EFFECT OF COSUBSTRATES ON PRIMARY BIODEGRADATION OF TRIPHENYLMETHANE DYES BY *PSEUDOMONAS*

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ABSTRACT

The effect of substrates (glucose, starch, lactose and sucrose) on primary biodegradation (decolourisation) of recalcitrant triphenylmethane dyes (Brilliant green and Crystal violet) by *Pseudomonas* sp was investigated. Biodegradation was enhanced in cultures with substrate supplementation. Glucose and starch were the best among the substrates tested. The percentage discoloration increased with increasing concentration up to maximum of $0.2mgL^{-1}$ for glucose and $0.3mgL^{-1}$ for starch. The percentage with glucose was $93.30 \pm 1.40\%$ for Brilliant green and $90.20\pm0.75\%$ for Crystal violet. With 0.30 mgL^{-1} of starch the percentage discoloration was $85.30\pm0.18\%$ for Brilliant green and $88.50\pm0.90\%$ for Crystal violet. Enhanced biodegradation was attributed to generation of redox equivalent (electron donors) as a result of metabolism of glucose and starch as cosubstrates. Decrease in percentage discoloration at concentration above the maximum for glucose and starch was attributed to saturation of the binding sites for redox equivalents to the chromophoric group (of quinone) of the dyes and/or metabolite repression of the peroxidase enzyme that catalyses the reductive cleavage of the chromophoric group. Cassava peeling (an agricultural waste) gave values of percentage decolourisation of $63\pm0.8\%$ for Brilliant green and $67.70\pm0.60\%$ for Crystal violet. The potential of agricultural wastes to enhance primary biodegradation of recalcitrant synthetic dyes is also revealed in this work.

Key words: *Pseudomonas* sp., Triphenylmethane dyes, primary biodegradation, cosubstrates.

INTRODUCTION

Synthetic dyes are used in the food, cosmetics, textile, pharmaceutical, leather, paper printing and aquaculture industries (Chen *et al.*, 1999; Chan-Jun *et al.*, 2001; Padmavathy *et al.*, 2003). World production of synthetic dyes is estimated at over 0.7 million tons annually, and about 10,000 different types of dyes and pigments are used worldwide (Padmavathy *et al.*, 2003). Synthetic dyes are classified into azo, anthraquinone, triarylmethane and phthalocynanine dyes based on their chromophoric groups and technological applications (Heinfling *et al.*, 1998). These dyes are highly resistant to microbial attack (Pagga and Brown, 1986; Cripps *et al.*, 1990).

It is estimated that between 10-90% of the dyestuff used during the dyeing processes do not bind to the materials being dyed, and are therefore lost in effluent stream (Zollinger, 1991; Pierce, 1994; Eaton, 1995, Reisch, 1996). Dye wastewaters are treated by various physicochemical methods. These methods are not only economically unfeasible but also transfer waste from one form to another and some of the products are more toxic than the parent compound (Churchley, 1994; Yeh and Thomas, 1995). Biological processes involve anaerobic and/ or

aerobic treatment processes (Bortone, 1995; O' Neil *et al.*, 2000; Oranusi and Ogugbue, 2003; Mendez-Paz *et al.*, 2005b). However, some dyes pass through these processes undegraded and may be more toxic than the parent molecule (Spadaro *et al.*, 1994).

Colour is the first sign of contamination recognized in dye wastewaters and has to be removed before discharge into the environment (Padmavathy *et al.*, 2003). Colour is not only aesthetically unacceptable but also inhibits luminosity thereby, affecting photosynthesis and consequently the productivity of the primary producers and growth of the consumers. In addition, the temperature increase leads to depletion of dissolved oxygen concentration (Young and Yu, 1997). With the increased use of a wide variety of dyes and synthesis of new ones to satisfy the ever-growing and changing needs of man, pollution by dye wastewaters is currently recognized globally as a serious environmental issue (Padmavathy *et al.*, 2003; Moreira *et al*, 2004; Mendez-Paz *et al.*, 2005a).

Arylmethane dyes also known as phenylmethane dyes are derived from methane in which some or all of the hydrogen atoms are replaced with aryl groups. Triphenylmethane dyes contain three aryl groups. These dyes are used in various industries including aquaculture (Sani and Banerjee, 1995; Culp and Beland, 1996).

