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Bacillus amyloliquefaciens AD 20 and *Bacillus altitudinis* AD 14 Isolated from a Dye Pond Decolorize Synthetic Textile Reactive Dyes

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Abstract

A screen of textile effluents, receiving waterbodies, and waste sites near a textile factory was undertaken to isolate new bacteria strains capable of dye degradation. Out of the 45 isolates, two dye decolorizers, *Bacillus altitudinis* AD14 and *B. amyloliquefaciens* AD20, obtained from the sediment samples were identified by cultivation and 16S rRNA gene sequencing. Decolorization testing was performed under static aerobic conditions in the laboratory. The two *Bacillus* species showed dye decolorization capabilities on media containing each of these four commercial textile azo dyes- Reactive Blue 4 Red (RBFR), Cibacron Brilliant Orange 4 Red (COFR), Cibacron Brilliant Yellow 6 Percent Green (CYPGS), and Turquoise Cibacron Green (TCG). At the end of a ten-day incubation period, *B. amyloliquefaciens* AD20 was more efficient in dye reduction than *B. altitudinis* AD14 on CYPGS and COFR at a magnitude of four-fold and two-fold, respectively, while *B. altitudinis* AD14 only outperformed it in the TCG dye media. The isolates performed best on medium containing RBFR; the principal dye used by the textile factory. Genome annotation revealed the absence of plasmids and the presence of putative genes associated with dye decolorization, such as laccase and azoreductases.

Keywords: **Decolorization, Enzymes, Reactive Blue 4 Red (RBFR), Cibacron Brilliant Orange 4 Red (COFR), Cibacron Brilliant Yellow 6 Percent Green (CYPGS), Turquoise Cibacron Green (TCG)**

1. Introduction

Synthetic dyes are widely used in the textile industry globally and are classified structurally as azo, anthraquinone, sulphur, heterocyclic, triarylmethane, and phthalocyanine [1-2]. Dyes are grouped as being reactive, direct, disperse, basic, or vat based on their method of application, [3-4]. Currently, the most common dyes in the textile industries worldwide are reactive dyes [5].

About 3 decades ago, the annual world production of textiles was 30 million tons requiring 700,000 tonnes of different dyes [1,6]. Currently, the annual world production of textiles fibres and apparel is about 110 million tons requiring 43 million tons of different dyes and chemicals [7-8]. Globally, about 79 – 93 billion cubic metres of water are used during textile processing with a large amount of this water discharged as coloured wastes [8-10]. This causes considerable environmental pollution [11-13]. Generally, the physicochemical properties of these wastes include a reduction in Dissolved Oxygen (DO), high Biological Oxygen Demand (BOD), high Chemical Oxygen Demand (COD), high salinity, increase in alkalinity, as well as a critical loss of biodiversity [14-15]. If current trends are not altered, the

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amount of textile wastes could reach 300 million tonnes by 2050 [7].

Reactive dyes have a high affinity for cellulosic fibre, which is why they are mostly used in textile industries [13,16]. The high affinity is due to the presence of reactive functional groups which attach to the cellulosic fibre in a strong covalent bond. The lack of biodegradability of most azo dyes makes modern treatment processes difficult as they are resistant to physicochemical destruction [17]. Current pollution control technologies, including harnessing various microbes in decolorization and biodegradation of azo dyes presents an emerging, effective, and promising approach [9, 11, 15, 18-27].

Laccase, a versatile enzyme, catalyses the breakdown of various macromolecules including azo dyes [28-30]. The first bacterial laccase was isolated from *Azospirillum lipoferum* by Givaudan et al. [31] and subsequently isolated from *Escherichia coli*, *Streptomyces*, *Marinomonas mediterranea*, and *Bacillus subtilis* [32].

Bacterial laccases have been associated with the decolorization of Congo Red [32], eriochrome black T [33], Alphazurine A [34], and Reactive blue 19 dyes [35]. Other bacterial enzymes involved in decolorizing dyes include lignin peroxidases and azoreductases, but they require hydrogen peroxide or a co-factor to function [36-38].

The earliest documentation of an azo dye degrading *Bacillus* sp. was reported by Horitsu et al. [39]. Since then, numerous reports have been on the dye decolorizing capabilities of different *Bacillus* species [15,16,24-25,27,40-41]. Laccases have been reported as dye degrading enzymes in different *Bacillus* species [32-35,42].

Azoreductases are enzymes that cleave mainly the azo class of dyes using either NADH or NADPH for direct reduction [43-44]. Azoreductases have been found to cleave Congo red, Methyl orange, Methyl Red, Basic Blues 14, Reactive Black 5, and Reactive Blue 59 dyes [43,45-46].

The objectives of this study were 1) to isolate bacterial dye degraders from surrounding waterbodies, effluents, and waste sites of a textile factory, 2) to determine the efficiency of the isolates in dye decolorization, 3) to detect the presence of lignin modifying enzymes and plasmids.

2. Materials and methods

Sample Collection

Wastewater samples collected from a textile company in Lagos State and its nearby water bodies included 1) Untreated effluent (UE) (effluents directly from textile mill). 2) Treated effluent (TE) (effluent after clarification and treatment according to industry standards for discharge) 3) DPS 1 (edge) and DPS 2 (about 3 ft from the edge) sediment samples from a dye pond. 4) Water sample (DPW) (water sample from the dye pond) (**Figure 1A**) Stream Control Sediment (SCS) and Stream Control Water (SCW) samples were sediment and water control samples obtained from a nearby stream 11 km SE of the textile factory not contaminated with dye waste which served as controls (**Figure 1B**). All samples collected were stored in sterile containers and transported on ice to the laboratory.

