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# UTILISATION OF AZO AND TRIPHENYLMETHANE DYES AS SOLE SOURCE OF CARBON, ENERGY AND NITROGEN BY *BACILLUS* SP

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## ABSTRACT

*Bacillus* sp isolated from soil contaminated with untreated textile mill effluent utilized an azo dye (methyl orange) and a triphenylmethane dye (malachite green) as sole source of carbon, energy and nitrogen. Highest percentage decolourisation (primary biodegradation) of 61.44% for malachite green and 75.85% for methyl orange was obtained in cultures without nitrate (sodium nitrate). In cultures with added nitrate (sodium nitrate) degradation decreased with increasing nitrate concentration. Data obtained at nitrate concentration of 0.20gL$^{-1}$ were 57.20% for malachite green and 52.46% for methyl orange. At nitrate concentration of 1.0gL$^{-1}$percentage decolourisation was 20.50% (malachite green) and 30.45% (methyl orange). The azo-nitrogen and phenyl-nitrogen of the dye substrates provided the nitrogen requirement of the organism in cultures without nitrogen. Decolourisation of dyes is a reduction process which requires redox equivalents (electron donors) that transfer electrons to the chromophoric group of dyes. Nitrate ($NO_3^-$) has higher oxidation character compared to the chromophoric group of dyes. The result obtained was attributed to competition between ($NO_3^-$) and the chromophoric group for the redox equivalents, which results in preferential reduction of ($NO3^-$) relative to the chromphoric group. Decolourisation was accompanied by increase in total viable count. Ring opening of the aromatic moiety of the dyes and demethylation of malachite green provided the carbon and energy source for the organism. The results show the potential of a bioprocess under nitrate-starvation condition for the treatment of dye wastewater.

**Key words:** *Bacillus* sp, primary biodegradation, methyl orange, malachite green.

## INTRODUCTION

Synthetic dyes are extensively used in the textile, food, cosmetics, pharmaceutical, paper, leather, photographic and aquaculture industries (Schnick, 1988; Chen *et al.*, 1999). The main classes of dyes are azo, anthraquinone and triarymethane dyes based on their chromophoric groups and application technologies. It is estimated that about 10,000 different types of dyes are used industrially worldwide (Carliell *et al.*, 1995). During the dyeing processes about 10-90% of the dyestuff do not bind to the fibres and, therefore, released into the sewage treatment system or the environment (Zollinger, 1991: Reisch, 1996; Abadulla *et al.*, 2000). This implies the wide occurrence of synthetic dyes in dye wastewaters.

Commercially useful dyes must posses a high degree of chemical and photolytic stability which implies that removal from effluent is difficult. Stability to microbial attack is also a required characteristic of dyes (Pagga and Brown, 1986). Thus, they are less amenable to biodegradation (Banat *et al.*, 1996) and contribute to contamination of the environment (Rafols and Narcelo, 1997; Riu *et al.*, 1998).

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Dye wastewaters can be treated by various physicochemical methods (Vandeevivere, *et al.*,1998; Arslan *et al.*,2000). However, these methods are not only economically unattractive because of high cost and high energy input but are environmentally unfriendly because they merely transfer the pollutant from one phase to another. For example, chlorination generates toxic organochlorides (Sarasa *et al.*, 1998).

Biological treatments of dye wastewaters under aerobic and/or anaerobic conditions have been reported (Cripps *et al.*, 1990; Banat *et al.*, 1996; Zissi *et al.*, 1997; Chen *et al.*., 1999,). However, these methods were under cometabolic conditions. Some dyes and /or their degradative by–products (aromatic amines from azo dyes and lecuco triphenylmethane from triphenylmethane dyes) are potentially toxic mutagenic and carcinogenic (Houk *et al.*, 1991; Burchmore and Wilkunson, 1993; Ganesh *et al.*, 1995; Henderson and Schmitt, 1997).

In dye-utilising industries, a wide range of structurally diverse dyes with diffent chromophoric groups are used in one and the same operation within a short period of time. This implies that effluents from these industries are extremely variable in composition (Correia *et al.*, 1995). This underlines the need to develop a bioprocess with

the potential to degrade dyes with different chromophoric groups.

This study is part of our on-going research to develop microbial culture with the potential to degrade synthetic dyes with different chromophoric groups (azo group and quinone group) as sole source of carbon, energy and nitrogen. It is our hope that such a bioprocess would detoxify these pollutants.

