Assessment of biodegradation and phytotoxicity of azo dyes

Abstract

Azo dyes are widely used colourants~~owing to their~~ , possess array of ~~possible~~ colours and are generally recalcitrant~~ce~~ to microbial degradation~~ owing to their toxicity~~. This study aimed to isolate azo dyes degrading bacteria from soil; treat methyl red and crystal violet dyes using the isolates, and assess the phytotoxicity of the treated and untreated dyes on sorghum. Ten bacteria were isolated from the soil, of which two were identified as efficient azo dyes degrading bacteria by efficiency in dye decolouration. The isolates were identified as *Escherichia coli* and *Bacillus subtilis* based on their biochemical characteristics. The isolates were able to degrade methyl orange dye by 16-75 % and crystal violet by 16-78%, depending on the concentration of the dye. The isolates were more efficient at degrading the dyes at a low concentration of 0.00025 mg/ml, as indicated by a higher percentage of decolourization (53-79%). The percentage phytotoxicity of the untreated methyl red ranged from 33-59%, while the toxicity of the untreated crystal violet ranged from 62-91%. The toxicity of the treated methyl red ranged from 9-34%, while the toxicity of the treated crystal violet ranged from 12-32%. The results revealed that the isolates were capable of degrading methyl red and crystal violet dyes, and could be useful in the bioremediation of azo dyes polluted soil.

Comment [A1]: Recast, language and content needs improvement

Keywords: Azo dyes, *Bacillus subtilis*, biodegradation, *Escherichia coli*, phytotoxicity

Introduction

Dyes are the coloured substances which fix firmly with the substrate. There are different types of dyes classified according to their structures and functions. Azo dyes are the largest group of dyes used in the industry, representing more than half of the annual production [1,2]. Azo dyes are the largest class of synthetic dyes with the greatest variety of colours [3]. Different varieties of azo dyes are in use today in the consumer goods industries because of the diversity of dye components available for the synthesis of a large number of structurally different azo dyes [4,5]. Azo dyes are used by a wide number of industries because these compounds retain their colour and structural integrity under exposure to sunlight, soil, bacteria and sweat; they also exhibit high resistance to microbial degradation [6].

There is a continual demand to develop longer-lasting, more applicable dyes for items such as synthetic fabrics [7,8]. Azo dyes are continually updated to produce colours that reflect the trends dictated by changing social ideas and styles. From an environmental standpoint, the expansion in the demand and application of azo dyes is a threat to the safety of life which is never going away unless alternatives are found or better ways to manage them are devised.

While most azo dyes themselves are non-toxic, a significantly larger portion of their metabolites are [6,9,10]. The potential for toxic effects of exposure to dyes and dye metabolites is not a new concern. Some dyes are carcinogenic and genotoxic because of the toxic potential of many aromatic amines [5,11]. Although several soil microorganisms can degrade dyes, they are often limited by the production of toxic by-products or the presence of toxic dye additives and also high salt concentrations [12]. Thus, these dyes are likely to persist in the environment and become toxic to biota.

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Comment [A3]: Lacks clarity

The treatment of dye effluents is of interest due to their toxic and aesthetic impacts on receiving soil and water bodies. Effective and economic treatment of a diversity of effluents containing azo has become a problem. Currently, much research has been focused on chemically and physically degrading azo dyes in wastewaters, such as chemical oxidation, UV oxidation, electrochemical or wet oxidation, activated carbon adsorption, reverse osmosis, or coagulation/flocculation [2,13,14]. Many of these technologies are cost-prohibitive, however, and therefore are not viable options for treating large waste streams.

While much research has been performed to develop effective treatment technologies, no single solution has been satisfactory for remediating the diversity of wastes from dye industries [15]. This is because no single treatment system is adequate for degrading the diverse dye structures. However, the chemical structures of dyes can be modified by microbial metabolism [16,17].

Dye substrates can serve as a source of carbon, energy and nitrogen for tolerant bacteria and fungi, as defined by their structural composition [3,18]. This makes the biodegradation of azo dyes a viable option for chemical and physical treatment, as it can bring about complete mineralization of the dye. Several species of species have been reported to effectively decolourize azo dyes in a wide range of salt solutions [19-21]. Microorganisms decolourize dyes by desulphonation of their aromatic moiety coupled with reductive cleavage of the azo bond to prohibit aromatic amine formation, thereby, detoxifying the aromatic amines [20,22]. Also, the adsorption of the dye molecules to microbial cells has been implicated in the decolourization of dyes [23]. Several factors could affect the degradation of dyes by microorganisms, including factors related to the microorganisms themselves.

The widely used phytotoxicity tests in vascular plants are the seed germination test (a direct exposure method) and the root elongation test [24,25]. Seed germination represents an important initial phase in the life cycle of plants. Any adverse effect on the seed germination process would be detrimental to the establishment and healthy growth of plants.

