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Biodegradation of Crystal Violet (hexamethyl-p-rosaniline chloride) by Oxidative Red Yeasts

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Crystal violet (hexamethyl-p-rosaniline chloride) an aniline dye, plays an important role in the classification of bacteria; thus, it is commonly used in the Gram stain method as well as in the culture media for the detection and enumeration of coliform organisms in water, food products, milk and other dairy products (Gerhardt et al. 1981).

A recent study has confirmed the presence of crystal violet and malachite blue in the sediment and water of the Buffalo River, near Buffalo, N.Y. (Nelson and Hites 1980). The isolation and identification of these dyes raise a question as to the possible persistence of the aniline dyes in the aquatic environment. In addition, results of a study by Black et al. (1980) also demonstrated that some of the aniline dyes could be mutagenic and carcinogenic to biota. Thus, these chemicals have been suggested to be responsible for the promotion of tumor growth in several bottom feeding species of fish (Diachenko 1979; Black et al. 1980). Consequently, knowledge of their ecological behaviour and fate is useful for the assessment of new and existing organic dyes.

Yeast and fungi, in particular, are directly involved in the removal and bioconcentration of toxic substances from the environment (Chacko and Lockwood 1967; Kwasniewska 1982). For this reason, current research efforts are directed towards the examination of the effects of contaminants on important aquatic species, such as yeast or yeast-like fungi (Kwasniewska and Kaiser 1983). They are similar to higher eukaryotes (such as mammals) in both cellular structure and metabolic behaviour. As a result, they offer certain biochemical, metabolic and genetic advantages over bacteria in toxicity research (Parry 1977). Furthermore, yeasts or yeast-like fungi are ubiquitous organisms (Ahearn et al. 1969; Hedrick and Soyugene 1965, 1967; Hedrick et al. 1966, 1968; Spencer et al. 1981) and have been shown to possess mechanisms to cope with various physical and chemical stresses. Such characteristics make yeasts useful and important organisms for the study of contaminant toxicity and biodegradation.

For various reasons comparatively little attention has been given to the use of yeast or yeast-like fungi in biodegradation of environmental contaminants.

In this report the biodegradation of crystal violet by oxidative red yeasts is described and the role of yeasts or yeast-like fungi in the removal of environmental contaminants is discussed. The fermentative strain of S. cerevisiae was also studied and could be considered a good mutant for dye in future studies.

MATERIALS AND METHODS

The crystal violet (hexamethyl-p-rosaniline chloride) was obtained from BDH Chemicals, Pool, England. The stock solution was prepared at the concentration of 0.1% (w/v) and was directly used in the experimental procedures.

Two growth media were used in the biodegradation experiments. The agar plate was prepared from the Littman Oxgall agar plate (Oxoid), and the broth medium was prepared in a laboratory with the following ingredients (gL^{-1}): glucose 10.0 g; peptone 10.0 g; Ox-Bile 15.0 g; and crystal violet 0.01 g. The medium was sterilized at 120°C for 15 minutes and the final pH of the medium was 7.0 (approximately). No antibiotics were used in either of these two media. The concentration of the test crystal violet in the growth media was at the level of 10 ppm.

In an earlier study by Kwasniewska and Kaiser (1983), the fermentative and oxidative yeast strains were observed to exhibit a different response to the same organic chemicals. Therefore, both oxidative red (Rhodotorulae) and fermentative S. cerevisiae were chosen for use in the biodegradation of crystal violet. The Rhodotorulae sp. originally was isolated from activated sewage sludge from a local municipal sewage treatment plant (Burlington, Ontario), Rh. rubra from the shore of Lake Ontario at Burlington, and commercially used S. cerevisiae from a grocery store.