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Decolourisation of triphenylmethane dyes leads to the formation of their leucoderivates. The toxicity, mutagenicity and carcinogenicity potential of leucoderivatives are less than that of the parent compound (Chan-Jun et al., 2001).

Brilliant green and Crystal violet are triphenylmethane dyes and are highly resistant to microbial degradation (Mc Donald and Cerniglia 1984; Bumpus and Brock, 1988; Ollikka et al., 1993; Pointing and Vrijmoed, 2000).Reductive cleavage of the chromophoric group (decolourisation) of recalcitrant synthetic dyes requires redox equivalents (Kudlich et al., 1997; Reyers et al., 1996; Wong and Yu, 1999; Johannes and Majcherazky, 2000; Van der Zee et al., 2000).

In Nigeria there is proliferation of small-scale and medium scale dye-utilising industries. Most of these industries discharge their wastewaters untreated either because of the high cost of treatment and /or are unaware of the potential adverse effects of the wastewaters on humans, animals and plants.

In order to develop a low-cost and low-technological bioprocess for the treatment of dye wastewaters, we carried out the decolourisation (primary biodegradation) of recalcitrant triphenylmethane dyes (Brilliant green and Crystal violet) with inexpensive cosubstrates. The metabolism of these substrates may generate redox equivalents (electron donors) for the reductive cleavage of the chromophoric group of the dyes. The results may be of value to our local industries that may need to treat their wastewaters before discharging into the environment.

MATERIALS AND METHODS

MICROORGANISM
Pseudomonas sp was from the Culture Collection, Department of Microbiology, University of Port Harcourt, Port Harcourt, Nigeria. It was originally isolated from textile mill effluent and adapted to growth on azo dyes (Orange II and Direct Blue 71) as sole source of carbon and energy (Oranusi and Ogugbue, 2001). Stock cultures were maintained on nutrient agar (Oxoid) slants at 4^0c in a refrigerator.

DYES
Triphenylmethane dyes – Brilliant green (ADH, England) and Crystal violet (Fisher Sci., Co., New Jersey, U.S.A) were used at their commercially available purity level of 95% dye content. Fig. 1 shows the chemical structures of the dyes.

Stock solutions of each dye were prepared by dissolving 0.03g in 50ml of deionised water. Sterilization was by membrane filtration (0.2μm pore size, Acrodisc).

COSUBSTRATES
The following substrate glucose, lactose, sucrose and soluble starch were used as cosubstrates. Four types of stock solutions of each substrate were prepared. Solution A contained one-gram in 100ml-distilled water, solution B contained 2g in 100ml-distilled water, solution C contained 3g in 100ml and solution D contained 4g in 100ml Solution were sterilised by autoclaving at 15 p.s.i. at 121^0C for 8min. except starch which was sterilized for 15 min.
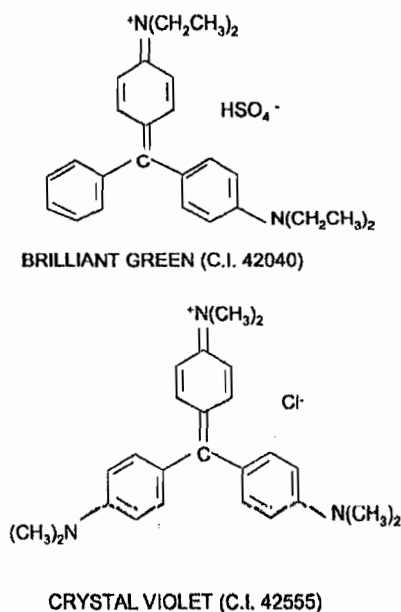

BRILLIANT GREEN (C.I. 42040)

CRYSTAL VIOLET (C.I. 42555)

FIG. 1: CHEMICAL STRUCTURES OF DYES USED (Adapted from Aldrich Chemical Co. Catalogue, U.S.A.)

MEDIUM
Mineral salts basal medium contained (gL^{-1}) NaCl, 2.0; KCl, 0.28; $NaNO_3$, 0.42; KH_2PO_4, 0.29; K_2HPO_4, 1.25; $MgSO_4$. $7H2O$, 0.42 mixed with EDTA 0.5ml and deionised water 1,000ml. Sterilization was by autoclaving at 121^0C for 15mins at 15p.s.i.

