth of *Hexagonia apiaria* during a week**

Mycelia growth increases with temperature between 15 and 35°C. Beyond 35°C, mycelia growth slowed to almost inhibition at 45°C. The optimal temperature of growth for this fungus is 35°C on solid culture medium. This fungus has a higher optimum temperature compared to many others strains of *Ganoderma lucidum* whose optimum temperature is between 25°C and 30°C (Jayasinghe *et al*., 2008). The white rot fungus *Phanerochaete chrysosporium* is very well known (Urek and Pazarlioglu, 2007; Kumaran and Dharani, 2011) and has an optimum growth temperature very high between 35°C and 40 °C (radial growth 19.61 and 17.08 respectively). Comparatively, the mycelia extension rate of *Hexagonia apiaria* increased at elevated temperatures up to 35°C (radial growth 12.89 for 35°C and 10.10 for 40°C) (Table 1). Low temperatures (15°C to 22°C) lead to a slowdown in the growth of the mycelium.

**Table 1. Radial growth rates (mm.day$^{-1}$) of two fungi on solid culture medium at temperatures from 15°C to 45°C**

| Temperature (°C) | Radial growth (mm day$^{-1}$) | |
|---|---|---|
| | *Phanerochaete chrysosporium* | *Hexagonia apiaria* |
| 15 | 3.89 | 2.95 |
| 22 | 7.35 | 4.57 |
| 25 | 9.63 | 5.64 |
| 30 | 13.92 | 7.95 |
| 35 | 19.61 | 12.89 |
| 40 | 17.08 | 10.10 |
| 45 | 12.57 | 2.90 |

Beyond 40°C, the rates of discoloration are less than 50%. The maximum discoloration was observed at 35°C (Figure 2).

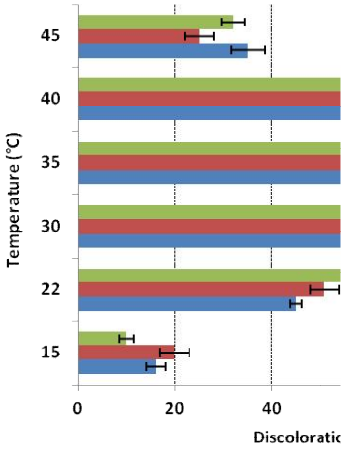



**Fig. 2. Discoloration of synthetic dyes by *Hexagonia apiaria* at different temperatures on solid culture medium during six day**

The effect of dye concentration (0.05 to 1.5 g/L) was investigated, keeping other parameters constant (temperature 35°C and pH 5). When dye concentration increased from 0.05 to 0.3 g/L, the discoloration slowly decreased from 100 to 90%; rates which were still very high (Figure 3). Although the maximum removal (100%) of colour was observed at 0.05 and 0.1 g/L, these concentrations are very low and were rapidly eliminated by this fungus. The most interesting optimum dye concentration was found between 0.2 and 0.3 g/L, with discoloration rates from higher than 95% and 90% respectively. For this study, all reactions of discoloration were positive (Figure 4). Dye discoloration activity was screened by simple solid culture medium method by amending the dyes OG, RB4 and CR at a concentration of 0.3g/L. The mycelia growth covered the solid culture medium completely on fifth day with discoloration zones. Further incubation led to complete discoloration on tenth day (not presented). Two

**Table 2. Fungal growth on solid medium containing dyes after six days**

| Fungal species | Orange G | | Reactive blue 4 | | Congo red | |
|---|---|---|---|---|---|---|
| | Discoloration scale[a] | Radial growth[b] | Discoloration scale[a] | Radial growth[b] | Discoloration scale[a] | Radial growth[b] |
| *Hexagonia apiaria* | +++ | +++ | +++ | +++ | +++ | +++ |
| *Phanerochaete chrysosporium* | + | +++++ | + | +++++ | + | +++++ |

[a]Discoloration scale measured on the 6th day of cultivation on solid culture medium containing 0.3g/L of each dye: + diameter of the discoloured zone 0–20 mm, ++ zone diameter 21–30 mm, +++ zone diameter 31–50 mm, ++++ zone diameter, 51–70 mm, +++++ zone diameter 71–90 mm.
[b]Radial growth rate measured on the 6th day of cultivation on solid culture medium containing 0.3g/L of each dye: + diameter of the mycelia colony 0–20 mm, ++ colony diameter 21–30 mm, +++ colony diameter 31–50 mm, ++++ colony diameter 51–70 mm, +++++ colony diameter 71–90 mm.