This study aims to isolate azo dyes degrading bacteria from the soil and assess their ability to degrade methyl orange and basic violet dyes, as well as to assess the phytotoxicity of the dye on sorghum (*Sorghum bicolor*) seeds.

Comment [A4]: Define the objectives, hypothesis and expected outcome with precision. Avoid too many redundant paras.

Materials and Methods

Sample Collection

The soil samples were collected from a laboratory-scale textile mill and printing press at the University of Port Harcourt, Port Harcourt. These samples were collected at a depth of 10cm in 10g amounts into a sterile Erlenmeyer flask.

Comment [A5]: How many samples

The dyes used are methyl orange (Acid orange 52) with 85% purity and the dye crystal violet (Basic violet 3) with 95% purity (BDH Chemicals Ltd, England). Stock solutions of each dye were prepared by dissolving 0.1g of dye into 100 ml of the mineral salt medium.

Isolation of Dye Degrading Bacteria

One gram of each dye was added into a 25ml mineral salt medium, followed by the addition of 1g of soil sample. The flask was shaken properly and incubated in a shaker incubator (Barloworld Scientific Ltd, U.K) at 50rpm at 30^0C. The cultures were observed daily for decolourization of the dye. After 14 days of incubation, 1ml of culture from each flask was serially diluted by Ten-fold serial dilution using physiological saline and spread plated onto mineral source agar medium in Petri dishes contained. Colonies which developed after

incubation at 30^0C were picked and repeatedly sub-cultured onto nutrient agar plates to obtain pure isolates.

Identification of Isolates

Isolates were identified following Gram reaction, motility, and spore formation, and based on their biochemical profiles following citrate, oxidase, methyl red, Voges-Proskauer, indole, TSI (Triple Sugar Iron), starch hydrolysis, sugar fermentation test.

Preparation of standard inoculum

Isolates were picked from the stock culture and each was inoculated into 10ml sterile nutrient broth contained in test tubes. The cultures were incubated at 37^0C for 24 hours. These served as the standard inoculums for the various tests.

Screening of Isolates for the Degradation of Methyl Orange and Crystal Violets Dyes

All bacterial isolates were screened for the ability to degrade methyl orange and crystal violet dyes in test tubes containing 20mls of stock solution. Isolates that were able to decolourize the dyes were considered as dye degrading bacteria.

Degradation of Dye

A loopful of each isolate from the standard inoculums was inoculated into 10ml of sterile nutrient broth in test tubes. Cultures were incubated at 37^0C for 24 hours. After incubation, 1ml from the culture was inoculated into 50ml of dye of different concentrations (0.00025mg/ml, 0.0005mg/ml, 0.001mg/ml and 0.002 mg/ml) contained in 250ml Erlenmeyer flask and incubated at 37^0C.

Degradation was monitored daily for four days as follows: Two millilitres were withdrawn from each flask starting from day one and was centrifuged at 1000rpm for 30 minutes in an 800 Electric Centrifuge (Xiangtian, China). The supernatant was decanted and analysed.

The optical density was read using Spectrophotometer (EMCLAM, Germany) at 505nm for methyl orange and 588nm for crystal violet.

$$\text{Percentage decolourization} = \frac{\text{OD of control} - \text{OD of sample}}{\text{OD of Control}} \; x \; 100$$

Phytotoxicity Assay

Phytotoxicity was conducted as per the method of Oyedeji and Immanuel [26] with modifications. Ten viable sorghum (*Sorghum bicolor*) seeds were pre-treated to prevent fungal growth by soaking them in sodium hypochlorite (3.5%w/v) solution for 8 minutes and subsequently rinsed ten times with sterile distilled water. Pre-treated seeds were placed on sterile Whatman No.1 filter papers in sterile Petri dishes. The Petri dishes were divided into three sets which consisted of set A (control experiment) which was irrigated with sterile distilled water; set B which was irrigated with untreated dye solution and set C which was irrigated with treated dye solution. The plates were irrigated daily with 5 ml of the dye solutions and were incubated at room temperature (25ºC).All experiments were carried out in triplicates. Seeds were considered to have germinated when root elongation was observed. The lengths of the radicles were recorded after 4 days. Toxic effect was measured in terms of percentage of phytotoxicity as:

Comment [A6]: Give the complete procedure

Comment [A7]:

Percentage of phytotoxicity = $\frac{\text{Radicle length of control} - \text{Radicle length of test}}{\text{Radicle length of control}}$ x 100

Results

Isolation and Identification of Dye Degrading Bacteria

A total of ten bacteria were isolated from the soil sample. Table 1 shows two capable of degrading methyl red and crystal violet dyes.

