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Decolourization of Azo Dyes by a Strain of *Micrococcus* Isolated from a Refuse Dump Soil

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Abstract: Bacterial degradation is a viable treatment option for azo dyes containing wastewater. However, a great drawback is the generation of potentially toxic and mutagenic end products (aromatic amines) by anaerobic bacteria. This study is part of efforts to develop textile effluent bio-treatment processes to produce reusable water by decolourization and degradation of azo dyes to non toxic metabolites. The ability of fourteen bacterial strains isolated from various environmental sources to decolourize textile wastewaters aerobically using a simulated effluent made with three textile reactive azo dyes; Reactive Yellow 42 (RY 42), Reactive Blue 13 (RB 13) and Reactive Red 58 (RR 58) were investigated. Three strains showed >95% decolourization of the synthetic effluent within 24 h. The effect of culture condition (pH, temperature and media) on the degradation of methyl red, a standard azo dye, by the isolate with the highest decolourization level; identified as *Micrococcus* sp., was also studied. The strain showed optimum decolourization at pH and temperature around 7 and 37°C, respectively. It preferred nutrient broth to minimal media and 0.02 g dry mass decolourized 50 mL, 56 mg L^{-1} solution of methyl red within 6 h under adequate oxygen supply. UV-visible spectra analyses of aniline sulphate (an aromatic amine) and those of the metabolic products of methyl red suggest that methyl red was first converted to aromatic amine(s) which was subsequently mineralized by the bacterium. The high azo dyes decolorization ability of the *Micrococcus* strain suggested that aerobic decolourization of azo dyes could be as effective as the anaerobic counterpart if suitable organisms are employed.

Key words: Aerobic decolourization, azo dyes, aromatic amines, methyl red, UV-vis analysis

INTRODUCTION

The textile industry is estimated to consume as much as two-third of the total annual production of dyes (Melgoza *et al*., 2004) Azo dyes are the largest and most versatile class of dyes and are commonly used to dye various materials such as textiles, leathers, plastics, cosmetics and food (Anliker, 1979; Blumel *et al*., 2002). They are the major group of dyes used in the textile industry and contribute between 50-65% of the colours in textile dyes (Chung and Stevens, 1993; Melgoza *et al*., 2004).

The inefficiencies in the dyeing process results in dyestuff losses between 2-50% to the waste water with the lower limit for basic dyes and the upper for azo dyes (Melgoza *et al*., 2004). Ultimately these dyes find there way to the environment and end up contaminating rivers and groundwater in the locale of the industries (Melgoza *et al*., 2004).

Colours in water bodies reduce light penetration, alter the pH, increase the Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) and thereby make aquatic life difficult (Ajayi and Osibanjo, 1980). Dye house effluents are therefore of serious environmental concern.

Several treatments of textile effluents to achieve decolourization have been reported. These include physiochemical methods such as filtration, specific coagulation, use of activated carbon and chemical flocculation. Some of these methods are effective but quite expensive (Do *et al*., 2002; Maier *et al*., 2004).

Although, biotreatment offers a cheaper and environmentally friendlier alternative for colour removal in textile effluents, it has its own demerits: Anaerobic decolourization of azo dyes produces aromatic amines which are toxic to aquatic life (Chung and Stevens, 1993), mutagenic to humans and cannot be degraded further under anaerobic conditions (Chung and Cerniglia, 1992; Goncalves *et al*., 2000). It has been reported that the only safe biodegradation method for azo dyes is combined aerobic treatment (Mabrouk and Yusef, 2008). However, there are very few reports of aerobic bacteria that can grow with azo compounds (Blumel *et al*., 2002).

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This study was carried out to isolate and screen indigenous bacterial strains for the ability to decolourize azo dyes aerobically and to investigate the influence of pH, temperature and media on this activity.

MATERIALS AND METHODS

Materials: Commercial grade Reactive Yellow 42 (RY 42), Reactive Blue 13 (RB 13) and Reactive Red 58 (RR 58), were kindly donated by one of the textile companies in Lagos, Nigeria. Other chemicals used were of analytical grade: Methyl red was supplied by Sigma Aldrich, Germany; yeast extract was from Sifin, Germany; while nutrient broth and nutrient agar were obtained from Lab. M, UK.