Media, Dyestuff, and Chemicals

Bacteria were cultured on Tryptone Soya Agar (TSA) (HiMedia Labs, India). The commercial synthetic textile reactive dyes donated by the textile company for use in this study were Reactive Blue 4 Red (RBFR), Cibacron Brilliant Orange 4 Red (COFR), Cibacron Brilliant Yellow 6 Percent Green (CYPGS) and Turquoise Cibacron Green (TCG) (**Figure 2**). RBFR was selected for the initial screening because it was the dominant dye used by the textile factory and the effluents and polluted water body seemed to retain their blue colour.

Stock solutions of Basal Medium (BM) were prepared by dissolving Na_2HPO_4 (2.13 g), NH_4Cl (0.5 g), KH_2PO_4 (1.3 g) and $MgSO_4.7H_2O$ (0.2 g) in 1 L of sterile distilled water. Basal Salt Agar (BSA) contained 1 L of BM and 20 g of Bacteriological agar (HiMedia Labs, India). Dye Agar Medium was prepared by adding a concentration of 200 mg/l of each dye to BSA.

During the preliminary screening, 200 mg of RBFR dye was homogenously dissolved into 1000 ml of BM, from which 5 ml aliquots were dispensed into test tubes and sterilized. The negative control comprised of 5 ml BM solution, and the positive control constituted of filter-sterilized 20 g of glucose in 1 L of BM dispensed in 5 ml volume into test tubes. Finally, an uninoculated control tube of RBFR + BM was prepared.

All four dyes were used for bacterial decolorization assay. For each dye broth, dye (200 mg) and glucose (20 g) were added to 1 L of BM and mixed thoroughly. Glucose was added to optimize the medium [25-26]. The resulting dye media were labelled as COBM (COFR + BM), CYBM (CYPGS + BM), RBBM (RBFR + BM) and TCBM (TCG + BM). After homogeneity, for each isolate to be tested, 100 ml of each dye medium was dispensed into 250 ml Erlenmeyer flasks and sterilized. All experiments were performed in triplicates.

Physicochemical parameters of Effluents and Wastewater

To ascertain the physicochemical properties of the disturbed site, the wastewater samples from UE, TE, DPW and CW were analysed. The pH and temperature of the samples were recorded using a multifunctional digital thermometer (Omron Digital Thermometer Alexandra Technopark, Singapore). Colour was measured using a UV spectrophotometer (DR

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3800 HACH Spectrophotometer, UK in Platinum Cobalt colour unit (Pt-Co).

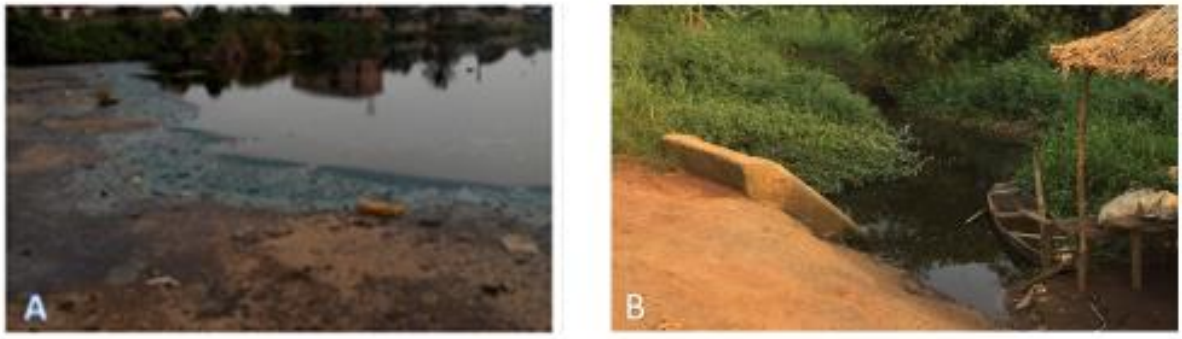

Fig. 1. (A) The edge of the artificial dye pond showing blue discoloration. (B). An unpolluted stream 11km east of the factory.