## MATERIALS AND METHODS

### SOURCE OF ORGANISM

Soil samples were collected in sterile plastic bags from soil contaminated by untreated textile wastewater from a small-scale textile mill in Port Harcourt, Nigeria. Samples were transported to the laboratory in an ice-packed chest box within 1h of collection.

Samples (10g) were transferred into triplicate 500ml Erlenmeyer flasks containing 250ml sterile physiological saline and acclimatised for 2 weeks at ambient temperature.

### DYES

Malachite green and methyl orange (Aldrich Chemical Co. U.S.A) were used at their commercially available purity level of 95% and 85% respectively. Fig. 1 shows the chemical structure of the dyes.

Stock solutions were prepared by dissolving 0.10g of each dye in 100ml deionised water. Strerilization was by membrane filtration (0.2μm pore size, Acrodisc).

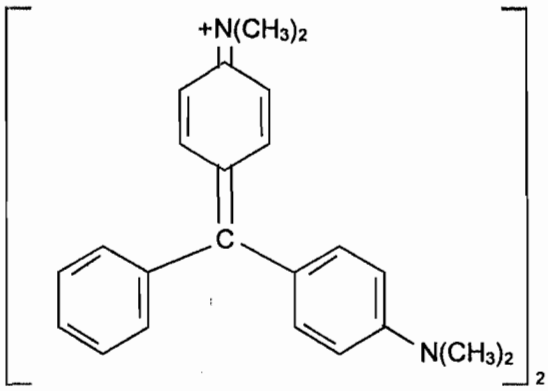
### MEDIUM

Mineral salts basal medium contained ($gl^{-1}$): NaCl 2.0, $MgSO_4$ .$7H_20$, 0.42, KCl 0.29, $k_2P0_4$ 1.27, $NaN0_3$ 0.42, $KH_2P0_4$ 0.85, EDTA 0.5ml and deionised water1,000ml.pH 7.0. Medium which contained malachite green was designated MGI and medium which contained methyl orange was designated MOI. Sterilization was by autoclaving at 121°C at 15psi for 15 min.

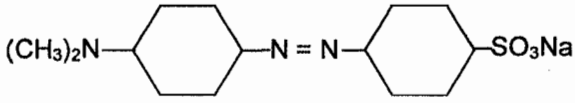
Agar medium was prepared by adding agar (Oxoid) at 1.5% (w/v) to the broth medium.

### ISOLATION AND EVALUATION OF DYE DECOLOURISERS

The principle of selective enrichment batch culture was used to select for dye decolourisers. The procedure was based on the method of Stanbury *et at.* (1995).




MALACHITE GREEN (C. I. 42000)




METHYL ORANGE (C. I. 13025)

**FIG. 1: CHEMICAL STRUCTURES OF DYES USED (ADAPTED FROM ALDRICH CATALOGUE, USA.)**

Ten grams of acclimitised soil sample was added into 100ml physiological saline contained in triplicate 500ml Erlenmeyer flask and shaken vigorously. Ten milliliters was withdrawn from each flask and inoculated into 100ml MGI broth contained in 250ml Erlenmeyer flask. Incubation was at 30±2°C with shaking.

Cultures were observed daily for increased turbidity (evidence of growth) and decolourisation of the medium. Ten mililitres of decolourised cultures were inoculated into fresh, sterile MGI broth and incubated. After four similar serial transfers, cultures were streaked on MGI agar plates and incubated at 30±2°C for five days. Single colonies which developed were picked based on cultural characteristics and Gram stained. Four isolates (coded A,B,C and D) were picked based on their Gram reaction and purified by repeated subculture.

The isolates were each inoculated into 10ml nutrient broth and incubated at 30±2°C for 24h. One milliliter of culture was inoculated into 25ml MGI broth or MOI broth contained in duplicate 250ml Erlenmeyer flask. Incubation was at 30±2°C with shaking for 6days. Decolourisation was ranked visually (intense +++; moderate++; slight+; no decolourisation -).

Intense and moderate decolourisation was observed with isolate A on MGI and MOI media respectively. Other isolates showed either slight or

no decolourisation for both dyes. Isolate A was, therefore, selected for subsequent experiments.

**IDENTIFICATION OF ISOLATE A**

Identification was based on Grain stain, spore test, motility and various biochemical tests as outlined in Cruickshank *et al.* (1980) and Holts and Bergey (1993).

