5HVHDUFK $UWLFOH Open Acces

&GGJDJFOU "FSPCJD %FHSBEBUJ4Q/I7PBNSPQP A *TPMBUFE GSPN 1FUSPMFVN 4MVEHF

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Introduction

Despite increased awareness and governmental regulations, large amount of organic pollutants are discharged in our water bodies. Sometimes this discharge is accidental (oil spills), but o en at times, especially in developing countries, it is deliberate (and criminal). It is well accepted that these organic pollutants pose a direct threat to aquatic and marine life and eventually us. Due to the extensive use of organic dyes by various industries, they have become an integral part of many industrial e uents. Most of these dyes are toxic and potentially carcinogenic in nature and hence their removal from the industrial e uents is a major environmental problem.

Removal of dyes from wastewaters using various methods has been suggested, such as coagulation, adsorption, advanced oxidation (AOPs) and the membrane processes [1-6]. All these approaches have some advantages and disadvantages as well as limitations. Besides these traditional physical techniques like adsorption on activated carbon, ultra ltration, reverse osmosis, coagulation by chemical agents and ion exchange on synthetic adsorbent resins have also been used by various groups [7-9]. However in many cases, these techniques are either very costly or economically unfeasible or may have technical constraints.

e versatility and adaptability of microbes for hazardous waste degradation is gaining a lot of attention lately. Literature survey shows the promise of e cient biodegradation of various classes of dyes using microorganisms, mainly due to the low cost, ability to produce less sludge and environmental compatibility [10]. In this regard, various microorganisms (e.g. Bacillus subtilis, Phanerochaete chrysosporium, Aeromonas hydrophila, Penicillium sp., Klebsiella pneumoniae, Proteus mirabilis and Pseudomonas cepacia) have been isolated and have been shown to be very promising for degrading di erent dyes [11-14].

In this paper, we present results on the isolation of twelve strains of dye degrading bacteria from petroleum sludge and characterized their abilities to degrade various types of organic dyes in aqueous solutions. Since most biodegradation of azo dyes are generally carried out under anaerobic conditions, part of the novelty of this work is that our strains were very active (in dye degradation) under aerobic (shaking) conditions. Additionally, they could also degrade dyes under anaerobic conditions. Analyses of degradation products of di erent dyes suggest that di erent degradation products are generated by our Sphingomonas strain depending on the availability of oxygen during the degradation process.

Materials and Methods

Toluidine Blue, Amido Black, Crystal Ponceau 6R, Trypan Blue, Methyl Blue, Orange G, Acid Red 40, Reactive Black 5 and Ponceau BS were obtained from Sigma-Aldrich. Eriochrome Black T, iazole Yellow G, Naphthol Green B and Congo Red were purchased from Fluka chemical and Malachite Green from BDH. Drimarene Black CL BGR, Drimarene Red CL 4BN and Drimarene Yellow CL 4R were obtained from Clariant chemicals. e chemical structures, dye class and the λ_{max} of all dyes are listed in Table 1. Media for culturing was obtained from Sigma-Aldrich. Nutrient Broth composition (Sigma) was as follows: 1 g/L D(+)-glucose, 15 g/L peptone, 6 g/L sodium chloride, 3 g/L yeast extract, 3 g/L, nal pH 7.5 ± 0.2 (25°C). All organic solvents (HPLC grade Acetonitrile and Methanol) were from Fisher Scienti c (UK). All chemical used in this work were of analytical grade and used without further puri cation.

Bacterial strain identi cation and phylogenetic analysis

e 12 bacterial strains (H1-H12) were identi ed using partial 16S rRNA sequencing of the crude DNA on a 3500 Genetic Analyzer, Applied Biosystems, USA. e obtained DNA sequences were compiled

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S.No	Name of dye	Class of dye	λ_{max} nm	Structure of dye
1.	Malachite Green	Triarylmethane		

in FASTA format and analyzed using BLAST (blastn) through NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). e phylogenetic analyses were performed using the online site http://www.phylogeny.fr [15,16] which produced the phylogenetic tree.