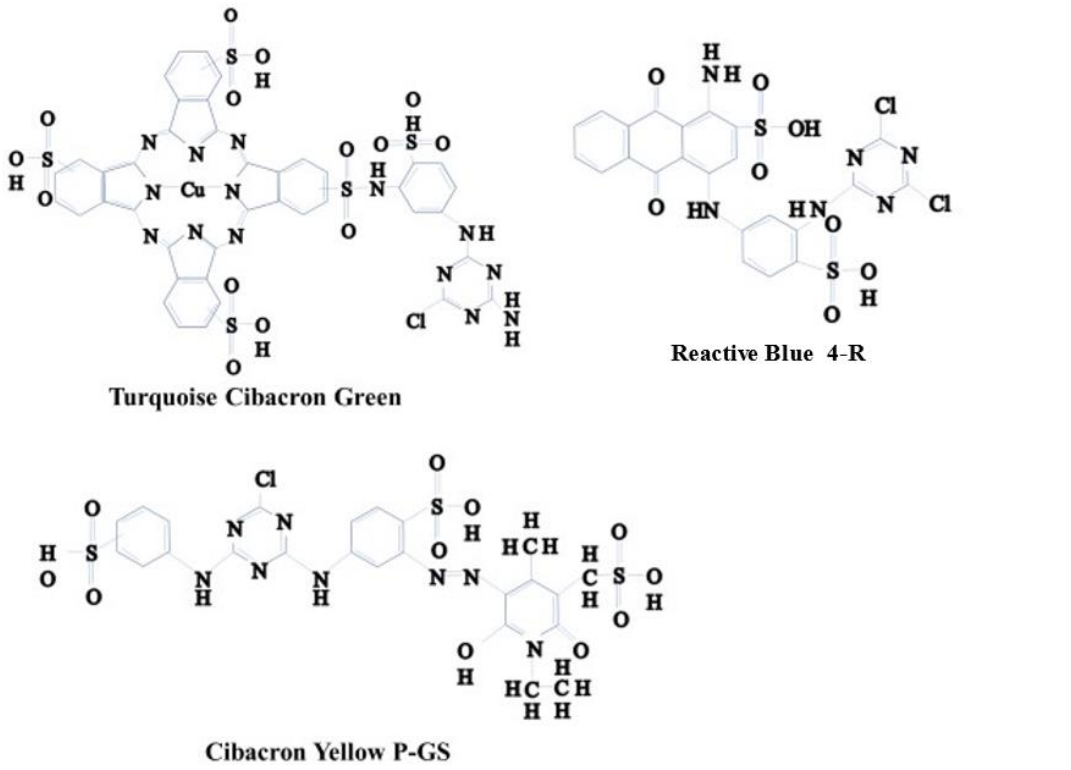

Fig. 2. Structural formulae of the textile dyes investigated.

Appearance was measured by filling a clean beaker with each sample and placing a fluorescent light source beneath the glass to observe the colour of the sample and the presence of particles. Turbidity was determined using a probe meter (2100AN Turbidimeter, HACH, UK), Total Suspended Solids (TSS) was measured using a UV Spectrophotometer with program number 630 for TSS (DR 3800 HACH Spectrophotometer, UK), Total solids were measured as previously described by Symons and Morey, [47-48]. The difference between TS and TSS determined total Dissolved Solids. Chloride was determined using Mohr's method (Argentometric titration) as described by Shukla and Arya, [49]. Nitrate, Phosphate and Sulphate measurements were determined as described by the HACH soil kit manual [50]. Dissolved Oxygen was measured using a probe (HI 2400 DO Meter HANNA Instruments, USA) dipped into the filled sample containers. Chemical Oxygen Demand was determined using the COD Cell Test Kit (COD Cell Test C3/25 Fisher Scientific, IR) and the COD Block Heater (Thomas Scientific 9855E85, UK). Biological Oxygen Demand was determined based on General/Textile industry calculations (COD/4).

The analysis of trace metals and heavy metals including Calcium, Copper, Iron, Chromium, Manganese, Lead, Cadmium, Nickel, and Silver was carried out using an Atomic

Absorption Spectrophotometry procedure using the iCE 3000 Series AA Spectrometer (Thermo Scientific, UK).

Isolation and Screening of bacteria from the textile effluents and sediments

To isolate culturable bacteria from all samples, each effluent sample (1 ml) and each sediment sample (1 g) were transferred into their respective Erlenmeyer flasks containing 9 ml of sterile distilled water to make 10^{-1} dilution and a series of dilution tubes were prepared up to 10^{-7}.

TSA Dilution plates were incubated at 37 °C for 24 hours. After incubation, distinct colonies were selected from the mixed culture plates to grow the bacterial isolates in pure cultures. Gram staining was done from pure culture plates for each of the isolates. All the isolates were subjected to the preliminary screening for dye decolorizing bacteria.

Preliminary screening for dye decolorizing bacteria was performed using the test tube assay method. A loopful of each bacterial colony was transferred into individual tubes containing RBBM, positive and negative control and uninoculated tubes. The tubes were swirled gently and incubated under static conditions at 37 °C. After 7 days, turbidity and colour reduction were determined to eliminate non-dye decolorizers.

The secondary screening was done by selecting a loopful from the turbid tubes and streaking on the agar plates of each of the four dyes. The plates were incubated for 7 days at 37 °C and observed.

Molecular identification of bacterial isolates

The two candidate decolorizers were selected and identified by 16S rRNA gene sequencing. DNA was extracted from the pure bacterial cells using ZR Fungal/Bacterial DNA MiniPrepTM kit (Zymo, USA), according to the manufacturer's instructions. Polymerase Chain Reaction (PCR) of the extracted genomic DNA from the isolates was done in a GeneAmp PCR system 9700 PCR thermal cycler. PCR amplicons were sequenced at the Bioscience Center, International Institute of Tropical Agriculture, Ibadan, Oyo using a 3130XL Genetic Analyzer (Applied Biosystems, CA, USA). Quality and assembling of the sequences were done using BioEdit (version 7.2.5) Sequence Alignment Editor [51]. The processed sequences for each isolate were compared to the GenBank nucleotide data library using the Basic Local Alignment Search Tool, BLAST software [52] at the National Center for Biotechnology Information (NCBI (http://www.ncbi.nlm.nih.gov) for strain identification. The sequences were submitted to GenBank.

Percentage Decolorization Rates of the Candidate *Bacillus* species on the Textile Dyes

To determine the decolorization capabilities of the selected isolates, each isolate was tested by transferring 200 µl of standardized (0.5 M McFarland) inoculum into the media flasks containing the respective dyes (COBM, CYBM, RBBM and TCBM) at a concentration of 250 mg/L. All flasks were incubated at 37 °C for 10 days and the assay was performed under aerobic static conditions [53-54]. The rate of decolorization was determined by a spectrophotometer. Absorbance values were read at respective wavelengths for each dye (COFR:480 nm, CYPGS:560 nm, RBFR: 600 nm, TCG: 520 nm). On Day 0, 2 ml was withdrawn from each flask and transferred at 24 hours' intervals for 10 days into microcentrifuge tubes. The tubes were centrifuged at 10,000 rpm for 10 min and 1 ml of the resulting clear supernatant was transferred into cuvettes and the absorbance values were read. Sterile BM only was used as a blank. The reduction in the colour intensity of the media indicates the decolorization capabilities of the bacterial species. The efficiency of decolorization of the dye was calculated using the following formula:

$$\% \text{ Decolorization} = \frac{A_0 - A_1}{A_0} x 100$$

Where, A_0 = Initial absorbance of the media, A_1 = Absorbance of the media at the interval of time.