**STOCK CULTURE**

In order to maintain the selective pressure isolate A tentatively identified as *Bacillus* sp was maintained on either MGI or MGO agar slats at 4°C in a refrigerator.

**STANDARD INOCULUM**

Cells from the stock culture were inoculated into 20ml nutrient broth contained in 250ml Erlenmeyer flasks. Incubation was 30±2°C with shaking for 24h.

**DETERMINATION OF OPTICAL DENSITY (OD) OF CULTURE SUPERNATANT**

Samples from experimental and control flasks were clarified by centrifugation in a bench centrifuge (Baird and Tatlock, England) at 6,000rpm for 30min. The OD of the supernatant was determined spectrophotometrically with spectrophotometer (Jenway 6110,UK) at $\lambda_{max}$ for each dye (614nm for malachite green and 505nm for methyl orange).

Percentage dye decolouration was calculated:

$$\% \text{ Decolourisation} = \frac{\text{ODinitial} - \text{OD final}}{\text{ODinitial}} \times \frac{100}{1}$$

**OPTIMUM pH**

The optimum pH 7.0 for malachite green and pH 6.5 for methyl orange were determined prior to carrying out subsequent experiments.

**EFFECT OF VARIOUS CONCENTRATIONS OF NITRATE (SODIUM NITRATE)**

The set-up for each dye substrate was as follows:

Four types of media (MG2, MO2, MG3 and MO3) were used. MG2 and MO2 media were same as the isolation media (MG1 and MO1) except that each contained 0.29g sodium chloride instead of sodium nitrate. These media served as positive controls. MG3 and MO3 media were same as MG1 and MOI media except that appropriate concentrations of nitrate as sodium nitrate ($gL^{-1}$): 0.20, 0.40, 0.60, 0.80 and 1.0 were added. Into each of triplicate 250ml Erlenmeyer flask which contained 90ml of MG2 or MO2 was added 0.20g sodium chloride (i.e. positive controls). Appropriate concentrations of sodium nitrate was added into triplicate 250ml Erlenmeyer flasks which contained 90ml of MG3 or MO3 medium. Serilization was by autoclaving. On cooling, 0.5ml of stock solution of malachite green or 1.0ml stock solution of methyl orange was added to each flask and inoculated with 10ml standard inoculums. Triplicate set of uninoculated MG2, MO2, MG3 or MO3 media were set-up as negative controls. Incubation was at 30±2°C with shaking for 6 days.

Samples (6ml) were withdrawn from each flask on the 1st day and after incubation and clarified by centrifugation. The OD of the supernatant was determined and percentage decolourisation calculated.

The cell sediment from each flask was resuspended in 10ml physiological saline and centrifuged. Supernatant from malachite green-containing medium was coded SMG and that from methyl orange-containing medium SMO. The optical density of the supernatant was determined spectrophometrically.

Highest percentage decoourisation was obtained in the positive control culture (no nitrate). Subsequent experiments were carried out without nitrate addition.

**GROWTH AND DECOLOURISATION OF DYES AT OPTIMAL CULTURAL (PH AND NITRATE) CONDITIONS**

**MALACHITE GREEN**

Ninety mililitres of MG2 broth without nitrate and pH 7.0 was added into each of triplicate 250ml Erlenmeyer flask and sterilized. On cooling, 10ml of standard inoculum was inoculated into each flask followed by the addition of 0.5 ml of stock solution of malachite green (final dye concentration 0.01mg $ml^{-1}$). Controls consisted of triplicate set of uninoculated flask. Incubation was at 30±2°C for 6 days with shaking.

Samples (6ml) were withdrawn on the 1st day and on daily basis and clarified by centrifugation. Optimal density (OD) of supernatant was determined spectrophotometrically. The cell sediment was resuspanded in 10ml physiological saline and centrifuged. The OD of supernatant was determined.

One mililitre was withdrawn from each flask and serially diluted by ten-fold serial dilution ($10^{-1}$ to $10^{-5}$) in phyusiological saline. Appropriate dilutions were inoculated onto triplicate nutrient agar plates by pour-plate method. Incubation was at 30±2°C for 24-48h. The number of colonies which developed was counted and results expressed as colony-forming-unit per ml. (cfu $ml^{-1}$).