Dye decolorization

A loopful of bacteria culture from glycerol stock was inoculated in a 50 mL sterile tube containing 15 ml nutrient broth and incubated at 37°C under shaking condition (200 rpm) for 24 h. For sampling, all dyes

Name of the Dyes	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12
Acid Red 40	-	-	-	-	-	-	-	-	-	-	-	-
Amido Black	++	++	+++	++	-	+++	++	++	++	-	-	-
Congo Red	+	++	+++	+++	-	+++	+++	++++	++	-	++	-
Crystal Ponceau 6R	-	-	-	-	-	-	-	-	-	-	-	-
Erichrome Black T	+	+	+	++	-	++	++	++	-	+	+	+
Malachite Green	+++	+++	++++	++	-	+++	+++	+++	++++	+++	++++	++++
Methyl Blue	++	++	++	++	-	+	++	++		++	++	++
Naphthol Green B	-	+	-	+	-	+	+	+	-	-	-	
Orange G	-	-	-	-	-	-	-	-	-	-	-	-
Ponceau BS	++	++++	++++	++++	-	++++	++++	++++	-	-	++	-
Reactive Black 5	+	+	-	++	-	-	+	+	-	-	-	-
Toludine Blue	++	+++	++	++	-	+	++++	+++	++	+++	++	++

H: Bacterial strains, (-) 0-20%,(+) 21-40%, (++) 41-60%, (+++) 61-80%, (++++) 81-100%

Table 2: Aerobic degradation of dyes by all twelve bacterial strains.

Optimization

e e ect of pH, concentration and carbon sources were studied and optimized. e decolorization of dye was observed at pH 5, 7, 9 and 11. Nutrient broth of pH 7 was used at various concentrations of dye. e e ect of carbon and nitrogen sources was examined by using di erent nutrient media such as Nutrient Broth, LB and 2xYT. All experiments were carried out as mentioned above.

HPLC and UV analysis for decolorization

High performance liquid chromatography (HPLC) was carried out as previously described [17]. Brie y, an Agilent PH 1100 liquid chromatography system, (Agilent, USA) with an Agilent Zorbax SB-C18 column 150 mm x 4.6 mm packed with 5 µm particle size, coupled to a diode array detector was used (Agilent, USA). e mobile phase consisted of solution A (0.1 M ammonium formate (pH 6.7) and solution B (1:1 acetonitrile/methanol) and gradient from 0% B to 80% B in 40 minutes and a ow rate of 1 mL/min was used to obtain the chromatographs. Percentage of dye decolorization was analyzed by using Epoch microplate reader from BioTek (USA). Sample from all tubes were examined at di erent times (0h, 2h, 4h, 6h, 8h and 24h) during the period of 24h. During all time intervals, each sample was centrifuged and the supernatant was used for absorbance measurements at $_{max}$ values of the dyes.

Result and Discussion

In initial screening we were interested in identifying bacterial strains that would be most e cient at degrading various classes of dyes. erefore, we tested our 12 bacterial strains, isolated from petroleum sludge, on twelve di erent dyes under aerobic conditions. e screening results are summed up in Table 2. All dyes showed reasonable degradation when exposed to various bacterial strains, except H5 strain which failed to show any decolorization of the dyes under investigation. Additional experiments with H5 showed that it could in fact degrade various dyes, but only under anaerobic conditions. Based on the results from this initial screening, isolate H8 showed most promising results in degrading various classes of dyes and was chosen for further study.