Detection of Laccase

To test for the presence of laccase enzyme, 5 g of α-naphthol pellets were dissolved in 100 ml of absolute ethanol to make the stock solution. Aliquots of 5 ml from the inoculated CYBM on Day 10 of decolorization testing was transferred to a fresh test tube. Then 200 µl of α-naphthol solution was added to the test tube containing CYBM and the inoculum and observed for 2 minutes. Laccase was confirmed by a deep purple precipitate at the bottom of the tube. Sterile CYBM media only and CYBM + the bacterial strain served as controls. CYBM was selected among the four dyes because of the clarity of the precipitate in the medium [55].

Exploration of *B. amyloliquefaciens* AD20 and *B. altitudinis* AD14 for putative Dye Degrading Genes and Plasmid-related sequences

The search for putative dye degrading genes and plasmid-related sequences was conducted within the genomes of B. amyloliquefaciens AD20 (PATRIC Genome ID 1390.742) [56] and *B. altitudinis* AD14 (PATRIC Genome ID 293387.135).

Determination of the presence of plasmids

To detect plasmid presence in the isolates DPS2a and DPS2b, the method was as described by Ehrenfeld and Clewell [57]. Pure overnight grown cultures of each bacterial cells grown in

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Tryptic Soy Broth were shaken and transferred into microcentrifuge tubes. The tubes were spun for 5 minutes at top speed and the supernatant was decanted. Cell pellets were resuspended in 200 µl of solution A (100 mM glucose, 50 mM Tris Hydrochloride (pH 8.0) and 10 mM EDTA). Lysozyme (10 mg/ml) was added, and the tubes were incubated for 30 minutes at 37oC. One percent sodium dodecyl sulphate (400 µl) was dissolved in 0.2 N NaOH and mixed with the cells by inverting the tubes. Potassium acetate (300 µl of 30 % KCH_3CO_2) (pH 4.8) was added to the solution and vortexed. The tubes were then incubated on ice for 5 minutes and cell debris was removed by high speed centrifugation for 5 minutes. The supernatant was transferred into a sterile microcentrifuge tube and extracted once with 1 ml of a phenol-chloroform mixture (1:1). After centrifugation of the mixture at top speed for 15 minutes, the upper clear solution was collected into a sterile tube. An equal volume of isopropanol/ absolute ethanol was added to precipitate the plasmid and washed twice with 70% ethanol and centrifuged for 1 minute at 14,000 rpm. The microcentrifuge tubes were then dried using a Centrivap DNA concentrator for 15 minutes with periodical monitoring. After drying, the plasmid DNA was eluted with 10mM Tris EDTA (pH 8.0).

For the plasmid gel electrophoresis, 0.8 % Agarose gel was prepared in a 100 ml 1X Tris Boric EDTA (TBE) buffer, heated, and on cooling, ethidium bromide was added. Ten microlitres of DNA samples mixed with 2 µl of loading dye, and a 100 bp DNA ladder (Hyperladder, Bioline, London, UK) was loaded on the wells and run at 100 V.

3.0 Results

Physicochemical Analysis of Samples

The physicochemical data of the samples were compared to industry guidelines from the state, (Lagos State Environmental Protection Agency (LASEPA)), and federal (Federal Ministry of Environment (FMEnv)) government agencies (**Table 1**) [58].

Isolation and Preliminary Screening of Decolorizing Bacteria

From the TSA serial dilution plates (10^{-4} and 10^{-5}), the forty-five bacterial colonies obtained from the mixed culture plates were grown in pure culture. From the five samples UE, TE, DPS1, DPS2 and CS, 6, 5, 9, 16, 9 colonies were obtained respectively. Preliminary screening of these bacteria species for dye utilization was performed in BSM infused RBFR dye for 7 days. Based on the extent of colour clearing in comparison with the uninoculated control tube, 7 isolates reduced the intensity of the dye. However, 3 isolates (DPS2a, DPS2b and TEa) were selected for additional screening based on their dye clearing efficiency (**Figure 3**). After the secondary screening, only 2 isolates (DPS2a, DPS2b) grew on the Dye Agar Medium (**Figure 4**). These isolates DPS2a and DPS2b were identified based on cultural characteristics and microscopy (**Figure 5**).

Molecular Identification of Decolorizing Bacteria

The 16S rRNA amplicon sequences of the isolates (DPS2a and DPS2b) had a 100 % nucleotide identity with *Bacillus altitudinis* GQJK2 and *Bacillus amyloliquefaciens* ALB79 respectively. The partial 16S rRNA sequences have been deposited in GenBank under accession numbers MT364383 and MT365797 respectively.