**Methyl Orange:**
The procedure was same as for malachite green except that MO2 medium (no nitrate addition and pH 6.5) was used. Final dye concentration (0.10mgml$^{-1}$).

## RESULTS

### ISOLATION AND SCREENING FOR DECOLOURISERS

The selective enrichment techniques ensured that only organisms capable of decolourising the dyes were isolated. Four of the isolates decolourised one or both dyes.

Table 1 shows the results on the decolourising potential of the four isolates. Intense and moderate decolourisation were obtained with isolate A in MG1 and MO1 media respectively. Slight decolourisation was obtained with isolate B in both media.

Media MOl and MG1 were slightly decolourised by isolates C and D respectively. No decolourisation was obtained with isolate C in MG1 medium and with isolate D in MOl medium.

### IDENTIFICATION

Table 2 presents the results of the Gram stain, spore test, motility and biochemical tests for isolate A. Based on the results obtained and with reference to Bergey's Manual of Determinative Bacteriology, isolate A was tentatively identified as *Bacillus* sp.

### EFFECT OF CONCENTRATIONS OF NITRATE (SODIUM NITRATE)

Fig. 2 depicits the data obtained on the effect of various concentrations of nitrate (sodium nitrate)on decolourisation of the dyes. There was decolourisation at all the concentrations. Decolourisation decreased with increasing nitrate concentration.

Highest percentage decolourisation (61.44% for malachite green and 75.85% for methyl orange) was obtained in the positive control cultures (no nitrate). In media which contained nitrate, decolourisation decreased with increasing nitrate concentration. For example, percentage decolourisation at

**Table 1: Screening For Decolourisation Of The Dyes By The Isolates In Broth Culture**

| Medium | Isolate | | | |
|---|---|---|---|---|
| | A | B | C | D |
| MG1 | +++ | + | - | + |
| MO1 | ++ | + | + | - |

**Key**: MG1 = medium with malachite green.
MO1 = medium with methyl orange
– = no decolourisation;
+ = slight decolourisation;
++ = moderate decolourisation and
+++ = intense decolourisation

**TABLE 2: Gram Stain, Spore Test, Motility And Biochemical Characteristics Of Isolate A**

| CHARACTERISTICS | REACTION |
|---|---|
| Gram reaction | Gram positive |
| Shape | Rod |
| Motility | + |
| Spore | present |
| Catalase | + |
| Oxidase | |
| Voges –Proskauer | + |
| Methyl red | - |
| Citrate utilization | - |
| Starch hydrolysis | + |
| Nitrate reduction | + |
| Indole production | - |
| Urease activity | - |
| Gelatin hydrolysis | - |
| Hydrogen sulphide production | - |
| Casein hydrolysis | + |
| Fermentation of sugars: | |
| Mannitol | + |
| Glucose | + |
| Fructose | + |
| Maltose | - |
| Sucrose | - |
| Lactose | - |
| Xylose | + |

**Key**: + positive reaction
– negative reaction

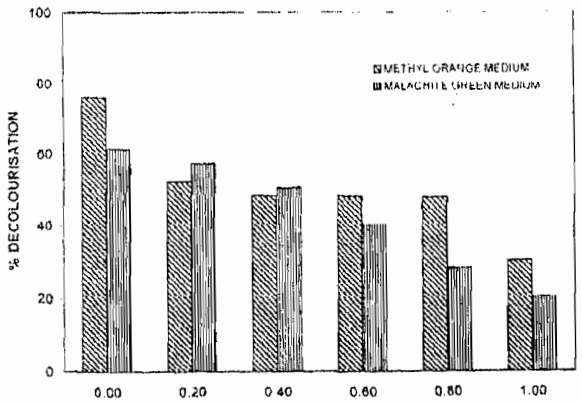



**FIG. 2: PERCENTAGE DECOLOURISATION WHEN *Bascilius* sp. WAS GROWN ON MEDIA WHICH CONTAINED VARIOUS CONCENTRATION OF NITRATEN (SODIUM NITRATE). DATA WERE MEANS OF TRIPLICATE DETERMINATIONS**

$0.20g^{L-1}$ was 57.20% for malachite green and 52.46% for methyl orange. Data at the highest nitrate concentration ($1.0g^{L-1}$) were 20.50% and 30.45% for malachite green and methyl orange respectively.

