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Biochemical characterization and 16s rRNA sequencing of different bacteria from textile dye effluents

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ABSTRACT

Environmental pollution has been identified as a major problem in the modern world. Dyeing effluents have become a vital source of water pollution. Release of coloured textile effluents is undesirable in the aquatic environment as they reduce light penetration, thereby affecting aquatic life and limits utilization of the water media. In Tirupur, the textile factories discharge millions of litres of untreated effluents into the drains that eventually empty into river, Noyyal. The release of coloured compound into water bodies is undesirable not only because of their impact on photosynthesis of aquatic plants but also due to the carcinogenic nature of these dyes and their breakdown products. The ability of bacterial strains isolated from the dye effluent of textile mill sites. Morphological and biochemical characterization was done to identify isolates and was found to be *Pseudomonas spp, Bacillus spp* and *Serattia spp*. The isolated strains were finally identified by 16S rRNA sequence analysis. Bacteria are generally identified by 16S rRNA sequencing. The rRNA is the most conserved (least variable) gene in all cells. They were identified as *Pseudomonas aeruginosa*, *Bacillus amyloliquefaciens* and *Serattia liquefaciens*. The sequences were deposited in GENBANK. The accession numbers were KU041528, KU041530 and KU041531 respectively. The identification was confirmed by 16S rRNA sequencing.

Keywords: Textile Dye Effluents, Bacteria, 16S rRNA, NCBI.

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INTRODUCTION

Water is life but now a-days due to the advancement in industrialization, it is spoiling a lot. Many contaminants present in wastewater, such as acids, bases, toxic organic and inorganic dissolved solids, and colors. Among them, colors are considered the most undesirable and are mainly caused by dyes [1]. At present scenario, environmental pollution is a major socio-economic and also a health problem. Among the various types of environment pollution, water pollution is a major concern. Colour is one of the most obvious indicators of water pollution. The discharge of highly colored synthetic dye effluents can be damaging to the receiving water bodies [2]. Noyyal river basin at the downstream of Tirupur Town is severely polluted due to discharge of partially treated effluent by the textile bleaching and dyeing units. These bleaching and dyeing units are located on either side of Noyyal River within 2 Km from the river. By average one unit generates 30 kilo litres per day (KLD) of trade effluent. The effluent reaching the river is in dark brown in colour. During summer period there is no water flow in river, only effluent flow can be noticed. In monsoon period the colour in river water can be noticed. Azo dyes, characterized by nitrogen to nitrogen double bonds (–N=N–), account for up to 70% of all textile dyestuffs produced and are the most common chromophore in reactive dyes [3]. The reactive azo dyes-containing effluents from these industries have caused serious environment pollution because the presence of dyes in water is highly visible and affects their transparency and aesthetics even if the concentration of the dyes is low. Most of these dyes are toxic and potentially carcinogenic and their removal from industrial effluents is a major environmental problem [4-6].

Microbes undergo high rates of dispersal and intermixing in nature but are constrained in their ability to colonize new habitats by environmental filtering and local competition [7]. Microbial diversity constitutes the most extraordinary reservoir of life in the biosphere that we have only just begun to explore and understand. Over the millennia, microbes have adapted to extremely diverse

environments, and developed an extensive range of new metabolic pathways or library of catabolic enzymes (Butler and Mason 1997; Ellis, 2000) [8-9]. Colony morphology may be an indicator of phenotypic variation, this being an important adaptive process adopted by bacteria to overcome environmental stressors. Growing on agar surfaces, microorganisms form colonies whose appearance helps the clinicians and researchers to identify genera or even species. One of the most intriguing aspects of this approach is the observation of similar colony patterns in different systems and the existence of distinct patterns when culturing a sole strain in analogous conditions. The large number of reckonable patterns turns the identification of colony morphologies a real challenge for microbiologists, clinicians and technicians. Preliminary characterization of the bacterial isolate regarding its morphological, biochemical characters and decolourisation activity as well as the molecular identity gives useful information with regard to the further application of strain for various purposes [10].