Sterilization techniques: All glasswares were washed with detergent, rinsed thoroughly with distilled water and oven sterilized at 80°C for 2½ h. All polypropylene tubes and tips used as well as media and solutions prepared were sterilized by autoclaving at 121°C for 15-25 min. Inoculations were done with flame sterilized loops and all experiments were performed wearing sterile disposable hand gloves.

Isolation and identification of bacterial strains: Bacteria were isolated from four sources around Lagos, Nigeria (between January and June 2007): D-strains were isolated from dye solution, E from effluent samples collected from the discharge and drainage pipes of a textile industry located at Oshodi, Lagos; P from water sample of a pond located at Redemption City, Ogun State Nigeria and R-soil samples taken from a municipal refuse dump in Redemption city, Ogun state Nigeria; All isolations were done in nutrient broth E (Lab. M, UK), using enrichment culture techniques, these were followed by streak plating and sub-plating for pure culture isolation. The organisms were identified to the generic level using the Cowan and Steel Scheme (Barrow and Feltham, 1999).

Preparation of solutions: The bacterial decolourization assays were conducted in 150 mL conical flasks. The basal nutrient was nutrient broth containing (g L^{-1}): 5 NaCl, 5 Peptone, 2 Yeast extract and 2 Beef extract, buffered to pH 7.2. 56 mg L^{-1} dyes solution was prepared by mixing 0.0187 g of each of the pre-dried reactive azo dyes (RY 42, RB 13and RR 58), using nutrient broth E and sterilized by autoclaving.

Decolourization under limited supply of oxygen: Decolourization experiments were done in 150 mL conical flasks containing 50 mL of the solution; 3 flasks were inoculated aseptically with ~0.02 g dry cell mass (Biomass was estimated using OD_{600}) of each of the 14 strains, the flasks were plugged with sterile cotton wool and incubated at 37°C.

Decolourization was measured spectrophotometrically at the simulated effluent pre-determined maximum absor bance (λ_{max}). Three mililiter of sample were collected at 1 h intervals, centrifuged at 4000 g for 15 min to remove biomass and the % decolourization was calculated as previously described (Olukanni *et al.*, 2006):

$$\text{Decolourization}\,(\%) = \frac{A_0 - A_t}{A_0} \times 100$$

Where:

A_o = Absorbance of the dyes solution

A_t = Absorbance of the treated dyes solution at specific time, t

Decolourization under normal supply of oxygen: Under limited supply of oxygen, it was observed that certain strains grew above solution (around the flask); it was suspected that those strains require more oxygen for their growth and decolourization activity. To investigate this, the experiment was repeated without plugging the flasks with cotton wool; the abiotic control was not plugged also. Strain R3, a *Micrococcus* strain which showed the highest decolourization under limited supply of oxygen and was among those that grew around the flask, above the decolourized dye solution, was used. Decolourization of methyl red solution, a well known azo dye, was done with the same strain in a similar experiment.

UV-visible spectral analysis: The UV-visible spectral analyses were done for both the biotic and abiotic solutions of the reactive dyes mixture and the methyl red at specific intervals using Shimadzu UV-visible spectrophotometer model UV-1650PC and changes in the absorption spectra (200-1000 nm) were recorded.

To investigate the formation of aromatic amines, a similar scan was done for 1% aniline sulphate (a standard aromatic amine) and compared with those of the metabolites generated from the decolourization of the reactive dyes mix (simulated effluent) and methyl red.

Effect of initial pH and incubation temperature on decolourization: Methyl red-nutrient broth (56 mg of dye L^{-1}) was prepared and adjusted to pH range of 4-10 using HCl or NaOH as appropriate. Approximately 0.01 g dry cell mass of the bacteria was added to 25 mL of the solution in 100 mL conical flasks. All experiments were done in triplicates. The results were compared to abiotic

controls which represented 0% decolourization. The extents of decolourization were determined after 6 h at the wavelength of maximum absorbance of the solution (λ_{max} = 482 nm).

The decolourization of methyl red in nutrient broth solution was also investigated at various temperatures between 25 and 45°C. Percentage decolourization was determined as described earlier in this study.

Effect of media composition on decolourization: To determine the effect of media composition on decolourization, the decolourization experiment was repeated using five media: Distilled water, DW; Nutrient Broth, NB; Minimal Media (Mills *et al*., 1978), MM; Minimal Media with Yeast extract and Starch (200 and 250 mg L^{-1}, respectively), MM+YS; Minimal Media with Starch only, MM+S. All media were adjusted to pH 7.2.