There was no decolourisation in the control flasks. The optical density of the supernatant (SMG and SMO) from recentrifuged cell sediment was negligible (< 0.001) and there was no colouration of the cells.

## GROWTH PROFILE AND DECOLOURISATION OF DYES AT OPTIMA CULTURAL (PH AND NITRATE) CONDITIONS

Fig. 3 and 4 depicit the growth profile and changes in optical density when *Bacillus* sp was cultured on the dyes as sole sources of carbon, energy and nitrogen.

In MG2 medium (Fig.. 3) total viable count (tvc) increased steadily from $1.50 \times 10^6$cfu $ml^{-1}$ on the 1st day to a maximum of $4.10 \times 10^9$ cfu $ml^{-1}$ on the 5th and 6th days of incubation. The tvc then decreased to $3.90 \times 10^9$ cfu $ml^{-1}$ on the 7th day. Increase in tvc was accompanied by concomitant decrease in optimal density (OD) of culture supernatant. Data obtained were 0.07 on the 1st day, 0.05 on the 4th day (28% decolourisation) and 0.03 on the 6th day (57.15% decolourisation).

In MO2 medium (Fig 4) the population density on the 1st day was $1.60 \times 10^6$ cfu $ml^{-1}$ and $9.50 \times 10^9$ cfu $ml^{-1}$ on the 5th day. Thereafter the population density decreased to $8.50 \times 10^9$ cfu $ml^{-1}$ on the 7th day. The OD on the first day was 0.09 and 0.04 on the 4th day (56.56% decolourisation). On the 6th and 7th days the OD was 0.022 (75.56% decolourisation).

There was neither growth nor decrease in OD of the supernatants in the control flasks.

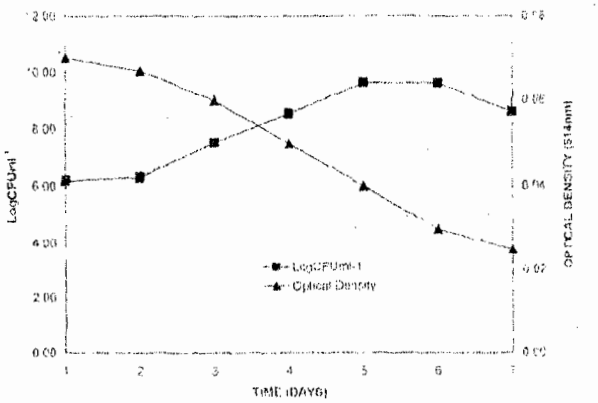



**FIG. 3: GROWTH PROFILE AND CHANGES IN OPTICAL DENSITY (OD) OF SUPERNATANT WHEN Bacillus sp. WAS GROWN ON MACAHITE GREEN AS SOLE SOURCE OF CARBON, ENERGY AND NITROGEN. DATA WERE MEANS OF TRIPLICATE DETERMINATIONS**

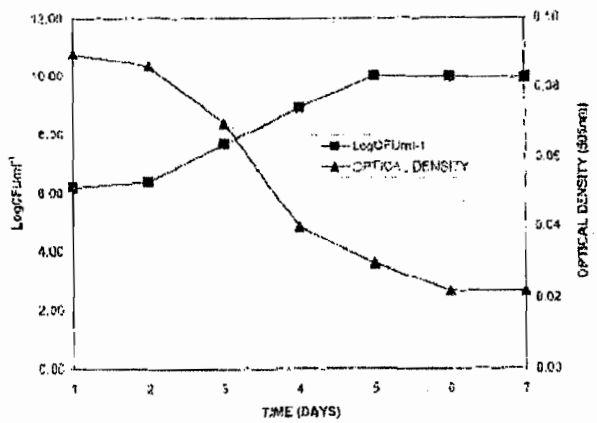



**FIG. 4: GROWTH PROFILE AND CHANGES IN OPTICAL DENSITY (OD) OF SUPERNATANT WHEN Bacillus sp. WAS GROWN ON METHYL ORANGE AS SOLE SOURCE OF CARBON, ENERGY AND NITROGEN. DATA WERE MEANS OF TRIPLICATE DETERMINATIONS**

## DISCUSSION

The isolates decolourised the dye substrates and decolourising efficiency varied among the isolate (Table 1). There was neither growth nor decolourisation in the control flasks. This showed that decolourisation was due to the metabolic activity of the organisms and not to abiotic factor.