Decolorization Efficiency of the Strains on Textile Dyes

A decrease in absorbance values was directly proportional to the reduction in dye concentration by the microbes. The absorbance values of 4 reactive dyes RBFR, COFR, TCG and CYPGS treated with the isolates DPS2a and DPS2b were obtained (**Table 2; Figure 6**). After 10 days, B. altitudinis reduced the concentrations of RBFR, COFR, TCG and CYPGS by 83%, 16%, 89% and 37% respectively while B. amyloliquefaciens also reduced dye concentration by 92 % (RBFR), 71% (COFR), 49 % (TCG) and 70 % (CYPGS) (Figure 7). The consortium of the two strains were able to decolorize RBFR by 94 %.

Detection of the laccase activity

Laccase production by the two strains was confirmed qualitatively by the detection of a purple precipitate at the bottom of the inoculated tubes because of the oxidation of α-naphthol in the CYBM (**Figure 8**).

Presence of Putative Dye degrading Genes and Plasmid-related sequences

WGS showed that *B. amyloliquefaciens* AD20 and *B. altitudinis* AD14 commonly had 2 copies each of the FMN-dependent NADH-azoreductase and a laccase gene. *B. amyloliquefaciens* AD20 had another azoreductase belonging to the class of NAD(P)H-dependent flavoenzymes (**Table 3**). No plasmids were detected in both *Bacillus* isolates by gel electrophoresis (**Figure 9**) and by WGS [56].

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Table 1. Physicochemical analysis of Dye Effluents

	Parameter	Samples				LASEPA standard	FMEnv Standard [58]
		UE	TE	DPW	SCW		
Physical	Color (Pt. Co. APHA)	3030	37	950	570	250	7
	Appearance	Darkish blue, suspended particles	Clear, suspended particles	Greenish blue, suspended particles	Yellowish, cloudy, suspended particles	Clear	
	Temperature (°C)	29	26	30	30	40	40
	pH	8.4	7.14	8.79	6.46	5.5-9.0	6-9
	Turbidity (NTU)	3.2	2.8	54.2	77		
	Total Suspended Solids (mg/L)	28	3	119	44	100	30
	Total Dissolved Solids (mg/L)	416	61	635	64	2100	2000
	Total solids (mg/L)	444	64	754	108	2200	
Chemical	Chloride (mg/L)	28.4	5.68	56.8	4.26	250	
	Nitrate	0.8	21.5	5.5	5.0		20
	Phosphate	0.56	0.52	1.32	4.63		5
	Sulphate	8	1	1	1		
	Dissolved Oxygen (mg/L)	3.97	6.28	0.00	6.64	≥2	
	C O D (mg/L)	314	2	489	53	200	80
	B O D (mg/L)	78.5	0.5	122.25	13.25	50	50
Trace/Toxic Heavy Metal	Calcium (mg/L)	0.06	0.04	0.36	0.33	200	200
	Copper (mg/L)	0.06	ND	0.01	0.001	3.0	< 1.0
	Iron (mg/L)	0.07	0.05	0.45	0.34	10	20
	Chromium (mg/L)	ND	0.01	0.002	ND	0.1	< 0.1
	Manganese (mg/L)	0.01	0.01	0.02	0.12	5.0	5.0
	Lead (mg/L)	ND	ND	ND	ND	0.1	
	Cadmium (mg/L)	ND	ND	ND	ND	2.0	
	Nickel (mg/L)	0.001	0.01	0.004	0.01	3.0	
	Silver (mg/L)	0.01	0.01	0.006	0.01	< 0.10	

UE- Untreated Effluent. TE- Treated Effluent, DPW –Dye Pond wastewater SCW control water from the Stream NTU: Nephelometric Turbidity Units; Pt. Co. APHA: Platinum Cobalt APHA method; HDL: Highest Desirable Level; MPL: Maximum Permissible Level; COD: Chemical Oxygen Demand

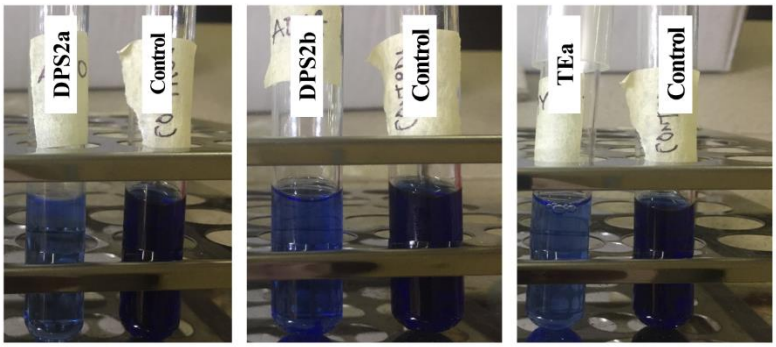

Fig. 3. Preliminary test of visible dye reduction in the RBFR-infused medium with isolates DPS2a, DPS2b and TEa in Tubes marked as DPS2a, DPS2b and TEa respectively after 7 days. Tubes labelled Control contain only sterile RBFR medium.

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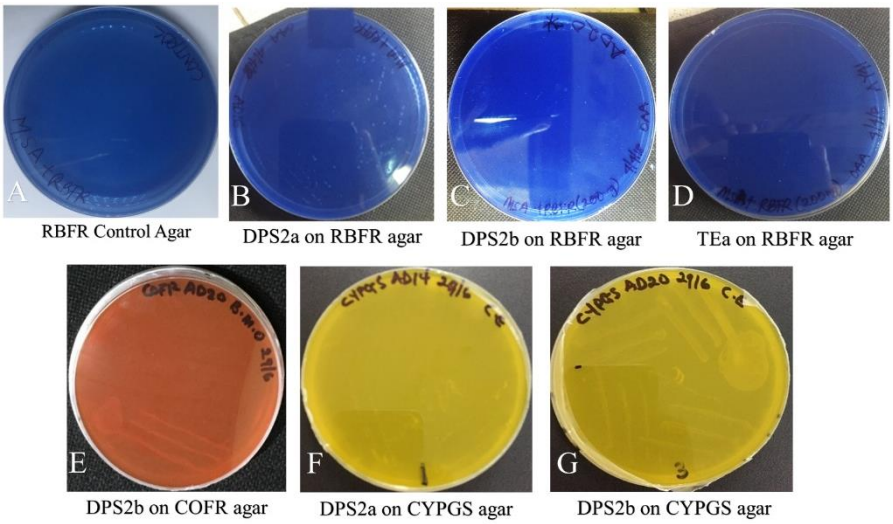