Each strain was cultured in 50 mL of the broth medium in a 150 mL Erlenmeyer flask on a gyratory shaker (120 strokes min^{-1}) at 22°C for 24 hours, and these culture stocks were used for seeding the test solutions and agar plates. For the biodegradation experiment, each inoculum was added aseptically to 250-mL Erlenmeyer flasks, each containing 100 mL broth medium with and without the addition of 10 ppm of crystal violet. At selected time intervals 5 mL of broth was withdrawn from each flask and was centrifuged at 10,000 x g for 30 minutes. The absorbance of the supernatant was read on a spectrophotometer at 600 nm. (The maximum absorbance of the crystal violet). Crystal violet degradation was measured in terms of primary degradation; i.e., by following the disappearance of crystal violet from the flask's broth. Experiments involving the use of agar plate method were also used to screen the ability of yeasts to biodegrade crystal violet.

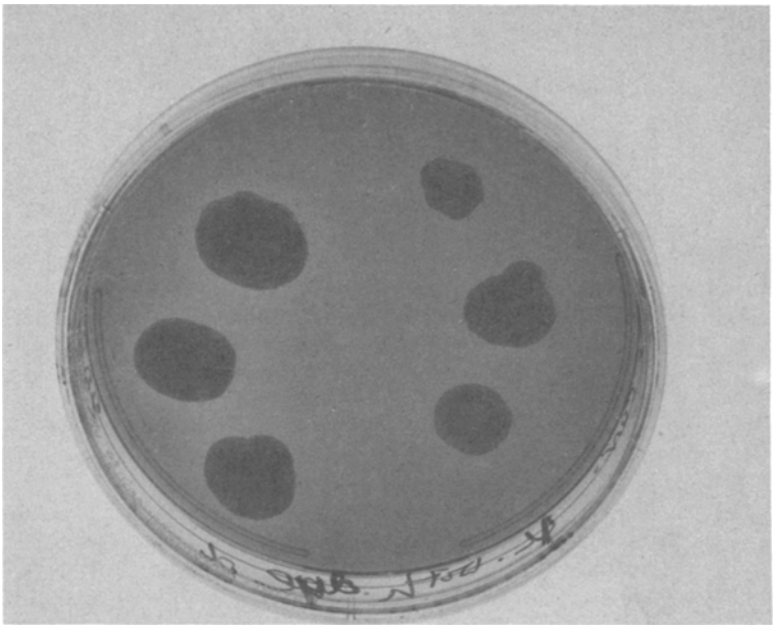

Figure 1. Biodegradation of crystal violet on the agar plate by the oxidative red yeasts (left) as indicated by the clear zone surrounding the colonies. No zones (right) were noticed around fermentative S. cerevisiae.

RESULTS AND DISCUSSION

Since many dyes are oxidation-reduction potential indicators per se, their colors would change with the varying pH of the growth medium. Thus preliminary experiments were initiated to investigate the stability of crystal violet in the culture growth medium.

This was done to ensure that biodegradation of crystal violet, as measured by the disappearance of the dye from the culture broth, was due to the biological reaction. Thus 0.1 mL of acid (6 N HCL), base (6 N NaOH), oxidizing agent (30% H_2O_2), or reducing agent (10% ascorbic acid) was added separately to test tubes, each containing 10 mL of liquid broth plus 100 µg of crystal violet. Then spectra were run on a double beam recording spectrophotometer from 400 to 700 nm. The spectra indicated that no reaction took place between crystal violet and the chemical reagents, implying that the crystal violet is an extremely stable dye, not affected by dilute acid, base, oxidizing or reducing agent. These simple preliminary experiments were essential to ensure that degradation of crystal violet in the growth medium was due to biological reaction brought on by the yeast's metabolism.