All decolourization experiments were done in triplicates and values were recorded as mean±standard deviation. Data were further analyzed using t-test for independent variables; readings were considered significant when p-value was <0.05.

RESULTS

Isolation and identification: A total of fourteen strains were isolated from the four sources: four from the dyes; four from textile effluents; three from pond water and three from the refuse dump. Three of these strains were gram positive rods, two gram positive spheres, six gram negative rods and three gram negative spheres. None of the organisms was acid-fast, none had spores and all were catalase positive.

The fourteen newly isolated bacteria were identified by morphological and biochemical characterization as two strains of *Acinetobacter* sp., six strains of *Bacillus* sp., four strains of *Pseudomonas* sp. and one strain each of *Staphylococcus* and *Micrococcus* sp. (Table 1). Further identification of strain R3 with the highest decoulorization showed that it is resistance to lysozyme, positive to Voges-Proskaüer test, non-nitrate reducing. It also gave yellow pigmentation on both nutrient agar and potatoes slope; a feature that distinguished *Micrococcus* from *Staphylococcus* (Barrow and Feltham, 1999).

Decolourization under limited supply of oxygen: All the fourteen newly isolated strains decolourized the reactive dyes solution but to various extents (Table 2). Under limited supply of oxygen 3 strains (D1, D2 and R3) showed >95% decolourization within 24 h, two of these (*Pseudomonas* sp.) were isolated from textile dyes, while the third (*Micrococcus* sp.), which showed the highest decolourization of 97.50±0.83 was of refuse dump origin.

Decolourization under normal supply of oxygen: Normal supply of oxygen reduced the duration of decolourization by the Micrococcus from 24 h to about 6 h. The plot of % decolourization against incubation period (Fig. 1) follows Michaelis-Menten's model .

UV-visible analysis: The initial UV-Visible spectrum of the mixture of reactive dyes (0 h) revealed two peaks in the visible region (412 and 549 nm) and one in the near UV region (291 nm); the 412 nm peak showed the maximum absorbance. After 24 h of decolourization experiment, the peaks in the visible region had been removed, while the one in the near UV remained (Fig. 2a).

Figure 2b showed the spectra of methyl red solution at 0 h and after 3 and 6 h decolourization. The spectrum of methyl red at 0 h showed two major peaks: one in the visible region (482 nm) and the other in the near visible region (312 nm) after 6 h the peak at the visible region as been removed. Interestingly, we noticed an initial increase

Table 1: Sources and identities of bacterial isolates

Identity	CHO (F/O/-)	Glucose (acid)	Oxidase	Catalase	Anaerobic	Aerobic	Motility	Spores	Acid fast	Shape	Grams reaction	Lab. No.
Pseudomonas	F	+	-	+	-	+	+	-	-	R	-	D1
Pseudomonas	O	+	+	+	-	+	+	-	-	R	-	D2
Bacillus	F	+	+	+	-	+	+	-	-	R	+	D3
Staphylococcus	F	+	-	+	-	+	-	-	-	S	+	D4
Acinetobacter	-	+	+	+	-	+	-	-	-	S	-	E1
Bacillus	F	+	+	+	-	+	+	-	-	R	-	E2
Bacillus	F	+	-	+	+	+	+	-	-	S	-	E3
Bacillus	F	+	+	+	-	+	+	-	-	R	-	E4
Pseudomonas	-	+	+	+	+	+	-	-	-	R	-	P1
Bacillus	-	+	+	+	+	+	+	-	-	R	+	P2
Bacillus	F	+	w	+	-	+	+	-	-	R	+	P3
Pseudomonas	O	+	+	+	+	+	-	-	-	R	-	R1
Acinetobacter	-	+	+	+	-	+	-	-	-	S	-	R2
Micrococcus	-	+	+	+	-	+	-	-	-	S	+	R3

+: Positive, -: Negative, w: weak reaction, R: Rod shape, S: Spherical, D: Dyes isolates, E: Textile effluent isolates, P: Pond water isolates, R: Refuse soil isolates and O/F: Oxidant/fermenter