Fig. 4. All media contain Dye and Basal Agar. Plate **A.** Uninoculated RBFR Agar. Distinct colonies of **B.** DPS2a on RBFR Agar. **C.** DPS2b on RBFR. **D.** No colonies of TEa on RBFR. Colonies of **E.** DPS2b on COFR Agar. **F and G** DPS2a and DPS2b on CYPGS Agar.

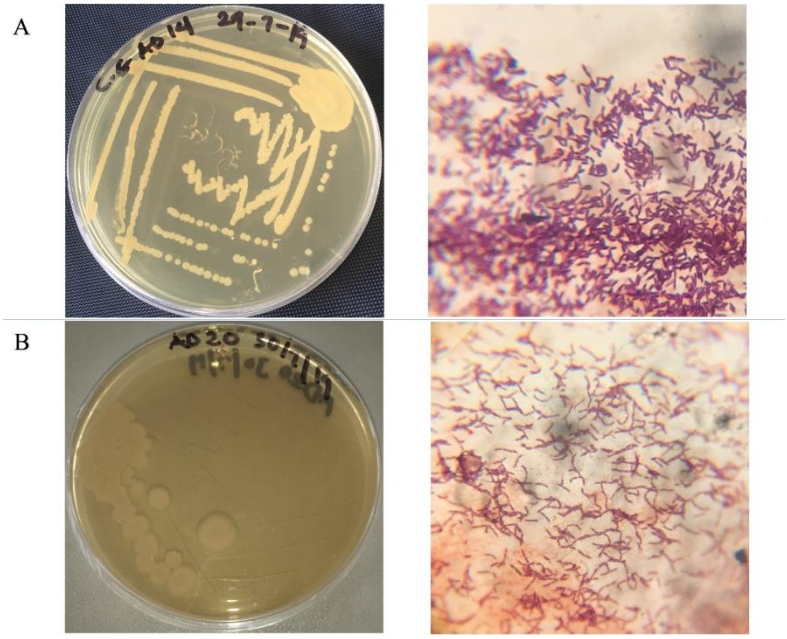

Figure 5. Pure culture TSA plates and corresponding micrographs of Gram-positive rods (at 1000X total magnification) of **A**. Isolate DPS2a and **B**. Isolate DPS2b.

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Table 2. Average Absorbance Values of DPS2a (*B. altitudinis* AD14) and DPS2b (*B. amyloliquefaciens* AD20) and in consortium decolorizing the synthetic dyes over a 10-day Incubation Period.

Isolates	DPS2a				DPS2b				DPS2a + DPS2b
Dyes	**RBFR[a]**	**COFR[b]**	**TCG[c]**	**CYPGS[d]**	**RBFR[a]**	**COFR[b]**	**TCG[c]**	**CYPGS[d]**	**RBFR[a]**
Initial Abs/Day 0	1.166	0.836	0.366	1.357	1.166	0.836	0.366	1.357	1.166
Day 1	0.287	0.783	0.194	1.166	0.2127	0.8187	0.317	0.817	0.19
Day 2	0.26	0.783	0.139	1.138	0.1567	0.779	0.317	0.718	0.171
Day 3	0.2537	0.7777	0.087	1.073	0.1517	0.7765	0.316	0.625	0.136
Day 4	0.246	0.7497	0.083	1.014	0.1437	0.705	0.309	0.591	0.127
Day 5	0.2263	0.747	0.079	1.025	0.1435	0.6705	0.26	0.542	0.12
Day 6	0.2207	0.744	0.073	1.01	0.132	0.6275	0.242	0.485	0.112
Day 7	0.1907	0.7345	0.071	0.983	0.1195	0.5475	0.237	0.436	0.103
Day 8	0.2175	0.7215	0.057	0.912	0.0995	0.362	0.225	0.418	0.095
Day 9	0.202	0.708	0.053	0.895	0.0985	0.287	0.209	0.409	0.072
Day 10	0.196	0.705	0.040	0.862	0.092	0.235	0.186	0.402	0.065

Wavelengths a – 600 nm, b – 480 nm, c – 520 nm, d – 560 nm.