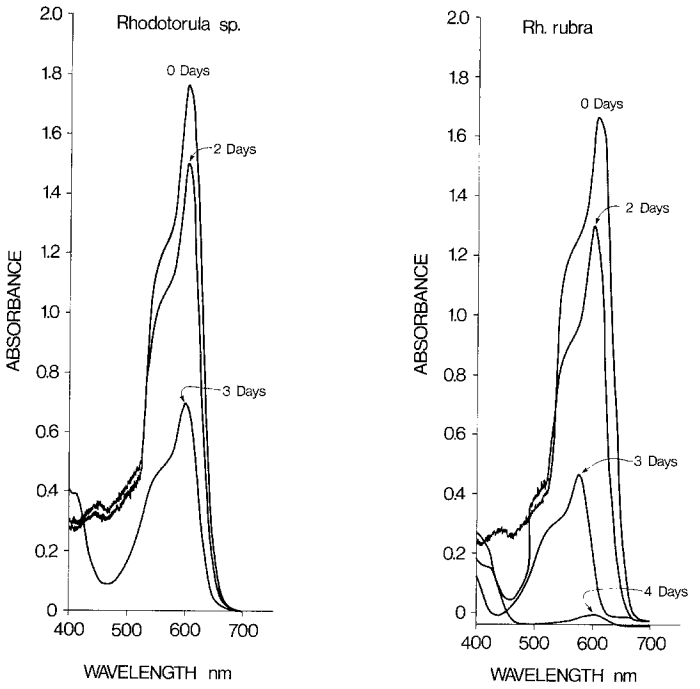

Figure 2. Spectra of crystal violet in the liquid broth medium of Rhodotorula sp. and Rhodotorula rubra after 0 to 4 days incubation.

In view of the complexity involved in the determination of crystal violet biodegradability, a simple and rapid method was needed to screen a large number of yeasts from environmental sources for their ability to degrade crystal violet. The agar plate method (Liu and Kwasniewska 1981) was found to be most useful in screening a yeast's biodegradation potential. The clear zones around the colonies of the oxidative red yeast Rhodotorulae as shown in (Figure 1) is a good indication that this yeast possessed a potential to degrade the crystal violet. However, the fermentative S. cerevisiae seemed not to exhibit any biodegradation potential toward the dye crystal violet, even after three weeks incubation. It may be of interest to note that the colonies of the fermentative yeast became deep purple in color after ten days incubation, without the formation of a clear zone around these colonies. This suggests that the fermentative yeast tends to bioconcentrate the crystal violet from the agar medium probably related to its cell content.

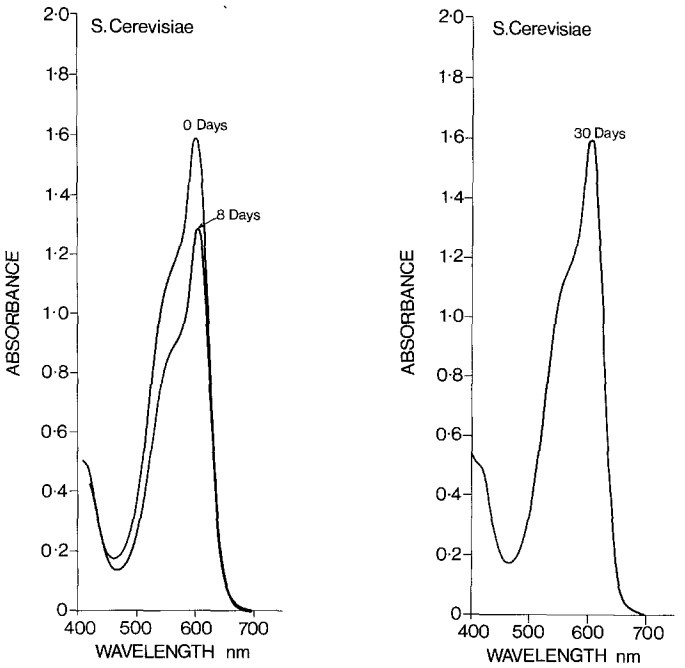

Figure 3. Persistence of crystal violet in the liquid growth medium of the fermentative S. cerevisiae after 30 days incubation.