Bacterial colonies display distinct properties such as size, color, shape, and texture, which fundamentally vary among different species. These features were the basic means for identifying, classifying, and characterizing bacteria in the early days of microbiology (Kaufmann and Schaible, 2005) [11] and are presently still exploited for clinical and research applications. Formation of bacterial colonies is a constructive survival strategy, enabling bacteria to utilize a greater variety of nutrients, endure rapid environmental changes, and resist antibiotic threats (Christensen *et al.,* 2002; Lewis, 2001; Moons *et al.,* 2009) [12-14]. The characteristics used to identify *Staphylococcus aureus* include Gram stain morphology, cell morphology, production of catalase, coagulase production, pigment production, susceptibility to lysostaphin and lysozyme, and anaerobic production of acid from glucose (Kloos and Schliefer 1999) [15]. There are several other commercially available methods that allow strains to be biochemically characterized. Therefore, and despite being described by several authors as old fashioned (Braga *et al.,* 2013; Weile and Knabbe, 2009) [16-17], colony morphology characterization can provide valuable insights into individual microbial diversity, both derived from genetic changes or reversible changes (Sousa *et al.,* 2011) [18].

The conventional biochemical tests used for identification of bacteria are time consuming, laborious and are not always conclusive. Modern pharmaceutical production and economic pressures can no longer accommodate this delay (Newby, 2000) [19]. In the past decade, nucleic acid sequencing methods have undergone tremendous advances (such as whole-genome sequencing as well as the determination of 16S rRNA, 16S-23S rRNA, spacer and 23S rRNA sequences), which minimize the time needed for identification of microorganisms. The ribosomal operons mainly 16S rRNA has proven to be a stable and specific molecular marker for the identification of bacteria. 16S rRNA sequencing analysis is widely used, (Bansal and Meyer, 2002) [20] and more useful in phylogenetic analysis compared to 16S-23S rRNA sequencing (Song *et al.,* 2004) [21] and also due to its rapidity, reliability, simplicity and reproducibility (Lane *et al.,* 1985; Patel 2001; Easter 2003) [22-24].

The 16S rRNA gene consists of highly conserved nucleotide sequences, interspersed with variable regions that are genus- or species-specific. PCR primers targeting the conserved regions of rRNA amplify variable sequences of the rRNA gene (Relman, 1999) [25]. Bacteria can be identified by nucleotide sequence analysis of the PCR product followed by comparison of this sequence with known sequences stored in a database (Clarridge, 2004) [26]. Sequences from tens of thousands of clinical and environmental isolates are available over the internet through the NCBI (National Center for Biotechnology Information) (www.ncbi.nlm.nih.gov). These sites also provide search algorithms to compare new sequences to their database. Thus the present study is focussed on the isolation, biochemical characterization and molecular identification of bacteria from the textile effluents through 16S rRNA based molecular technique.

MATERIALS AND METHODS

Study Area

Tirupur town is situated between 11°10"N to 11°22"N latitude and 77°21"E to 77°50"E longitude. It is located on the bank of Noyyal River, a tributary of river Cauvery. Orathupalayam dam was constructed on the Noyyal River which was irrigate an area of 500 acres in Erode district and 9875 acres in Karur district. The river Noyyal is a seasonal river and it originates from Vellingiri hills in the Western Ghats of Coimbatore district. It flows through Coimbatore, Erode and Karur districts and finally joins into Cauvery River near Noyyal village. It flows over a distance of 175 kilometers. The catchment area of the river is 3.49 lakh hectares.

Sample collection

Effluent sample was collected from the textile Dye effluent located in the Tirupur region, Tamil nadu (Figure 1). Bacteria were isolated from Tirupur textile effluent and cultured in agar media at 37°C. Nutrient broth cultures were maintained at -20°C in glycerol till further use.

Figure 1: Sample collection Area

Isolation of pure cultures

1 ml of diary effluent was added to 10 ml of sterile distilled water. Mix vigorously and take 1 ml from this tube, add to another tube containing 9 ml of sterile water to get the dilution of 10^{-1}. From this tube 1ml was taken and added to another tube containing 9 ml of sterile water to get the dilution of 10^{-2}. This serial dilution procedure was repeated up to 10^{-9} dilutions. From each dilution 0.1 ml was placed on nutrient agar medium by spread plate method and the plates were incubated at 37°C for 24h to allow microbial growth. Colonies with different morphology were selected, pure cultures were developed on nutrient agar plates and stored at 4°C.