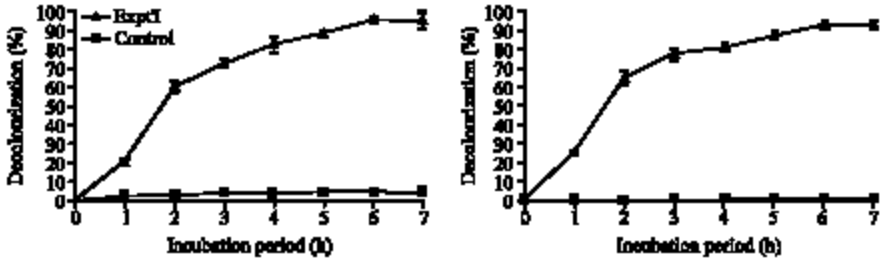

Fig. 1: Decolourization of azo dyes by *Micrococcus* strain R3: (a) reactive dyes mixture and (b) methyl red

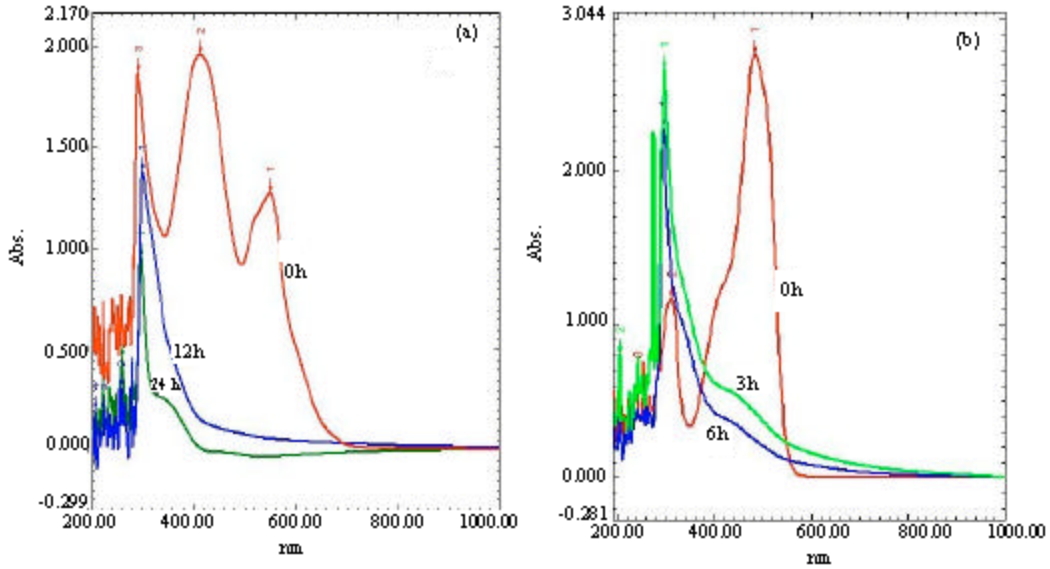

Fig. 2: UV-visible Spectra of (a) reactive dyes mix and (b) methyl red solutions and their metabolites

Table 2: Initial decolourization of mixture of reactive azo dyes under limited supply of oxygen

	Decolourization* (%)	
Lab. No.	12 h	24 h
D1	45.83±3.00	95.00±1.67"
D2	61.67±1.44**	96.94±2.10"
D3	35.28±4.88	52.22±4.28
D4	20.56±1.27	27.78±1.73
E1	52.22±3.94	72.50±3.63
E2	19.72±2.55	40.56±3.37
E3	43.61± 4.88	65.83±2.20
E4	27.78±7.92	57.50±3.63
P1	36.67±3.33	39.72±3.15
P2	41.94±8.67	51.39±3.37
P3	26.11±2.41	30.28±1.27
R1	29.17±8.21	43.61±2.93
R2	29.72±3.94	41.39±2.68
R3	71.67±7.50**	97.50±1.83"

*Values are means of triplicates±SD. **Shows significantly higher activity compared to other strains except strain D2 ($p<0.05$). "Shows significantly higher activity compared to other strains except strains D1 and D2 ($p<0.05$)

in the absorbance values of the near visible peak from 1.154 at 0 h to 2.749 at 3 h, followed by a reduction to 2.255 at 6 h, respectively.

When the UV-visible spectra of the metabolites of the reactive dyes mixture and methyl red were compared to that of the aniline sulphate (Fig. 3), the three peaks were observed in the near-visible region.