The results in Figure 2 indicated that the oxidative yeasts Rhodotorula sp. and Rh. rubra were capable of degrading the dye crystal violet in the liquid broth. After four days incubation the absorbance of the supernatant at 600 nm became nonmeasurable, indicating the complete biodegradation of crystal violet by both oxidative yeasts. Visual examination of the cell pellet did not reveal any absorption of the dye by the yeast biomass. The cell's pellet was subjected further to sonification in 70% ethanol and the ethanol extract showed no absorbance at 600 nm. The above would strongly suggest the rapid biodegradation of crystal violet by the oxidative yeast.

The fermentative S. cerevisiae was not capable of degrading the dye crystal violet in the liquid medium even after 30 days of incubation (Figure 3). The decrease of the absorbance in the liquid broth after eight days incubation was probably due to the absorption of the dye by the yeast S. cerevisiae cells as, after 30 days incubation, the absorbance spectrum became identical to that of the day zero. The reason for that is very likely due to the release of the dye from the dead cells. The inability of the fermentative yeast S. cerevisiae to

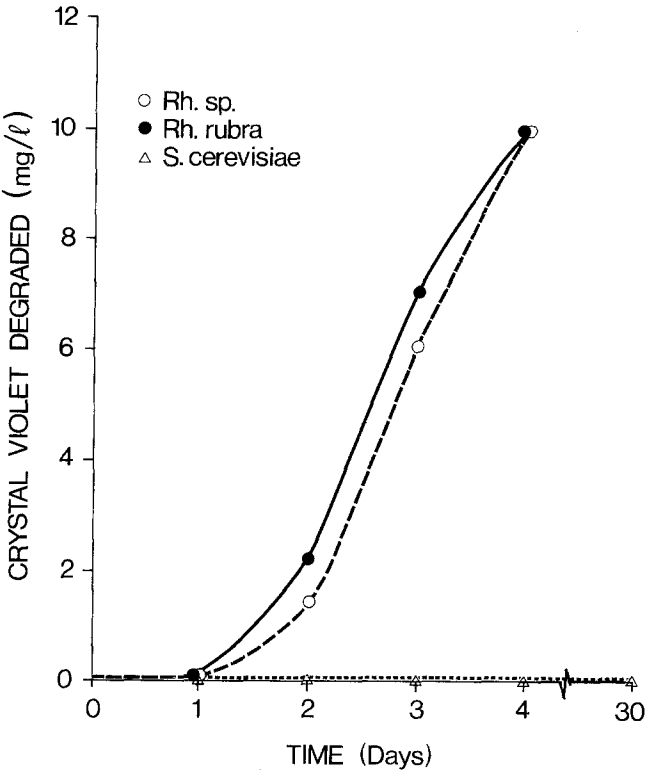

Figure 4. Time courses of crystal violet biodegradation by various red yeasts in liquid broth medium.

biodegrade the dye crystal violet was apparently not due to any toxic effect from the dye. The yeast culture was found to grow equally well in both the control and the test flasks in terms of cell dry weight and cell morphology (phase contrast microscopy examination). The data in Figure 4 clearly demonstrated that both oxidative yeasts Rh. sp. and Rh. rubra had a high biodegradation potential against the crystal violet. After two days incubation, approximately 15 to 22% of the added crystal violet was degraded. There was a linear degradation of crystal violet biodegradation by these two yeasts between day two and day four, indicating that the oxidative yeasts had acquired the enzymes necessary for the degradation of crystal violet. In fact, no trace of crystal violet could be detected in the liquid broth medium after four days incubation.

In contrast, the fermentative S. cerevisiae did not appear to possess any biodegradation potential against crystal violet. After thirty days incubation, nearly 100% of crystal violet could still be detected in the growth medium. From the literature search, no information is available regarding the biodegradation of crystal violet by microorganism including bacteria. The results of this study would suggest that yeast can be used as a tool for the study of the biodegradation of contaminants, particularly those aromatic compounds.

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