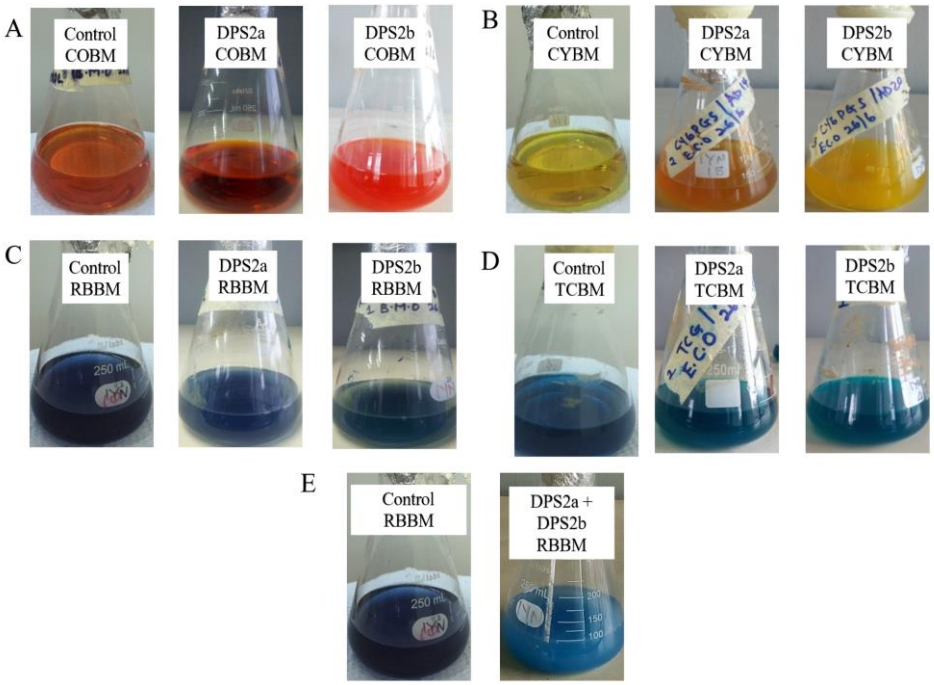

Fig. 6. Decolorization flasks containing the different dye basal media inoculated singly with the isolates (DPS2a/DPS2b) A. COBM B. CYBM C. RBBM D. TCBM and both isolates (DPS2a & DPS2b) E. RBBM in comparison with uninoculated flasks after a ten-day incubation.

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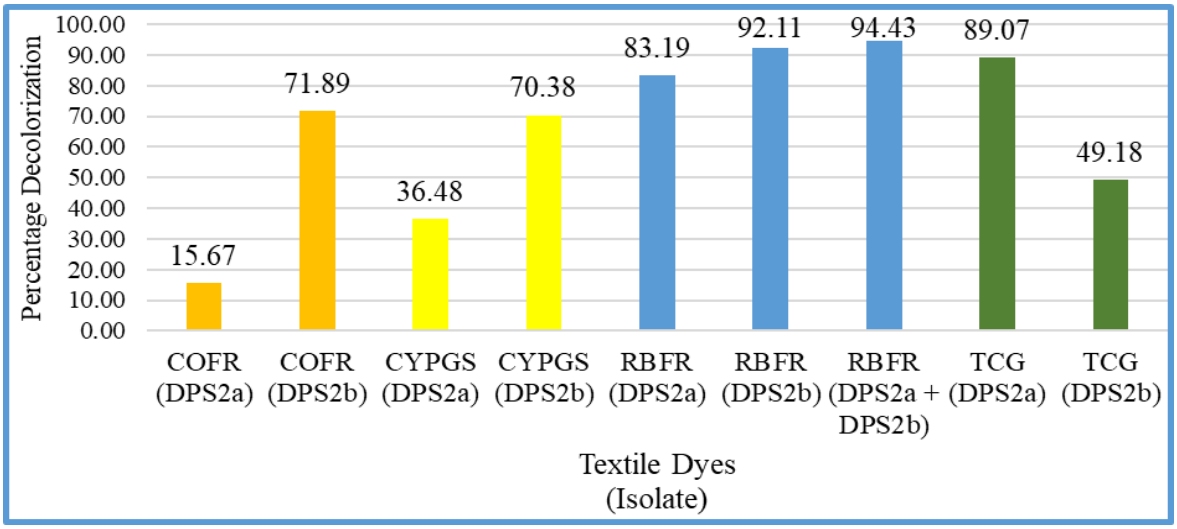

Fig 7. Average percentage decolorization rates of the four synthetic textile dyes-COFR, CYPGS, RFBR and TCG by *B. altitudinis* AD14 and *B. amyloliquefaciens* AD20 after a 10- day incubation.

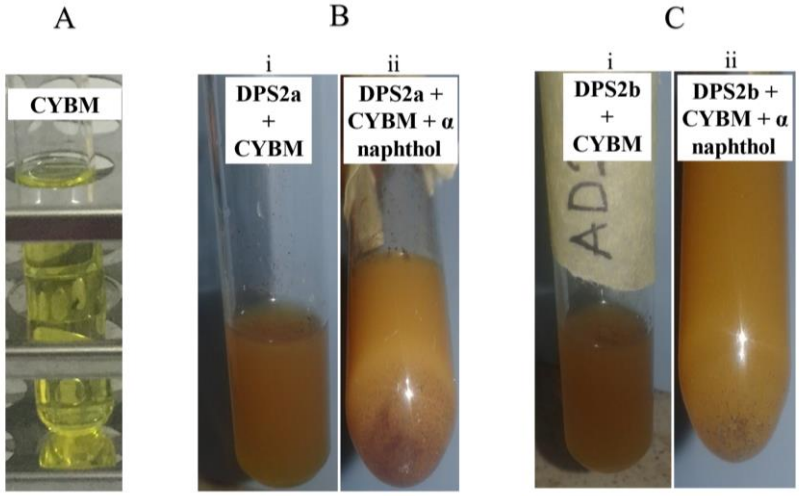

Fig. 8. Qualitative Detection of Laccase based on the addition of drops α-naphthol to CYBM cultures of the isolates. The formation of an insoluble violet precipitate at the base of the tube suggests the presence of laccase. **A**. uninoculated CYBM **B**. (i) DPS2a + CYBM (ii) DPS2a + CYBM + α-naphthol **C**. (i) DPS2b + CYBM (ii) DPS2b + CYBM + α-naphthol

