

Dye Degradation by Fungi: An Exercise in Applied Science for Biology Students

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Abstract: An easily implemented practical exercise in applied science for biology students is presented that uses fungi to degrade an azo-dye. This is an example of bioremediation, the employment of living organisms to detoxify or contain pollutants. Its interdisciplinary nature widens students' perspectives of biology by exposing them to a chemical engineering process. It also demonstrates the use of biological organisms to degrade a relatively complex chemical, Amaranth. This azo-dye, which is vibrant red across a broad pH range, is safe to employ in the student laboratory. Various species of white rot fungi of the genus *Trametes* are able to degrade Amaranth to differing degrees, permitting comparative studies. This laboratory exercise involves observations that occur over the course of 3 days, but may be shortened or lengthened, as desired. Results involving decreases in color intensity may be obtained by making comparisons to color standards or by using a spectrophotometer. In our example, we suggest using species of white rot fungi, including *Trametes versicolor* and *Trametes pubescens*. The complexity of the lab is easily modified to challenge students of diverse backgrounds because the degree of difficulty of the exercise is adjustable to suite biology non-majors or majors in introductory through advanced courses.

Keywords: Bioremediation, applied science, interdisciplinary exercise, biology non-majors, biology majors, mycology, white rot fungi, fungal culturing, azo-dye

Introduction

Major areas of concern when instructing college and university biology students include how to effectively demonstrate that basic information leads to applications (Clarkeburn et al., 2000) and how interdisciplinary approaches (Barisonzi and Thorn, 2003; Zoller and Scholz, 2004) improve the understanding of biology (Fields, 2001; McCarthy, 2004). Students should be engaged in interesting exercises that teach them the value of basic knowledge (Hughes, 2001; DebBurman, 2002) in society (Key and Nurcombe, 2003). This laboratory exercise was originally designed for a course in bioremediation, the use of organisms to dispose of environmental contaminants, but is equally adaptable to introductory courses involving aspects of environmental biology.

The theme of the lab exercise is the investigation of how white rot fungi can be used to degrade a dye similar to those used in the textile industry, where the dye waste problem is estimated at over eight hundred thousand tons annually (Wesenberg et al., 2003). The exercise follows the course of dye decoloration by species of the genus *Trametes* involving the degradation of the azo-dye, Amaranth. A degradation pathway has been proposed by Champagne and Ramsay (2005) in which the azo bond may be cleaved first (Martins et al. 2003).

Because these fungi grow into spheres in liquid culture, they can be counted to provide an estimate of biomass. Dye is added to the cultures and the loss of color is monitored over a period of days; thereby, color change is an indicator of the rapidity of the process while making comparisons between species. Details of the laboratory preparation for instructors are provided. The students' part of the exercise requires less than one hour for initial setup, and measurements at each time point take about 15 minutes. Required materials and organisms are readily available from scientific suppliers. Conventional laboratory glassware in the form of flasks, liquid measurement and transfer apparatus, and a platform shaker are required. Instructors need some training in microbial culturing techniques, although the student laboratory exercise may be performed without the use of sterile technique.

Materials and Methods

Recommended Protocol

The following is a protocol that we have been using in our course. These may be modified as suggested in the Discussion section.

Fungal Species

Species of the genus *Trametes* can be obtained from the American Tissue Culture Collection (<http://www.atcc.org/>) e.g. *Trametes versicolor*

ATCC Number: 64311 (turkey tail fungus), and by searching the catalogues at CABRI (Common Access to Biological Resources and Information) website (<http://www.cabri.org/CABRI/home/index.html>) e.g. *Trametes pubescens* Strain Number: CBS 396.90 from CBS (Centraalbureau voor Schimmelcultures) Utrecht, The Netherlands. We recommend obtaining strains of *Trametes versicolor* and *Trametes pubescens* which decolor Amaranth relatively quickly, whereas other species, such as *Trametes hirsuta*, are less active. A lab exercise involving two or more species enables the students to rank the species' effectiveness in bioremediation.

Storage and Culture of Fungi

Preparation of fungi by the instructors for the practical exercise requires the use of sterile technique. Plastic Petri plates and 50 mL screw capped tubes can be purchased sterilized in plastic sleeves. Glassware and other implements such as metal spatulas need to be sterilized prior to their use by autoclaving, or by washing in 70% ethanol:water and rinsing with sterile water, ideally in a sterile air laminar flow cabinet. Common means of sterilizing fungal culture media and water include boiling, pressure cooking, and autoclaving. In the latter case, culture media may be autoclaved in glass flasks capped with aluminum foil lids.

Fungal strains are grown on semi-solid Kirk's 1.2 AT medium containing Malt agar (30 g/L) in 150 mm Petri plates, each containing 10-15 mL. The Kirk's 1.2 AT medium contains 1.2 g/L ammonium tartrate (see Table 1; Kirk et al. 1978). Seal the plates' lids with Parafilm™ (Fisher Scientific) or plastic wrap. Allow the fungi to grow for 3-7 days at 25-30 °C after which these plates can be kept in a refrigerator for up to two months.

To prepare fungi for the student laboratory, small amounts are removed with a sterilized spatula from these storage plates as pieces of agar approximately 5 mm in diameter. These are placed in the center of fresh plates containing the same medium and incubated at 25 to 30°C.