Comment [A8]: Revise , draw the findings suitably for all the figures

Table 1: Biochemical characteristics of methyl red and crystal violet degrading bacteria

	Gram stain	Shape	Spore	Motility	Oxidase	Citrate	Indole	MR	VP	Starch hydrolysis	TSI	Gas	Glucose	Sucrose	Lactose	Mannitol	Probable Organism
IsolateA	_	Short rod	-	+	-	-	+	+	-	-	A	+	A/G	A/G	A/G	A/G	*E. coli*
IsolateB	+	Rod	+	+	-	+	-	-	+	+	A	+	A/G	A/-	-/-	A/-	*Bacillus subtilis*

Keys: A= Acid, G= Gas, - = negative), + = positive

Percentage degradation

Figure 1 shows results for biodegradation of the dyes by decolourization by Isolate A. Isolate A degraded methyl orange dye by19-75% and crystal violet by16-66%.

Figure 2 shows results for biodegradation of the dyes by decolourization by Isolate B. Isolate B degraded methyl orange dye by 16-53 % and crystal violet by18-79%.

Figure 3 shows phytotoxicity results for dyes treated with isolate A and untreated dyes. The toxicity of untreated methyl red ranged from 33-59%; while the toxicity of crystal violet ranged from 62-91%. The toxicity of treated methyl red ranged from 9-34% while toxicity of crystal violet ranged from 12-32%.

Figure 4 shows phytotoxicity results for dyes treated with isolate A and untreated dyes. Toxicity of untreated methyl red ranged from 40-89%; while the toxicity of crystal violet ranged from 64-93%. Toxicity of treated methyl red ranged from 22-56% while toxicity of crystal violet ranged from 10-52%.

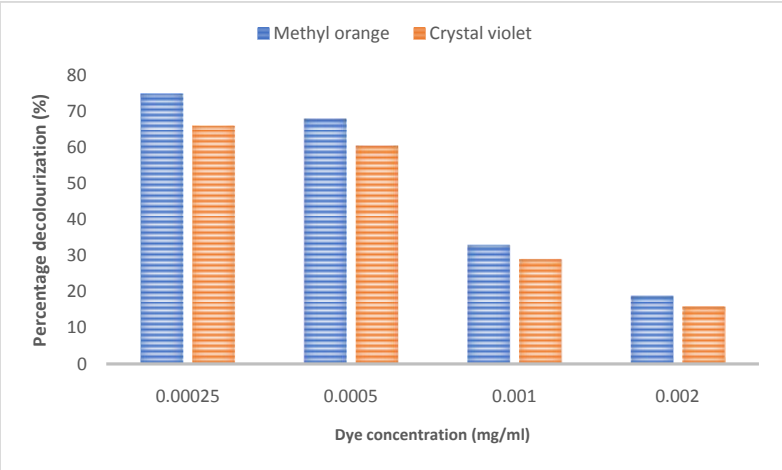

Fig. 1: Percentage degradation of methyl orange and crystal violet dyes by Isolate A

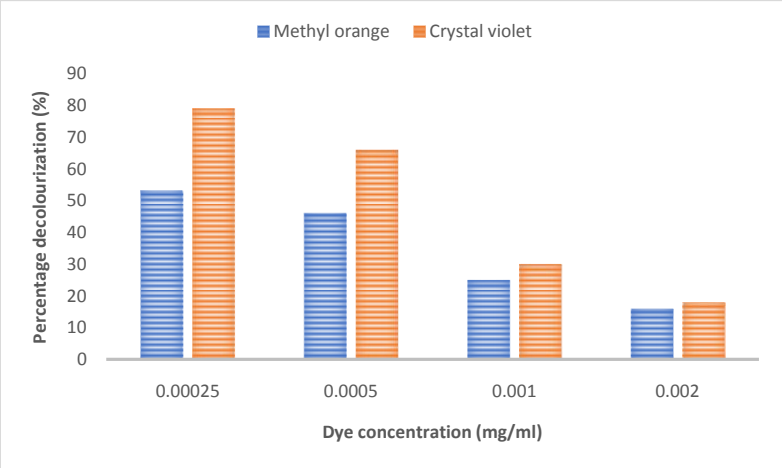

Fig. 2: Percentage degradation of methyl orange and crystal violet dyes by Isolate B