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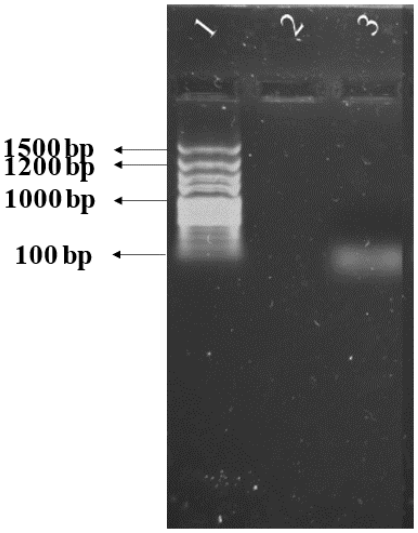

Fig. 9. 0.8% Agarose gel of analysis of plasmid DNA. Lane 1: 100 bp DNA ladder, Lane 2: *Bacillus altitudinis* AD14 (no band indicating the absence of a plasmid), Lane 3: B*acillus amyloliquefaciens* AD20 (no band within the plasmid size range but an RNA band is present).

Table 3. Putative Dye -Degrading Enzymes identified in *B. altitudinis* AD14 and *B. amyloliquefaciens* AD20

Organism	Function	Start	Stop	PGFam
B. altitudinis AD14	FMN-dependent NADH-azoreductase (EC 1.7.1.6)	102559	103194	PGF_10547233
	FMN-dependent NADH-azoreductase (EC 1.7.1.6)	195434	196078	PGF_10547233
	Laccase (EC 1.10.3.2)	121040	122569	PGF_00053829
B. amyloliquefaciens AD20	Azoreductase (EC 1.7.1.6)	1035246	1034722	PGF_00069238
	FMN-dependent NADH-azoreductase (EC 1.7.1.6)	947729	948355	PGF_10547233
	FMN-dependent NADH-azoreductase (EC 1.7.1.6)	264265	263630	PGF_10547233
	Laccase (EC 1.10.3.2)	32466	30928	PGF_00053829

Discussion

In this study, a screen for dye-reducing bacterial species from textile factory effluents, water bodies and sediments in the textile factory's vicinity. Dye-degrading bacteria have often been linked to effluents or waste dumpsites. *Bacillus* species isolated from dye wastewater sites have been shown to possess dye decolorizing capabilities [15,16,24-25,27,40-41].

Physicochemical analyses of the samples revealed that for the parameters assessed, the treated effluent (TE) samples were within the industry standards set by the state (LASEPA) and the federal agency (FMEnv). It was not surprising that the untreated effluents and dye pond wastewater exceeded the COD and BOD limits, and the DPW also fell below the DO limits. The comparison of untreated effluent (UE) and treated effluent (TE) samples showed that there was a marked reduction in colour, BOD and COD but the dye pond should be an area of concern because its levels of BOD, COD and DO indicate toxicity. In addition, as expected the levels of TSS, TDS and TS in the dye pond were higher than in the natural dye-free stream.

The levels of heavy metals in the samples investigated were below statutory limits and, in some cases, not detected. This lack of evidence for the presence of these heavy metals might be due to the sensitivity limits of the equipment used for their testing. Numerous studies have shown that heavy metals are present in dye effluents [59-61].

The two isolates used for the decolorization experimental analysis were identified to be *B. altitudinis* AD14 (DPS2a) and *B. amyloliquefaciens* AD20 (DPS2b) by molecular16S rRNA gene sequencing.

Singly, *B. altitudinis* AD14 and *B. amyloliquefaciens* AD20 cleared the RBFR dye with a percent efficiency of 83 %; and 92 % respectively and in a consortium (94 %). DPS2a reduced TCG approx. 1.5 times over DPS2b, whereas DPS2b cleared CYPGS and COFR with two-fold and four-fold efficiencies respectively over DPS2a. Overall, *B. amyloliquefaciens* AD20 (DPS2b) possessed more decolorizing capabilities than *B. altitudinis* AD14 (DPS2a).

The two decolorizing bacterial strains from this study oxidized α -naphthol to give a deep purple precipitate indicating the presence of laccase. The detection of the presence of a lignolytic enzyme, laccase in the final CYPGS decolorization medium of each of the strains denotes that a possible biocatalytic activity might be linked to the breakdown of the complex dye [55]. We did not check for the presence of other lignolytic modifying enzymes, so their activities in the decolorization process cannot be ruled out.

It is well known that laccase from *Bacillus* spp. degrade synthetic dyes [30, 33-35, 42] including Remazol Brilliant Blue R (RBBR), Alizarin Red, Congo Red, Methyl Orange and Methyl Violet using the laccase enzyme [30].

The putative dye degrading enzymes identified in these strains catalyze the decolorization of a wide range of azo dye classes. Azoreductases degrade Direct, Acid and Basic dyes [43], FMN-dependent NADH azoreductases and bacterial laccases can degrade Reactive dyes [45-46]. Both *Bacillus* strains from our study did not bear any plasmids. WGS provided supporting evidence for these [56].

Conclusions

This is the first study on the bacterial decolorization of these four dyes RFBR, CYPGS, TCG, COFR used by one of the principal textile factories in Nigeria. Our current investigation has revealed the dye degrading potential of *B. altitudinis* AD14 and *B. amyloliquefaciens* AD20. Based on their decolorization efficiencies in the laboratory, especially with RFBR, they show promise for biotechnological applications. Considering that the two identified decolorizers were isolated from the same dye pond, other non-cultivable dye degraders could have been missed.

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