Optimal temperature and pH: The results of the experiments conducted at different incubation temperature showed that the optimal incubation temperature for the decolourization of methyl red by the isolated *Micrococcus* strain R3, is at 37°C (94.89±4.79% decolourization in 7 h). It was also observed (Fig. 4a) that the strain decolourized the solution well at the ambient environmental temperature of 30°C (86.11±4.5% in 7 h).

Figure 4b shows the effect of initial pH on decolourization of the reactive azo dyes mix and methyl red at optimal temperature of 37°C. The *Micrococcus* strain R3 effectively decolourized the methyl red solution at pH 6, 7 and 8, with % decolourization of 84.60±1.16, 94.19±1.75 and 93.94±2.00, respectively. The optimal pH appears to be between 7 and 8.

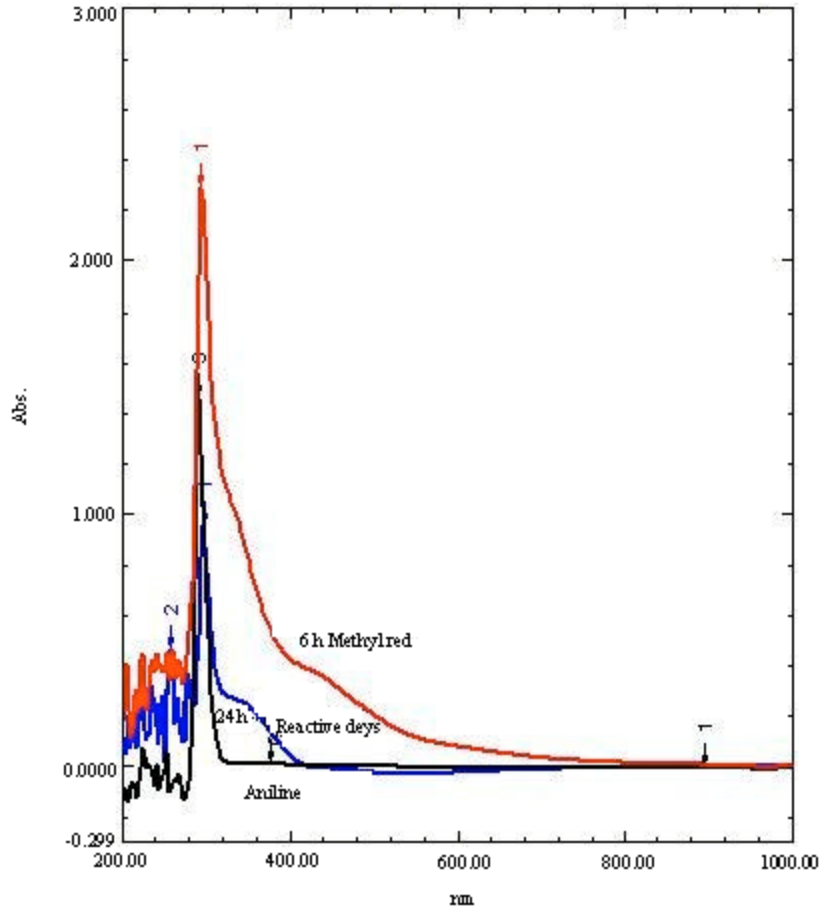

Fig. 3: Overlaid spectra of aniline sulphate and metabolites of methyl red and reactive azo dyes

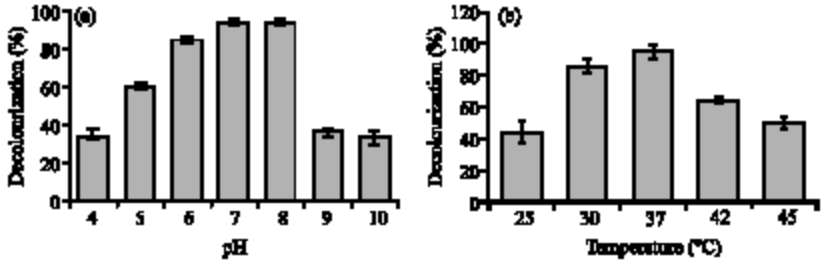

Fig. 4: (a) Effect of pH and (b) incubation temperature, on decolourization of methyl red

Media preference: Table 3 shows the effect of different media types on decolourization of methyl red. Among the media types, the most effective decolourization was achieved using nutrient broth with 98.40±0.42% decolourization within 6 h.

