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Microbial Diversity Analysis in Malachite Green Dye Treating Sequencing Batch Reactor

Naeem-ud-Din Ahmad[1], Khizar Hayat Bhatti[2*], Ijaz Ali[3], Khalid Khan[1], Sher Aslam[4], Amin Shah[5], Ejaz Hussain Siddiqi[2], Muhammad Akbar[2] and Liu Ying[6]

[1]Department of Biological Sciences, Government Superior Science College, Peshawar-25000, Pakistan.
[2*]Department of Botany, ICBS, HH Campus, University of Gujrat, Gujrat-50700, Pakistan.
[3]Institute of Biotechnology and Genetic Engineering, Peshawar-25000, Pakistan.
[4]Department of Agriculture, University of Haripur, KPK, Haripur-22620. Pakistan.
[5]Department of Biological Sciences, University of Sargodha, Sargodha-40100, Pakistan.
[6]Institute of Microbiology, Chinese Academy of Sciences, Haidian District, Beijing-100080, China.

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ABSTRACT

Microbial diversity was investigated in an optimized sequencing batch reactor (SBR), treating malachite green containing wastewater, with the decolorization efficiency of 89 % and chemical oxygen demand (COD) removing ability of 93%. Both culture-independent 16S rRNA gene method and culture-dependent plate-dilution method were utilized. Phylogenetic trees were sketched by neighbor-joining method using bioinformatics tools. Culture-independent method showed the SBR community affiliation with the Alpha, Beta, Gamma and Delta proteobacteria, in addition to the moderate resemblances with Verrucomicrobia, and some uncultured bacteria. The culture-dependent isolates, however, identified only with the Beta and Gamma proteobacteria. Some sequences had less than 95% homology to the data in GenBank indicates the possibility of novel bacterial species.

KEYWORDS: Bioinformatics Tools; Malachite Green Dye; Microbial Diversity; Phylogenetic Tree; Sequencing Batch Reactor

INTRODUCTION

The use of dyes has been tremendously increased due to industrialization and modernization. The textile industry alone utilizes thousands of different dyes and pigments and million tones of synthetic dyes are produced annually across the globe. However, in the process of dying, direct loss of the dye(s) in the waste water was reported as much high as 10-15%. Dyes can adversely affect photosynthetic activity in the plants and aesthetic value of the receiving water bodies, because they have high tinctorial value while, dye even less than 1 ppm gives noticeable color [1].

Elimination of dye out of the wastewater is relatively an intricate issue due to complicity of treating procedures for contaminated wastewaters by conventional means [2]. However, majority of the dyes are synthetic and exhibit complex structure that enable them to resist biodegradation [3]. Additionally, physicochemical methods are very expensive and yet not safe to our existing environment. Alternatively, biological processes gather immense potential to be popularized in the public [4]. Phugare, et al. (2011) reported a bacterial consortium for degradation, detoxification and decolorization of textile effluents, successfully [5].

Malachite green (MG) is a triphenylmethane (TPM) that is popularly used in textile industry. It is also considered a valuable biological stain. In aquaculture, it is used as antifungal, bactericide and parasite regulator. The malachite green dye is phytotoxic, a respiratory poison, and a potent teratogen. This dye has been included in US National Toxicology Program as candidate chemical for cancer testing [6]. Due to low cost and efficacy MG is likely to be used in many areas of the globe, especially the third world.

The sequencing batch reactors (SBR) have been in use for degrading industrial intractable chemicals with remarkable success owing to diversity of physical and biological conditions applicable them [7]. In bio-treatment activated sludge are used for adsorption rather than effective degradation [8]. Numerous biomaterials have been investigated for characterization of their adsorptive capacity for the target dyes and pollutants [9]. Tan, et al. (2009) found that activated sludge caused decolorization at textile sewage treatment plant ranging from 70 to 90%. [10].

The biomass in wastewater bioreactor is an important source of highly complex mixture of microbial community. As a general rule, the contaminants in wastewater support flourishing growth of highly resistant organisms that continue to exist and adjust owing to their genetic make-up. Zhang, et al. (2013) cited evidence that to tackle the shock load of pollutants, the indigenous bacterial community adjusted by adapting gradually itself in the harsh atmosphere [11].