On cooling, appropriate amount of stock solution of each dye was added depending on the experimental set-up. The medium which contained Brilliant green was coded MSBG, while MSCV medium contained Crystal violet. The pH of each medium was adjusted to the optimal value (data not shown) obtained from preliminary experiments (pH 6.5 for MSBG and pH 6.0 for MSCV).

STANDARD INOCULUM
Cells from the stock cultures were inoculated in triplicate into 20ml nutrient broth (Oxoid) contained in 250ml Erlenmeyer flasks. Incubation was at 28 ± 2^0C for 24h.

DETERMINATION OF OPTICAL DENSITY (OD) OF SUPERNATANT
Samples withdrawn from experimental flasks were centrifuged at 6,000rpm for 30min in a bench centrifuge (Brau Scientific and Instrument Co., England). The optical density of the resulting supernatant was determined spectrophotometrically (spectrophotometer 6100, Jenway, England). At λ max for each dye substrate (λmax. 625 nm Brilliant green; 588nm Crystal violet).

SCREENING FOR DECOLOURATION OF DYES
a) DYES AS SOLE SOURCE OF CARBON AND ENERGY
Into each of duplicate set of 250ml Erlenmeyer flask which contained 90ml of either MSBG or MSCV medium was added one milliliter of stock solution of each dye. Each flask was inoculated with 10ml standard inoculums. Controls consisted of duplicate set of uninoculated flasks containing 100ml of either medium and 0.5ml of dye stock solution. Incubation was at 28 ± 2^0C with shaking at 150rpm for 10 days. Flasks were observed daily for turbidity (evidence of growth) and decolourisation (primary biodegradation) of culture medium. Percentage decolourisation was determined as follows:

$$\% \text{ decolourisation} = \frac{\text{initial OD - final OD}}{\text{initial OD}} \times \frac{100}{1}$$

Where initial OD = optical density at time zero and final OD = optical density at each sampling. Slight decolourisation was observed in the inoculated flasks after 10 days of incubation. Based on these results, we decided to incorporate cosubstrate to assess their potential to generate redox equivalents (election donors) to enhance the biodegradability of the dyes.

B) DYE SUBSTRATES SUPPLEMENTED WITH COSUBSTRATES
The set-up was same as in (a) above except that one millilitre of a sterile solution A of each cosubstrate was added to each of the inoculated and uninoculated flasks. In addition, flasks which contained no cosubstrate were also set-up. Incubation was at 28 ± 2^0C with shaking at 150rpm for 10 days.

After incubation, samples (6ml) were withdrawn from each flask and clarified by centrifugation. The percentage discoloration of the resulting supernatant was determined. Based on the results obtained glucose and soluble starch were used for subsequent experiments.

DETERMINATION OF THE EFFECT OF VARIOUS CONCENTRATIONS OF COSUBSTRATES
A) GLUCOSE
Into each of six 250ml Erlenmeyer flasks which contained 90ml of either MSBG or MSCV medium was added one millilitre of stock solution of appropriate dye (final dye concentration after inoculation = 0.006mg ml^{-1}). Either 0.5ml or 1ml of appropriate sterile stock solution (A, B, C or D) of glucose was added to give various concentrations (0.05, 0.10, 0.20, 0.30 and 0.4mg ml^{-1}) after inoculation. Triplicate flasks for each concentration were each inoculated with 10ml of inoculum. Controls for each concentration consisted of uninoculated triplicate flasks.

Experimental flasks and controls were incubated at 28 ± 2^0C with shaking at 150rpm. Aliquots (6ml) were withdrawn from each flask on zero day and on the 8th day of incubation. Percentage decolourisation of the supernatant was determined spectrophotometrically after clarification of the samples by centrifugation.

B) SOLUBLE STARCH
The set-up was same as for glucose except that soluble starch was substituted for glucose.

C) CASSAVA PEELINGS
Decolourisation with cassava peeling was also determined. This is a low-cost and abundant agricultural waste. Cassava peelings were blended in a blender and dried to a constant weight in a hot air oven at 100^0C for 3h. The dried sample was sieved. The set-up was same as for soluble starch except that concentrations of 0.1 and 0.2mg ml^{-1} were used.