**Table 3. Discoloration of dyes (0.3g/L) on solid medium by white rot fungus after six days**

| Fungus | Orange G | | | Reactive blue 4 | | | Congo red | | |
|---|---|---|---|---|---|---|---|---|---|
| | Discoloration rate (mm/day) | Inhibition rate (%) | Growth rate (mm/day) | Discoloration rate (mm/day) | Inhibition rate (%) | Growth rate (mm/day) | Discoloration rate (mm/day) | Inhibition rate (%) | Growth rate (mm/day) |
| *Hexagonia apiaria* | 8.3 | 2.2 | 9.3 | 7.6 | 10.0 | 7.0 | 7.8 | 7.0 | 7.6 |

**Table 4. Qualitative enzymes production of fungi**

| Fungalspecies | Strain no | Cellulase CMC | Laccase (Guaiacol) | Phenoloxydase (Gallic acid) | Laccase (ABTS) | Peroxidase | Tyrosinase |
|---|---|---|---|---|---|---|---|
| *Hexagonia apiaria* | 51323 | ++ | ++ | +++ | +++ | ++ | - |
| *Phanerochaete chrysosporium* | MARD78 | +++ | - | +++ | - | +++ | - |

## Optimization of dye discoloration

In order to maximize the discoloration of the textile dyes OG, RB4 and CR, experiments were conducted at different temperatures and concentrations of dyes. To explore the effect of temperature, experiments were performed at different temperatures (15°C, 22°C, 30ºC, 35ºC, 40°C and 45ºC) keeping other conditions constant (pH 5, dye concentration 0.3g/L). For these three dyes, it was observed that the rate of discoloration increased with increasing temperature, with discoloration rates more than 70% between 30°C and 40°C.

strains of white rot fungi were screened for discoloration and radial growth rates on solid culture medium containing 0.3g/L of each dye (OG, RB4 or CR) (Table 2). Both strains discoloured differently the media during six days. *Phanerochaete chrysosporium* discoloration of the dyes was not homogeneous (not presented) on solid media compared to *Hexagonia apiaria*. The two fungi grew rapidly on solid media in the presence of the dyes and a positive correlation between the radial growth and the discoloration rate was found especially in the case of *Hexagonia apiaria*.

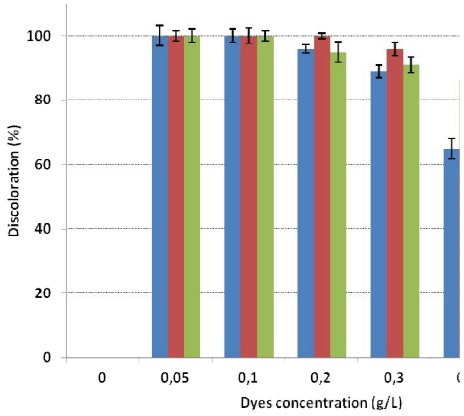
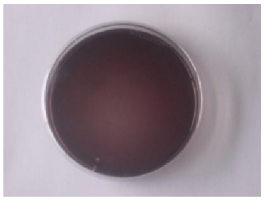



**Fig. 3. Discoloration of synthetic dyes by *Hexagonia apiaria* at different concentrations on solid culture medium during six days**

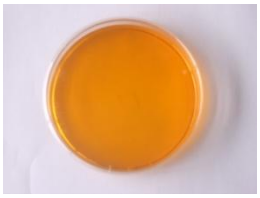
The dyes evaluated in this study were degraded by fungus *Hexagonia apiaria*. The growth and degradation efficiencies of the test fungi as determined based on their discoloration ability in solid media are shown in Table 3. The rate of discoloration OG is superior to those of RB4 and CR. Nevertheless, the inhibition of growth is low in the presence of different dyes. Both fungi produce cellulolytic and ligninolytic enzyme activities on solid media (Table 4). As described in literature (Urek and Pazarlioglu, 2007), *Phanerochaete chrysosporium* does not produce laccase, unlike *Hexagonia apiaria*. The highest level of ligninolitic activities such as laccase, peroxidise and phenoloxidase produced by *Hexagonia apiaria* as observed in Figure 5, indicates a strong enzymatic activity and therefore a high ability to degrade dyes and xenobiotics.

## DISCUSSION

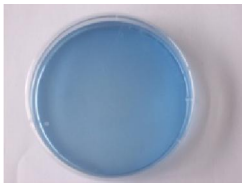
The present study is an effort to develop a potential fungal isolate as an effective discolouring agent of industrial synthetic dyes OG, RB4 and CR. This fungus showed maximum grow that a temperature of 35°C on the solid culture media. This temperature is close to those observed in the sampling site in Gabon (30°C-40°C). Many other decay fungi possess optimal growth temperatures in this range (Hakala *et al*., 2004; Urek and Pazarlioglu, 2007; Duarte *et al*., 2012). The effect of temperature and concentration on degradation of dyes (RB4, CR and OG) was studied at pH 5 and it was observed that at 0.3g/L dye concentration and 35°C, discoloration by fungus