Corresponding Author: Khizar Hayat Bhatti, Department of Botany, ICBS, HH Campus, University of Gujrat, Gujrat-50700, Pakistan. Email address: khizar.hayat@uog.edu.pk

The operational conditions and the presence of pollutants may lead to the dominance of certain groups of bacteria. Likewise, physical conditions can be manipulated for maximum COD (chemical oxygen demand), NH_4^+–N and Mn^{2+} removal from the waste material using appropriate strategies [12].

Stable microbial communities are very important and effective in bioremediation; therefore, phylogeny and properties of the concerned bacterial groups are very important parameters for the adjustment of the operating conditions and enhanced efficiency of the bioreactors.

A great deal of knowledge about the microbial community in a dye contaminated wastewater treating reactor would be useful in association with operational conditions necessary to eliminate the pollutant efficiently from the containments. Keeping in view its importance in general and specifically this aspect inspired the researchers to evaluate the microbial community under the selective pressure of malachite green in the SBR.

2. MATERIALS AND METHODS

2.1. The Bioreactor. An activated sludge based SBR from a previous experiment for nutrients removal was used in the current study. The reactor was optimized at HRT for at least one day at dye concentration of 35 mg/L, with gradual increase in the dye concentration during one month. The system achieved the decolorization efficiency as much as 89 % and chemical oxygen demand (COD) removing ability was recorded to be 93%. Nitrogen removal rate of this system was however; relatively lower i.e., about 70 %. Under such conditions, the SBR water samples were collected, and the biomass obtained was used for microbial community analysis. Some of the parameters of the reactor are given in Table 1.

Table 1. Some parameters of the SBR in the current study.

1	Biomass, as mixed liquor suspended solids (MLSS)	5.5 gm/L
2	SVI Sludge Volume Index	50 ml/gm
3	pH	7.6 to 8
4	Dissolved Oxygen	0.5 - 1.5 mg/L
5	Temperature	25-30 °C
6	HRT	One Day
7	Malachite Green Initial Concentration	35 mg/L

2.2. Analysis of the Microbial Community. In the present study, both culture-dependent and culture independent 16S rRNA gene library-based molecular strategy were used for phylogenetic analysis. This strategy was employed explore the bacterial community association with MG treatment using SBR.

2.3. Genomic DNA Extraction from Reactor Sludge. The bacteria were collected in appendorf tube after centrifugation at 12,000 rpm for 5-7 min. The pellet was dissolved in 100 uL 20% SDS and 1 mL TES buffer (100 mM Tris, 100 mM ETDA and 100 mM NaCl). Thereafter, 0.6 gm of glass beads (Sigma) were added and mixed thoroughly using vortex for 5 minutes followed by centrifugation for 1 minute at 12,000 rpm. The supernatant from the previous step was treated with the same volume of phenol/chloroform/isoamyl alcohol (25:24:1). After Phenol: Chloroform extraction, DNA was precipitated with absolute ethanol (double volume) and 5M sodium acetate (1/10 volume). RNA was degraded by adding 5 ul RNase (10 mg/ml). The DNA was run on 1% agarose gel in 1 x Tis-Acetate-EDTA (TAE), 40 mM acetic acid, 40 mM Tris, 1mM EDTA, pH=8). The gel was stained with ethidium bromide (5 ug/ml) after electrophoresis. Before 16S rRNA gene PCR amplification, the genomic DNA was stored at –20°C.

2.4. PCR-Amplification, Clone Library Construction and Sequencing. For the amplification, universal primers of bacteria 27F (3′-AGAGTTTGATCATGGCTCAG-5′) and 1492R (3′-TACGGYTACCTTGTTACGACTT-5′) were used. The PCR products were purified with PCR Kleen spin columns (Bio-Rad Inc.). Nucleotide sequences were determined from the purified ~800-bp partial fragment of the 16S rDNA gene. From DNA of the bacterial community, 16S rRNA gene fragments were amplified. The parameters for PCR cycles were as follows: initial denaturation at 95°C for 5 minutes, thereafter, 30 cycles of denaturation at 94°C (1 min), annealing at 55 °C (1min), extension at 72°C (90 sec), and final extension at 72°C (10 min). The amplified products were purified using Wizard® SV Gel and PCR Clean-up system (Promega®) as described by manufacturers. The PCR products of size 1400 bp were analyzed on 1% (w/v) agarose gel electrophoresis. The products were recovered by using gel purification kit (EZN China). The PCR products were ligated in pBS-T Vector (Tiangen Biotech, Beijing China) and subsequently transformed into *E. coli* Top10. The positive clones were identified and were allowed to grow at 37°C in a rotary shaker at 120 rpm in 0.5 ml of LB broth with 50 mg/ml of Ampicillin. The clones were sequenced and blast analysis was carried out.