RESULTS

RESULTS OF SCREENING FOR DYE DECOLOURISATION
Fig. 2 depicts the results obtained when *Pseudomonas* sp. was cultured in either MSBG or MSCV medium with and without cosubstrate supplementation. The highest percentage decolourisation was obtained with glucose (74.50% for Brilliant green and 62.5% for Crystal violet). The lowest decolourisation (8.0% for

Brilliant blue and 10.0% for crystal violet) were obtained in media without cosubstrate supplementation.

Decolourisation was accompanied by increased turbidity of the medium (evidence of growth). There was neither growth nor decolourisation in the control flasks. This shows that decolourisation was a result of metabolic activity of the organism.

EFFECT OF VARIOUS CONCENTRATIONS OF COSUBSTRATES ON DECOLOURISATION

Decolourisation increased with increasing concentration of glucose or starch for both dyes up to a maximum concentration of 0.20mg ml^{-1} for glucose (Table 1) and 0.30mgml^{-1} for soluble starch (Table 2). Decolourisation then decreased as concentration increased above these maximum concentration for both cosubstrates.

Maximum decolourisation (92.30% for Brilliant green and 90.20% for Crystal violet) was obtained at glucose concentration (0.20mgml^{-1}). Corresponding data for soluble starch were 91.50% (Brilliant green) and 86. 20% (Crystal violet) at concentration of 0.30mg ml^{-1} (Tables 1 and 2).

The data obtained with cassava peeling were 55.60% and 63.70% decolourisation at concentrations of 0.10mg ml^{-1} respectively in medium which contained Brilliant green. In medium which contained crystal violet the data obtained were 45.30% and 67.70% at concentrations of 0.10mg ml^{-1} and 0.20mg ml^{-1} respectively.
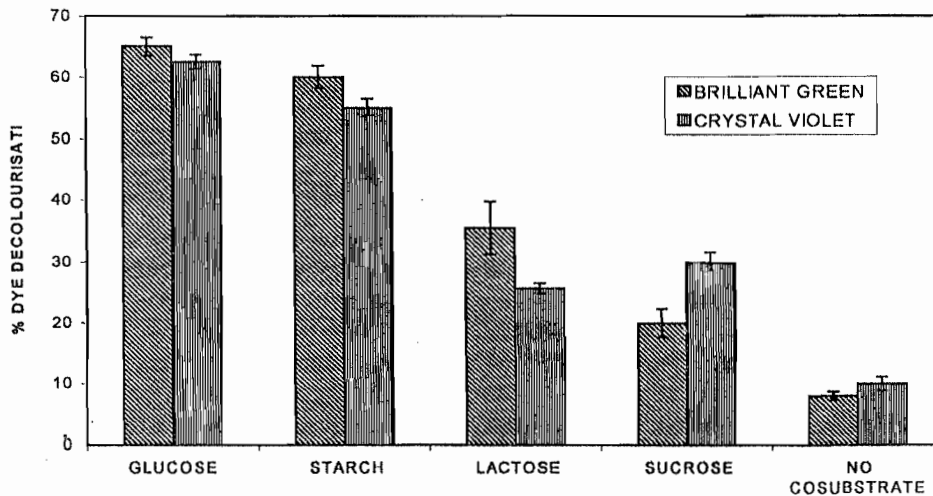

FIG. 2 PRELIMINARY SCREENING FOR DECOLOURISATION OF THE DYES WHEN *Pseudomonas* sp. WAS CULTURED ON MSBG and MSCV MEDIUM WITH AND WITHOUT COSUBSTRATE SUPPLEMENTATION

TABLE 1 PERCENTAGE DECOLOURISATION OF BRILLIANT GREEN AND CRYSTAL VIOLET IN MEDIA WITH DIFFERENT CONCENTRATIONS OF GLUCOSE

MEDIUM	CONC. OF GLUCOSE (mgml^{-1})				
	0.05	0.10	0.20	0.30	0.40
MSBG	71.60±1.25	75.50±1.55	92.30±1.40	85.30±0.80	80.40±3.10
MSCV	50.20±2.40	60.30±1.40	90.20±0.75	88.50±0.90	65.20±2.20

MSBG - mineral salts brilliant green medium
MSCV - mineral salts crystal violet medium

TABLE 2 PERCENTAGE DECOLOURISATION OF BRILLIANT GREEN AND CRYSTAL VIOLET IN MEDIA WITH DIFFERENT CONCENTRATIONS OF SOLUBLE STARCH

MEDIUM	CONC. OF SOLUBLE STARCH (mgml^{-1})				
	0.05	0.10	0.20	0.30	0.40
MSBG	60.40±1.50	75.10±2.15	83.20±1.40	91.50±1.75	80.10±0.90
MSCV	45.20±2.30	64.60±1.65	76.30±1.40	86.20±0.70	76.20±1.20

MSBG - mineral salts brilliant green medium
MSCV - mineral salts crystal violet medium

DISCUSSION

The results obtained (Fig. 2 and Tables 1 and 2) show that the dye substrates were decolourised (primary biodegradation) as the chromophoric group (quinone group) was cleaved. The cleavage generated colourless intermediates.