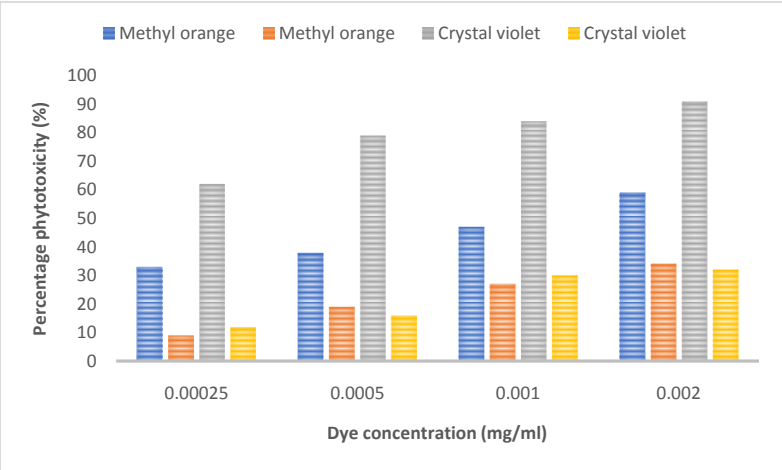

Fig. 3: Phytotoxicity of dyes treated with isolate A and untreated dyes

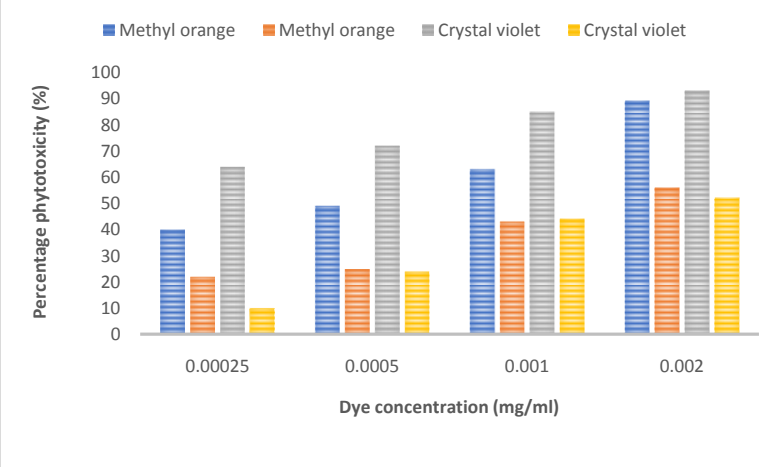

Fig. 4: Phytotoxicity of dyes treated with isolate B and untreated dyes

Discussion

Two azo dyes degrading bacteria were isolated from small-scale textile mill-contaminated soil. The isolates were identified as *E. coli* and *Bacillus subtilis*. Both isolates decolourized the methyl blue and crystal violet dyes. Decolourization of substrates is often associated with the degradation of the dye as decolourizing efficiency was dependent on the growth of the isolate in the flask [11,20]. There was neither growth nor decolourization in the control flasks. This evidence that the decolourization was due to the metabolic activity of the organisms and not due to any abiotic factors, in agreement with Oranusi and Ogugbue[20]. The dyes served as carbon, energy and nitrogen sources for the isolates as alluded to by Oranusi and Mbah [18].

Several bacterial species have been reported to degrade azo dyes including species of Proteus, Pseudomonas, Sphingomonas,Actinobacillus, Mycobacterium, Bacillus and *E. coli*[11,19,20]. *E. coli* and *Bacillus subtilis* have been reported to degrade methyl blue, a cationic dye, in

earlier studies, on account of their high metabolic diversity [20,27]. The efficiency of azo dye biodegradation by the two isolates can be influenced by factors such as the initial dye concentration, the presence of co-substrates, and the cultivation conditions such as pH, temperature, and oxygen availability. Therefore, cultural conditions must be optimized to increase the efficiency of dye degradation. Aromatic amines generated by the reductive cleavage of azo dyes are potentially toxic, mutagenic and carcinogenic [5,11]. The reduction in the toxicity of the dyes after treatment as the isolates utilized the dyes as a source of nitrogen might have detoxified the parent compounds or any intermediates formed as alluded to by Chivukula and Renganathan [28] and Abadulla*et al*. [29].

In the present study, the general effect of the two dyes on germination followed the same patterns. The percentage of phytotoxicity of both dyes increased with an increase in concentration. Although higher concentrations of the dye exert a phytotoxic effect ranging from 47-93%, the lower concentration of the dye also exerted an adverse phytotoxic effect on the seed by 33-64%. However, the treated dye exerted toxicity of 9-22% at the lowest concentration of 0.00025 mg/ml and toxicity of 32-56% at the highest concentration of 0.002 mg/ml. This reemphasized that indiscriminate disposal of azo dye-containing waste into soil and water bodies poses a major threat to the environment.

Conclusion

The present study showed that microbiological treatment of dyes is an efficient way to detoxify methyl red and crystal dyes. The toxic effect of dyes reported in this study suggests the need for remediation of textile dyes in the industry effluents before discharging them into the environment.Whatever treatment is adopted should ensure complete conversion of dye components to avoid the build-up of toxic metabolites.

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