2.5. Culture-Dependent Method. 1 ml of the mixed liquor from the aeration stage was vortexed and diluted till 10^{-7}. The mixture (0.1 mL) was used to inoculate LB plates, which were incubated at 30 °C until observable growth occurred. Macroscopically unique colonies with different morphologies grew on the culture plates. These colonies

were sub-cultured to ensure their purity. Isolates were selected from the heterotrophic plating and sequenced accordingly.

2.6. Bioinformatics Analysis. The obtained sequences were aligned with CLUSTAL_W program [13]. Same segments of the 16S rRNA gene sequences were selected for phylogenetic analyses using default parameters in the MEGA 5 (Molecular Evolutionary Genetics Analysis) [14]. Phylogenetic distances were calculated by using neighbor-joining (NJ) method [15]. The bootstrapping supports for the trees were calculated from a sample of 1000 replicates [16].
Sequences with similarity of more than 97%, were considered as identical and used for further phylogenetic analysis as an operational taxonomic unit (OTU). A sequence randomly selected from each OTU was BLAST-searched to ascertain the taxonomic position with its related sequences deposited in the gene bank (NCBI).

The coverage was calculated according to the following equation:

$$C = [1-(nl/ N)] X 100\%$$

Where; nl = number of OTUs consisting of only one clone, N= number of all clones in the 16S rRNA gene library [17].

3. RESULTS AND DISCUSSION

In the current study, the sequences identifying with ά-, *β-, γ- §-proteobacteria* and *Verrucomicrobia* groups were obtained are shown in Table 2. The *β-and γ-proteobacteria* were in high abundance, valuing 24% and 45% of the total clones. *Alpha and Delta-proteobacteria* and *Verru microbia*, were 4%, 9%, and 2% respectively. A moderate amount of clones, about 9%, ranked with the uncultured bacterial strains with sequenced data in the NCBI. The 16S rRNA gene sequences BLAST searched for matching neighbors of the OTUs is given in Fig. 1).

The similarity of six culture independent clones to the known sequences in the GenBank was lower than 95%. It is assumed that similarity values to the known sequences below 95% may be regarded as evidence of the discovery of novel species [18]. Thus, the current study suggests that there is an ample evidence of possibility of novel bacteria in the SBR. The dominant genera recorded were *Hydrocarboniphaga*, *Acinetobacter* and *Ralstonia* are shown in Table 2.