Bacteria have been reported to be highly substrate specific to the dye to which they are adopted (Pasti-Grigs *et al.*, 1992, 1996). The bacterium (*Bacillus* sp) isolated in this study decolourised two dyes with different chromophoric groups (azo bond in methyl orange and quinoine group in malachite green). This may be due to the source (soil contaminated with untreated textile effluent) which contained dyes of various chromophoric groups. Members of the genus *Bacillus* have been reported to decolourise azo dyes (Wuhrmann *et al., 1980*; Zissi *et al,* 1997).

Decolourisation of synthetic dyes is the result of the cleavage of the chromophoric group which generates colourless metabolic intermediates. The intermediate metabolites of the dye substrates are aromatic amines and leucomalachite green (Haugh *et al.,* 1991; Chivukula and Renganathan, 1995; O' Neil *et al.,* 2000; Cha *et al.,* 2001). The results obtained (Table 1 and Figs. 2-4) show that the chromophoric groups of the dyes were cleaved.

The cleavage of the chromphoric group of dyes is a reduction process which requires redox equivalents (electron donors) that transfer electrons to the chromophric group (electron acceptors) of dyes (Russ *et al.,* 2000). Electron acceptors, for example, $NO_2^-$ and $NO_3^-$ have higher oxidative character compared to the chromophoric group. (Wuhrmann *et al.,* 1980; Carliell *et al.,* 1995). The higher percentage decolourisation obtained in the positive control cultures (no nitrate) and decreasing percentage decolourisation with increasing concentration of nitrate (sodium nitrate) (Fig. 2) may be attributed to competition between $NO_3^-$ and the chromophoric group of methyl orange and malachite green for the redox equivalents (electron donors). Decolourisation of azo dyes have been reported to be enhanced in nitrogen-limited than in nitrogen-sufficient culture (Cripps *et al.,* 1990; Hu, 1998).

Adsorption of dye molecules on microbial cell surface has been implicated in decolourisation of dyes in microbial cultures (Dohanyas *el at.,* 1978;Yuxin and Jian,1998). The optical densioty of SMG and SMO supernatants were negligible (<0.001) and the cell sediment was not coloured. These confirm that decolourisation (Table1 and Figs.2-4) was not due to adsorption on the cell surface.

The dye substrates were the sole source of carbon, energy and nitrogen in MG2 and MO2 media. The data (Figs. 3 and 4) show that the dye substrates provided the carbon, energy and nitrogen as evidenced by increase in total viable count (cfu $ml^{-1}$) in these media. Ring opening of the aromatic moiety of the dyes and/or demethylation of malachite green (Fig. 1) were the sole sources of carbon and energy. Utilisation of the azo-nitrogen of methyl orange and phenyl-nitrogen of malachite green provided the nitrogen requirements of the organism. Bacterial utilization of azo dyes as sources of carbon and energy under cometabolic conditions have been reported (Yatome *et al.,* 1993; Dykes *et al.,* 1994) and as nitrogen source (Cough Lin *et al.,* 1997). This study is perhaps one of the few reports in the literature on utilization of azo and triphenylmethane dyes as a source of carbon, energy and nitrogen in monosubstrate utilisation mode.

Aromatic amines and/or leucomalachite green generated by the reductive cleavage of methyl orange and malachite green respectively and the parent molecules are potentially toxic, mutagenic and caranogenic (Rafii *et al.,* 1990; Garnesh *et al.*1994; Spadaro *et al.*, 1996; Fessard *et al.,* 1999). The utilization of these dyes as sole sources of carbon, energy and nitrogen may detoxity the parent compounds or their metabolic intermediates. Detoxification of azo, triarymethane, and anthroquinone dyes by microbial cultures or laccase enzyme was attributed to conversion of azo-nitrogen and ary-nitrogen to non-toxic metabolites (Chivukula and Renganathan, 1995; Abudulla *et al.,* 2000).

This study has demonstrated (i) the feasibility of developing a bioprocess capable of utlising synthetic dyes belonging to different chromophoric groups as sole source of carbon, energy and nitrogen. Such a process may detoxify the dyes (ii) treatment of dye wastewaters requires nitrogen-deficient conditions.

We are continuing our studies on the potential of the organism to degrade other synthetic dyes under nitrogen-starvation conditions and the toxicity of the parent compounds and their degradation products to organisms of ecological and economic importance (nitrifying bacteria and shrimps).

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