Biodegradation (decolourisation) of synthetic dyes proceeds by the reductive cleavage of the characteristics chromophoric group of the dyes by enzymes –azo reductases, laccases and peroxidases (Banat et al., 1996; Chen et al., 1999; Oranusi and Ogugbue, 2001, 2003; Padmavathy et al. 2003; Mendez-Paz et al., 2005 a, b). Reductive cleavage of azo dyes is catalysed by the action of redox mediators which transfer redox equivalents (electron donors), for example, NADH, NADPH or FADH to the chromophoric of group the dyes (Russ, et al., 2000). Redox equivalent may be added to the culture medium or generated during the metabolism of suitable cosubstrates (Wong and Yu, 1999; Johnanes and Majcherezk, 2001; Padmavathy et al., 2003; Mendez-Paz et al., 2005). All the cosubstrates tested generated redox equivalent (electron donors) as evidenced by the higher decolourisation values in cultures which contained the cosubstrates compared to the cultures without cosubstrate supplementation (Fig. 2).

The metabolism of glucose generated higher amounts of redox equivalents compared to the other substrates which resulted in higher percentage decolourisation relative to the other cosubstrate (Fig.2) The metabolism of the disaccharides (lactose and sucrose) and starch (a polymer of glucose) requires initial breakdown to their respective monomers. The quantity of glucose intermediate from these substrates may not be high enough to generate enough redox equivalent compared to glucose alone as a cosubstrate. Glucose is a monosaccharide and is the preferred metabolisable substrate compared to the disaccharides (lactose and sucrose) and starch (a polymer of glucose). This may result in the generation of higher amount of redox equivalents and hence, higher decolourisation (Fig. 2). Carliell et al.(1995) and Donlon et al.(1997) reported enhanced azo dye reduction by glucose compared to other substrates.

The low percentage decolourisation obtained without cosubstrate supplementation (Fig. 2) may be attributed to the generation of low levels of redox equivalents. Synthetic dyes contain impurities which are precursor intermediates during the manufacturing process. The quantity of these impurities is propriety information. The dye content of the dye substrates (Brilliant green and Crystal violet) is 95% while, the impurities account for 5% (Aldrich Chemical Co., U.S.A.). The impurities and/or their metabolic intermediates may act as redox equivalent thereby, initiating decolourisation. Vander Zee et al.(2000) reported that 1-amino-2 naphthol (an intermediate of the azo dye-Acid Orange 7) acted as a redox mediator during the reductive cleavage of the dye.

The data obtained at high concentration of glucose (Table 1) and starch (Table 2) may be due to high amounts of redox equivalents generated with increasing concentration and, also to increased mass transfer effect resulting in greater contact between the substrate and the biomass. Mendez-Paz et al., (2005a, b) attributed the enhanced biodegradation of Acid Orange 7 to mass transfer at high concentrations of glucose.

At glucose and starch concentrations above the maximum ($0.2 mgml^{-1}$ for glucose and $0.3mg ml^{-1}$ for starch), decolourisation decreased (Table 2). The data suggest catabolite inhibition of the synthesis of the enzyme (peroxidase) which mediates the cleavage of the chromophoric group of the dyes and /or saturation of the 'binding' site of the redox mediators as a result of increased generation of redox equivalents at high concentrations of the cosubstrates. The data contained with cassava peelings show that it is a suitable and cheaper alternative substrate for decolourisationn of dye wastewaters.

This study has shown that (a) recalcitrant triarylmethane dyes can be degraded by the addition of suitable cosubstrates in the medium and (b) cassava peeling is a potential cosubstrate. This is a low-cost alternative cosubstrate for the small-scale and medium-scale dyers for the treatment of their wastewaters containing mixtures of azo, anthraquinone and other triarylmethane dyes.

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