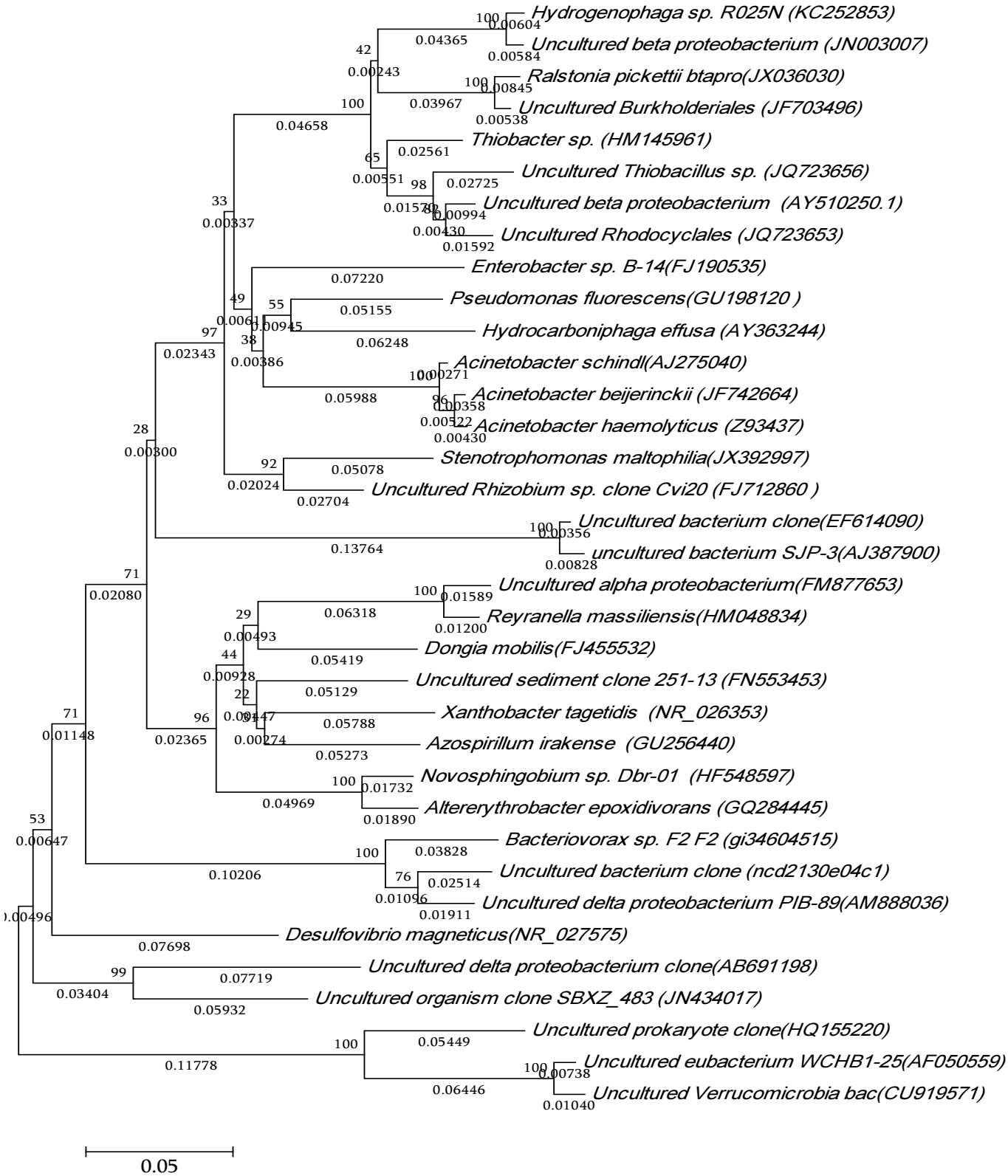
In the current study, phylogenetic analysis and molecular strategy based on 16S rRNA gene were used to explore the bacterial community. Distribution of the obtained sequences in the bacterial groups and the phylogenetic location are shown in (Figures 3-5, and Tables 2 & 3). The similarity values of the clone sequences with the data base sequences between 91 and 100 %.

Table 2. Proportion of various bacterial genera present in the dye wastewater of the sequencing batch reactor

Genus	%
Caulobacter	6
Azospirillum	3
Burkholderia	3
Thiobacillus	3
Hydrogenophaga	3
Ralstonia	13
Pseudomonas	6
Acinetobacter	22
Stenotrophomonas	3
Hydrocarboniphaga	25
Bacteriovorax	6
Desulfovibrio	3

3.1. Culture-Independent Method. Total 66 clones were obtained with 27-F and 1492-R primers, out of which 29 operational taxonomic units (OTUs) were recognized. The coverage was calculated to be 79 %. Phylogenetic studies using the clone sequences suggested that there was microbial diversity among the following main divisions; *Alpha- Beta- Gamma- and Delta- proteo bacteria* and *Verruco microbia.* Phylum Proteobacteria is divided into five major subphyla, which are represented by aerobic, anaerobic, photoautotrophic, photo-heterotrophic and chemo lithotrophic bacteria. In this study, representatives of all the subphyla were found except Epsilon proteo-bacteria.

3.2. Culture-Dependent Method. Twenty seven (27) isolates were selected from the SBR and their 16S rRNA genes were sequenced accordingly. The comparison of the similar sequences of the reference organisms on BLAST search was made. However, microbial diversity obtained by culture dependent method was poor as given in Table



3. There are some bacteria which are easily cultured in the laboratory. However, there exists persistently large number of bacteria which are unidentified and unculturable as well [19]. It is estimated that only 1-10% of all soil bacteria are considered to be cultured [20].

The culture dependent sequences identified only with the *Alpha & Gamma Proteobacteria*, and the *Actinobacteria*, which is strikingly different from the results with the culture-independent method where the *Beta*, *Delta* and *Gamma Proteobacteria* were in abundance shown in Fig. 2. There were 21% of isolates matched the *Rhodobacteriales,* and the same percentage identified with *Sphingomonadales* sequences present in the GenBank, while *Rhizobiales* accounted for 16% of the isolates are given in Table 3.