The strain showed high level of decolourization when Yeast (2 gL^{-1}) and Starch (0.25 gL^{-1}) were used as co-substrate in the minimal media (MM+YS); 76.07±2.68% decolourization in 6 h. The absence of yeast in a similar medium (MM+S) however reduced the decolourization to almost zero (0.11±0.14%).

Table 3: Effect of media composition on methyl red decolourization

	Decolourization* (%)		
Media	6 h	12 h	24 h
NB	98.40±0.42**	97.39±0.47**	97.72±0.77**
MM+YS	76.07±2.68	76.72±3.72	74.50±2.08
MM+S	0.11±0.14	0.13±0.18	0.02±0.03
DW	0.89±0.43	0.83±0.29	0.74±0.34
DW+Y	2.22±0.42	2.69±0.30	1.52±0.23

*Values are means of triplicates±SD. **Values are significantly higher than other values at all the incubation periods ($p<0.05$). NB: Methyl red in nutrient broth, MM+YS: Methyl red in minimal medium with Yeast extract and Starch as co-substrate, MM+S: Methyl red in minimal medium with starch only as co-substrate, DW: Methyl red in distilled water, DW+Y:- Methyl red in distilled water and yeast extract

Within 6 h only 0.89±0.43% decolourization was obtained with Distilled Water (DW), but when 2 g L^{-1} yeast was added (DW+Y) the decolourization increased to 2.22±0.42%.

DISCUSSION

Fourteen aerobic bacteria able to decolourize azo dyes with varied efficiency were isolated. One of the isolates, R3, tentatively identified as a strain of *Micrococcus* which showed the highest decolourization over 24 h under limited oxygen availability was able to achieve virtually the same level of activity in 6 h under normal supply of oxygen. This shows that the azo dyes were not only decolourized aerobically but that oxygen is required for the activity of strain R3. This observation contrasts with most publications which indicate that azo dye reduction is an exclusively anaerobic process (Ganesh *et al*., 1994; Jimenez *et al*., 1988; Pagga and Brown, 1986; Pagga and Taeger, 1994; Shaul *et al*., 1991) but is in tandem with the findings of some previous researchers (Zimmermann *et al*., 1982; Padmavanthy *et al*., 2003; Chen *et al*., 2004). The preponderance of biodegradation reports are on anaerobic treatment probably because earlier reports indicated that azo dyes were resistant to bacterial degradation under aerobic conditions while anaerobic decolourization was perceived to be easy to achieve (Sudharkar *et al*., 2002; Moutaouakkil *et al*., 2003). The aerobic decolourization ability or the organism might not be unconnected to the source and catabolic versatile nature of the micrococcus genera. However, it is now generally recognized that aerobic biotreatment is more ecologically friendly because aromatic amines which are recalcitrant to anaerobic biodegradation are mineralized to less toxic or innocuous products.

The spectra analyses of the reactive dyes mix and the methyl red showed the presence of aromatic amine impurities in the dyes. This is however not uncommon (Brown and De Vito, 1993). The presence of aromatic amine impurities was further supported by the lower absorbance value of the near-UV peak of the methyl red (a laboratory grade reagent) when compared to that of the reactive dyes mixture.

The observed disappearance of the peaks in the visible region of the spectra for both the reactive dyes and methyl red showed that the dyes were actually decolourized. The initial increase in the absorbance value of the near-UV peak of the methyl red between 0 and 3 h followed by a decrease at 6 h suggests that the azo dyes were converted to aromatic amine(s) prior to mineralization.

The UV-visible spectrum of aniline sulphate agreed with the report of Vander and Villaverde (2005), that aromatic amines absorb around 260-300 nm. The presence of similar peaks in the spectra of the metabolites of the reactive dyes and methyl red further substantiate the formation of aromatic amines as products of the decolourization

Present findings point to the influence of nutrient composition on decolourization and buttresses prior reports indicating the requirement for a co-substrate acting as an electron donor in the azo dye reduction process (Telke *et al*., 2008). The high level of decolourization with yeast extract supplemented minimal media might not be unconnected to the supplement introduced. Yeast extract has been reported to be a suitable electron donor in azo dyes reduction (Nigam *et al*., 1996; Telke *et al*., 2008).

As a conclusion, this study reports the isolation of a strain of *Micrococcus* with a potential for the safe treatment of azo dye containing effluents.

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