Mycelial growth should be evident within one or two days and it will reach the edge of the plate in a few days. Before it does, take hollow tubes such as plastic drinking straws (sterilized in 70% ethanol:water) and press into and extract round agar plugs from the growing edge of each fungal species. Use different tubes for each species to place 20 to 25 plugs as inoculum into 100 mL liquid cultures of Kirk's 1.2 AT medium (no Malt agar) in 250 mL Erlenmeyer flasks (or similar containers) capped with aluminum foil lids down to at least 5 cm below the lip. Flame the lids with an alcohol or Bunsen burner and then place into a rotary shaker at 30 rpm or an oscillating shaker at 15 rpm for 4 days at 25-30 °C. The plugs will grow into spheres and should not

break up under gentle agitation. The number of spheres is used to determine the amount of fungus in all subsequent samples.

Using a spatula or small spoon remove the fungal spheres from the flasks and place them into an open sterile Petri plate and use this as a source to inoculate the decoloration chambers.

Setup of Student Samples

Clear plastic fifty mL sterile screw-cap tubes (Fisher Scientific) are used as decoloration chambers. One to six fungal spheres are transferred into each chamber containing 15 mL of liquid Kirk's 0.2 AT medium (containing 0.2 g/L ammonium tartrate) to which 20 ppm Amaranth dye is then added from a 100 X stock of Amaranth (0.2% in water), i.e. 150 µL in 15 mL. These tubes are tightly capped and placed on their sides in groups of four to six, held together with elastic bands. Incubation is at 25-30°C with gentle shaking (15 rpm). In 1-5 days, the dye will decolor because low nitrogen and the azo-dye, itself, act to induce the lignin-degrading enzymes, peroxidase and laccase. When all species have decolorated the Amaranth dye, they are ready for the student practical exercise.

Amaranth Decoloration Experiments

Each group of students is provided with a pair of samples for each species of *Trametes* in fifty mL tubes. Three or four groups may be permitted to pool their results if statistically valid comparisons are desirable. One tube of each pair is heat treated in a boiling water bath for one hour, allowed to cool, and employed as a negative control to see if any subsequent decoloration could be attributed to the non-biological composition of the media or the physical conditions of the experiment. These heat-treated controls may be prepared ahead of time. All tubes are then treated as follows. Amaranth was added as a 100 times stock (0.2%) to bring the concentration to 20 ppm, i.e. 150 µL in 15 mL, and the samples are returned to the shaker incubator at 25-30 °C.

Decoloration, i.e. degradation, can be monitored in a simple fashion by comparing the color of the tubes to a series of tubes of Kirk's media containing 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 ppm Amaranth dye using the same volume of 15 mL in the fifty mL tubes. This provides the advantage of not needing to remove a sample for measurement by spectrophotometer (see below), which means that sterile technique is not a concern, although it is recommended to open the tubes to provide fresh air to the cultures once a day. The sample times should include an immediate reading which assures that a proper amount of dye was added, and as many hourly readings over a 2 to 3 day period as is reasonably possible. When complete decoloration has occurred

in the fastest decoloring species after 1-2 days, a second addition of Amaranth may be added to the tubes and another set of data collected, if desired.

Alternative Spectrophotometric Method

Spectrophotometry can be employed as an alternative method to estimate Amaranth concentrations at an optical density of 523 nm and using Kirk's medium as a blank. Ensure that the volumes removed are small (less than 50 μ L) to permit sampling, which may require more than ten time points over a three day period. Precision pipetting is critical for these small volumes. Also, avoid any flocculent material. Hamilton syringes sterilized with 70% ethanol or automatic micropipettors using sterilized disposable tips are recommended.

Discussion

We employ this lab in our course on bioremediation. It requires minimal resources and is easily set up. For example, in the absence of a shaking incubator, manual agitation of the cultures can be performed at room temperature. This can be as simple as a gentle rocking every 1-2 hours during the working day. In our course, all of the students attend the initial exercise period that involves the addition of dye to the fungal cultures and the first two readings, *i.e.* zero and one hour time points. After this, members of each group share the time-point readings, which they coordinate with the instructors.

The complexity of this lab can be modified to suit biology non-majors and majors at introductory to advanced levels. It can be simplified by reducing the number of fungal species. Conversely, the exercise can be made more complex by increasing species number, varying media composition, and modifying growth conditions such as using different pHs and temperatures. For example, the media may contain

different levels of nitrogen (Swamy and Ramsay, 1999a) and metals such as copper and manganese (Swamy and Ramsay, 1999a; Galhaup and Haltrich, 2001). Other dyes may also be employed such as Tropaeolin O and Reactive Blue 15 (Ramsay and Nguyen, 2002), although precautions will be needed to avoid potential toxicity. In this way the laboratory exercise can be adapted to suit the experience level of the students.

Measuring enzyme activities over time can also be added to this lab. The relatively simple assays for laccase and peroxidase are described in Johannes and Majcherczyk (2000) and Wariishi et al. (1992), respectively.

The students may be required to tabulate the results (see Table 2), plot them and answer questions such as: What is the rate of dye degradation? or Which species is the most effective at dye degradation? Statistical analysis with more replicates can provide additional complexity. In an advanced course the student may be requested to submit a formal laboratory report containing the following sections: introduction, methods and material, results, and discussion.

Students appreciated seeing a useful bioremediation process that utilized recognizable wood fungi such as the turkey tail fungus (*Trametes versicolor*). They are exposed to a simple form of microbial batch culture in a biological engineering application and the effects of powerful degradation enzymes. This lab also highlights the enormity of the global dye-waste problem. The students indicated interest in implementation of this dye degradation at a larger scale and the biochemistry associated with the process. This exercise is interdisciplinary in nature and can be employed in biology, botany, environmental science and chemical engineering for majors and non-majors as well as in more focused bioremediation courses.

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