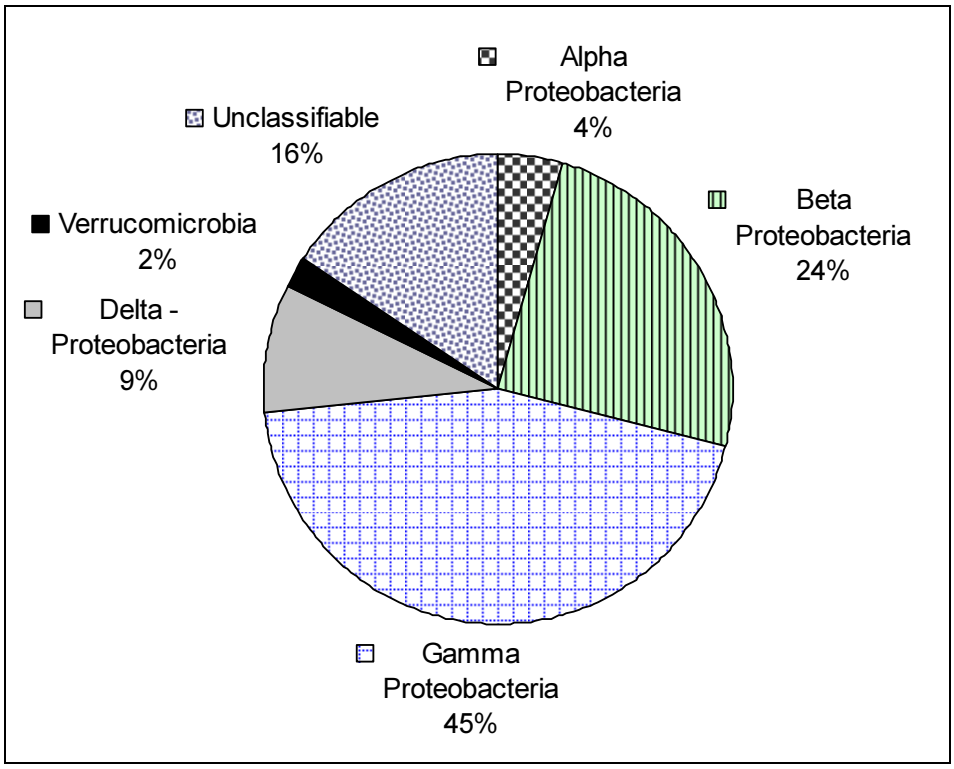


Fig. 2. Phylogenetic Distribution of Microbial Community (Culture Independent, 27Fand 1492R) in the SBR Involved in the Biotreatment of Textile Dye MG.

Alpha Proteobacteria was represented by 58 % of the isolates. *Gamma Proteobacteria* was represented by *Xanthomonadales* acouting for the 5% of the isolates. Similarly, *Actinobacteria*, matched as actinomycetales, was represented by 5% of the isolates. These groups are well represented in the polluted environments and members like *Rhodopseudomonas palustris* (*Alpha proteobacteria*) has been shown to be efficient COD remover in a columnar bioreactor immobilized on fiber [21]. Similarly, *Citrobacter freundii, Acinetobacter* sp., and *Klebsiella oxytoca* were isolated from the culture of activated sludge cultures on 4-nitroaniline by Khalid [12].

Some of the isolates identified with the well-known biodegraders, the notable being *Dokdonella koreensis, Rhodobactor, Sphingomonas* and *Paracoccus species.* In addition, an estrogen degrading strain DQ044639 was also detected by culture dependent method in the present bioreactor.

Table 3. BLAST Analysis of the gene sequences of 16S rRNA detected in the SBR by culture dependent method

Taxonomic Group	Closest Relatives of the Sequences BLAST-searched	Source in NCBI	Homology %
Alpha Proteobacteria			
	Sinorhizobium sp	AM084032	99
Rhizobiales	*X.tagetidis*	X99469	99
Rhodobacterales	*Rhodopseudomonas palustris*	AB017261	99
	Catellibacterium nectariphilum	AB101543	99
	Phyllobacteriaceae bacterium	AM403241	99
	Rhodobacter sphaeroides	D16424.1	100
	Haematobacter missouriensis	DQ342315	97
Sphingomonadales	*Sphingomonas* sp. DS4	EF494189	99
	Sphingomonas taejonensis	AF131297	99