Among the many dyes used in various applications, azo dyes are most extensively used in industries because they are easy to synthesize and are thus cost e ective. Unfortunately, most azo dyes are toxic, carcinogenic and mutagenic in nature [18]. e azo bonds present in these compounds are resistant to breakdown, which can result in the accumulation of these molecules in the environment. However, as has been reported previously and shown in Table 2, they can be degraded by speci c strains of bacteria under aerobic and/or anaerobic conditions. Subsequent experiments were carried out with additional azo dyes with strain H8, under aerobic conditions. e % decoloration observed with a set of twelve diverse azo dyes are presented in Table 3. Based on the % decolorization achieved, these azo dyes were divided into three main groups. e results are shown in Figure 1. Dyes belonging to group A showed maximum decoloration, whereas, group C dyes showed minimum decoloration. An interesting part of this investigation is that, when same dyes were examined under static (anaerobic) conditions,

they showed almost the same amount of decolorization as compared to shaking (aerobic) condition - the comparative results are shown in Figure 2. At the end of second screening, three azo dyes namely, Amido Black, Congo Red and Ponceau BS (all from group A) were chosen for further analysis as they showed more than 75% decoloration.

Partial sequencing of the 16S rRNA gene showed that the 12 bacterial strains belonged to four different bacterial genuses: *Bacillus* (H1, H2, H4, H5 and H6), *Sphingomonas* (H3 and H8), *Alphaprotobacterium* (H7) and *Pseudomonas* (H9, H10, H11 and H12). Table 4 shows the individual bacterial strains, their GenBank accession number, and their identity (based on 16S rRNA sequence similarity to published sequences). The phylogenetic analysis of these twelve strains (and a few representative bacteria) is also shown in Figure 3. It is interesting to note that of the four genera of bacterial strains that we isolated from petroleum sludge, three of them (*Bacillus*, *Sphingomonas*, and *Pseudomonas*) have been previously shown to be able to degrade various classes of organic pollutants [19,20]. For example, Ding and colleagues have shown that a *Sphingomonas* strain isolated from $Fe(OH)_3$ enriched microbial electrochemical reactor was able to efficiently degrade Acid Orange 7 dye under anaerobic conditions [20].

Citation:

[30,32]. Although interesting and useful, most of the above mentioned studies have been carried out on di erent strains and di erent dyes, and di erent degradation conditions (aerobic and anaerobic). In this manuscript, we present preliminary results from our systematic degradation report their studies under anaerobic conditions, bacterial azo dye degradation can proceed under both aerobic and anaerobic conditions, or in mixed-batch mode [28]. It has been shown that the rst step in azo dye degradation under both aerobic and anaerobic conditions proceeds via the reductive cleavage of the azo bond, leading to subsequent generation of aromatic amines [29].Under anaerobic conditions, and depending on the ring substituents and microbial strains, these amines are further converted to various metabolites such as 1-amino-2-naphthol, sulfanilic acid and nitroaniline [30]. However, if the degradation is being carried out under aerobic conditions, then microbial oxygenases can work on the aromatic amines to produce mono and dihydroxyaromatic compounds [31]. ese compounds can be very di erent than those produced under anaerobic degradation conditions, and include metabolites such as 3-aminobenzenesulphonates, 2-aminonapthyl sulfonate, and hydroxysalicylic acids [32,33]. Interestingly, one of the metabolites, 1-amino-2-naphthol, has been reported to be produced under both aerobic and anaerobic conditions

and anaerobic conditions. Lastly, preliminary HPLC analyses showed that depending on the presence or absence of oxygen, very di erent metabolic products can be produced, the identities of which will be reported in a future publication.

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show 5 peaks in aerobic and 2 peaks in anaerobic condition (Figure 8). Surprisingly, Amido Black chromatograms showed the apparently same metabolites under both aerobic and anaerobic conditions (Figure 9). ese results show that the degradation pathway for Ponceau BS and Congo Red are probably very di erent under aerobic and anaerobic conditions based on their HPLC pro le, but Amido Black may proceed via a common scheme under both aerobic and anaerobic conditions. Future studies are planned to identify these metabolites that are produced under di erent degradation conditions.

Conclusion

In summary, we report here that petroleum sludge is a rich source of microbes that could be used to degrade various classes of dyes, including diverse azo dyes, which are normally recalcitrant to degradation. Furthermore, we identi ed one of the strains as Sphingomonas sp which could degrade up to 7 di erent azo dyes, including three (Amido Black, Congo Red, and Ponceau BS) very e ciently under both aerobic

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