**Congo red control (0.3g/L)**

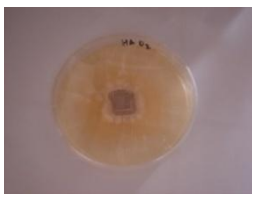


**Orange G control (0.3g/L)**

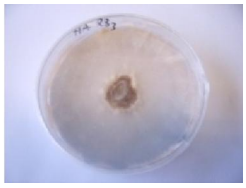


**Reactive blue 4 control (0.3g/L)**

**Congo red discoloration on solid medium**

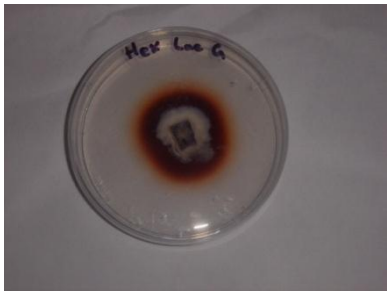


**Orange G discoloration on solid medium**

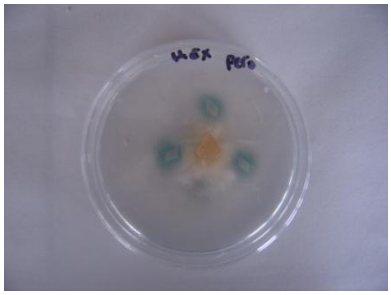


**Reactive blue 4 discoloration on solid medium**

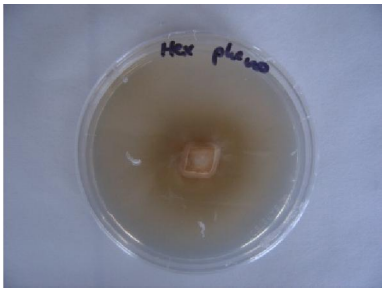
**Fig. 4. Screening of dyes degrading by *Hexagonia apiaria***



**Observation of red colour around a colony with guaiacol indicates laccase activity**



**Observation of the intense blue colour in wells with $H_2O_2$ indicates peroxidase activity**



**Observation of the brown zone around a colony indicates phenoloxidase activity**

**Fig. 5. Screening** for **ligninolytic enzymesof *Hexagonia apiaria* on solid media**

*Hexagonia apiaria* was optimum during six days. In literature, different studies had shown the capacity of white-rot fungi to degrade classes of synthetic dyes at concentrations below 0.2g/L (Tekere *et al*., 2001; Rigas and Drista, 2006; Bibi and Bhatti, 2012). Nevertheless, some studies have shown the ability of different fungal strains like *Dichomitus squalens*, *Ischnoderma resinosum* and *Pleurotus calyptratus* and *Datronia sp* to degrade dyes at high concentrations (Eichlerová, 2005; Vaithanomsat *et al*., 2010). The optimum concentration of our study (0.3g/L) is above those normally used, with very high rates (over 90% discoloration), showing effective discoloration of the three dyes. The discoloration was assessed as the disappearance of colour from the Petri dishes during the growth of the fungal mycelium. For the three dyes, the applied fungus showed positive results for biodegradation and the initial colours of these dyes were modified. A small fraction of dyes (RB4 and CR) was also accumulated by the applied fungus and its mycelia turned into blue sky colour for RB4 or red colour for RC. In generally, white rot fungi produce several enzymes that have been related to their ability to degrade natural polymers such as lignin and cellulose, but can also attack different synthetic chemicals usually recalcitrant to biodegradation (Eichlerová, 2005; Prachi and Anushree, 2009; Vaithanomsat *et al*., 2010). The colour removal observed in our study may be due to the production of extracellular enzymes by the applied fungus, during the biodegradation of tested dye. Moreover, the microbial degradation of various hazardous dyes like CR, RB4 and OG by the different fungi has been investigated earlier with different conditions and degradation pathways (Eichlerová *et al*., 2005; Axelsson *et al*., 2006; Saranraj *et al*., 2010). Breakdown of most of xenobiotics by fungi is closely linked with ligninolytic metabolism. Discoloration of dye is related to the process of extracellular oxidases, particularly laccases, manganese peroxidases and liginin peroxidases which have been reported to discolour dyes. In the present study, the discoloration of dyes by *Hexagonia apiaria* appeared to be due to the production of extracellular enzymes by this fungus in the medium containing the dye. The change in colour might be due to the biochemical (metabolic) reactions of fungal strain.

**Conclusion**

The qualitative assessment by screening in solid culture medium with different substrates showed the ability of the fungus *Hexagonia apiaria* to discolour diverse synthetic dyes (RB4, CR and OG) on solid media. This study is one of the first to report the discoloration capability accompanied by the production of ligninolytic enzymes, mainly laccase, phenoloxidase and peroxidases, by the fungus *Hexagonia apiaria* on solid media. The optimum discoloration parameters obtained were 35°C and 0.3g/L. A thigh concentration of 0.5g/L, the discoloration rate was higher than 60%. Slowdowns were observed with concentration of 1.5g/L. The results also indicate that ligninolytic enzymes from *Hexagonia apiaria* possesses a significant dye degradation capacity in solid culture media and can further be applied in bioremediation of toxic industrial dyes.

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