Gammaproteobacteria			
Xanthomonadales	Dokdonella koreensis strain *NML 01-0233*	EF589679	100
Actinobacteria	*Microbacterium hydrocarbonoxydans*	AJ698726	98
Unclassified			
	Uncultured bacterium clone LR A2-35	DQ988316	100
	Uncultured bacterium clone SLB728	DQ787731	97
	Uncultured bacterium clone aab65g10	DQ814239	99
	Uncultured bacterium clone WBB38	EU184871	100
	Estrogen Degrading Bacterium	DQ066439	99

The microbial community phylogenetic affiliations show the abundance of *Beta* and *Gamma-Proteobacteria* in the malachite green treating bioreactor is shown in Fig. 3. This finding is similar to that of Zilouei [22], who found these groups to be dominating the microbial community used for phenol removal process. Similarly, microbial strains capable of reducing the "azo dyes" including *Pseudomonas luteola, Sphingomonas spp., Klebsiella pneumoniae, Penicillium spp., Streptococcus faecalis, Aspergillus spp.* have been described [23].

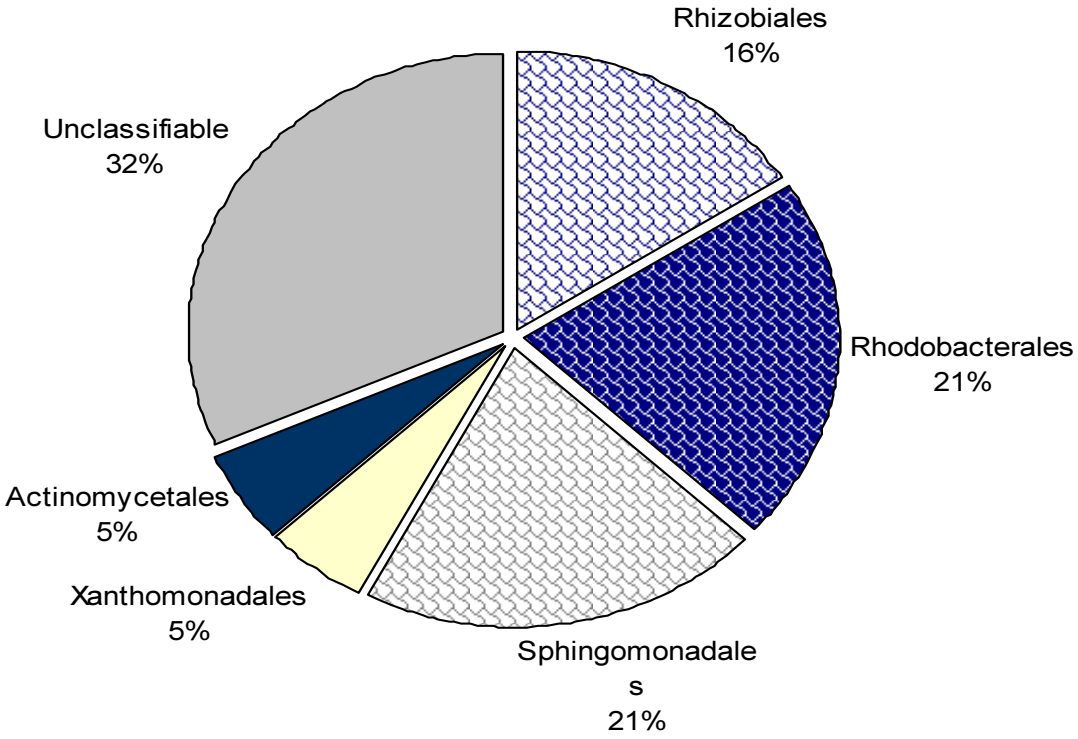


Fig. 3. Phylogenetic Distribution Percentage, As Illustrated By the Culture Dependent Method.

4. Conclusion. The microbial community analysis showed a relative dominance of pollutants- degrading functional groups of bacteria. Majority of the isolates matched with the β- and γ-proteobacteria. The detected diversity by culture-independent method was fairly better than that of culture dependent method. The dominant genera detected in the present study were *Sphingomonas, Microbacterium, Hydrocarboniphaga*, *Acinetobacter* and *Ralstonia.* The present study is a great deal of contribution to the knowledge about the microbes in the polluted environments.

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