Morphological & Biochemical Characterization

The isolated strains were identified by morphological and biochemical characterization according to Bergey's Manual of Determinative Bacteriology (Krieg and Holt 1984) [27]. Morphological features were identified by growing the isolated cultures on EPS medium and Gram staining was performed for each isolate. Different Biochemical tests were carried out includes Indole, Voges Proskauer, Citrate utilization tests, catalase test, starch hydrolysis, H_2S production test, Urease test and Carbohydrate fermentation of various sugars Bergey's manual (1984) [28].

DNA isolation

Five ml of overnight culture were washed and suspended in TE buffer, PH 8.0. Genomic DNA was isolated as per the method of Schmalenberger [29].

PCR conditions

All the reaction mixtures contained 1X PCR buffer (10 mM Tris HCl, pH-9.0, 50 mM KCl and 0.01% gelatin); 100 mM concentration of each dNTPs and 0.75 unit of Taq polymerase. The final concentration of $MgCl_2$ was adjusted to 1.5 mM in PCR-Ribotyping. In PCR-ribotyping $MgCl_2$ concentration was adjusted to 3 mM. Each primer concentration was 20 pmol for PCR-Ribotyping. PCR-ribotyping was carried out according to the method of Franciosa. The primers for the amplification of DNA spacer regions between the 16S-5S genes were F (5' -TTG TAC ACA CCG CCC GTC A-3') and R (5' -GCT TAA CTT CCG TGT TCG GTA TGG G-3'). The amplification was carried out by after initial denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 1 min, 35°C for 1 min and 72°C for 2.5 min, with a ram time of 2 min between 35 and 72°C; a final extension was performed at 72°C for 5 min.

Agarose gel electrophoresis

The PCR products (10 ml) were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide (0.5 mg/ml) at 100 V for 1 h. in 1X TBE (Tris-Boric acid-EDTA) buffer. The gel images were digitized through UV gel image acquisition camera (Kodak, Japan).

16S r RNA sequencing analysis

The PCR amplified DNA was sequenced in ABI 3730 DNA Analyzer (Applied Biosystems). The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment required editing of the obtained sequences were carried out using Geneious Pro v5.

Phylogenetic analysis

The 16s rDNA sequences of *B.a* isolates aligned with the sequences of similar species which were retrieved from the Gene bank database. The sequences were converted in to FASTA format. The retrieved sequences were fed with ClustalW for multiple sequence alignment to observe sequence homology. Evolutionary tree was inferred by using the neighbour-joining method [30]. Dendrogram was constructed by neighbour-joining method using PHYLIP software package.

RESULTS AND DISCUSSION

Table 1: Morphological characterization of Bacteria

S.No	Morphological test	*P.aeruginosa*	*B.amyloliquefaciens*	*S. liquefaciens*
1	Shape	Rods	Rods	Rods
2	Gram staining	Gram negative	Gram Positive	Gram negative
3	Motility	+	+	+
4	Endospore Formation	-	+	+

Table 2: Biochemical characterization of Bacteria

S.No	Biochemical test	*P.aeruginosa*	*B.amyloliquefaciens*	*S. liquefaciens*
1	Indole Test	-	+	-
2	Citrate Test	+	+	+
3	VP Test	-	-	+
4	Oxidase	+	+	-
5	Nitrate reductase	-	-	+
6	Starch	-	-	+
7	Casein	-	+	+
8	Gelatin	-	+	+
9	Urea	-	-	-
10	MR	+	+	-
11	Gas production	-	-	-

+-Positive, – Negative.

Morphological characterization

In the present investigation morphological features were observed for the isolates grown on Nutrient agar medium (Figure 2). All bacterial isolates were rod shaped in nature with the help of compound microscope. In bacterial isolates two strains was Gram negative which accepted the gram stains (Table 1). Remain one strain was gram negative which cannot accept the gram strains. All the bacterial strains have motility characterization and endospore formation.

Biochemical characterization

In the present investigation *P. aeruginosa* showed positive for Citrate utilization test, Oxidase test and negative for Indole test, VP test, nitrate reductase test, starch test, casein test, gelatin test, urea test, MR test and gas production. *B. amyloliquefaciens* showed positive for Indole

test, citrate test, oxidase test, casein test, gelatin test and MR test. *B. amyloliquefaciens* showed negative for VP test, nitrate reductase test, starch test, urea test and gas production. *S. liquefaciens* showed positive for citrate test, VP test, nitrate reductase test, starch test, casein test and gelatin test. *S. liquefaciens* showed negative for indole test, oxidase test, urea test, MR test and gas production (Table 2).

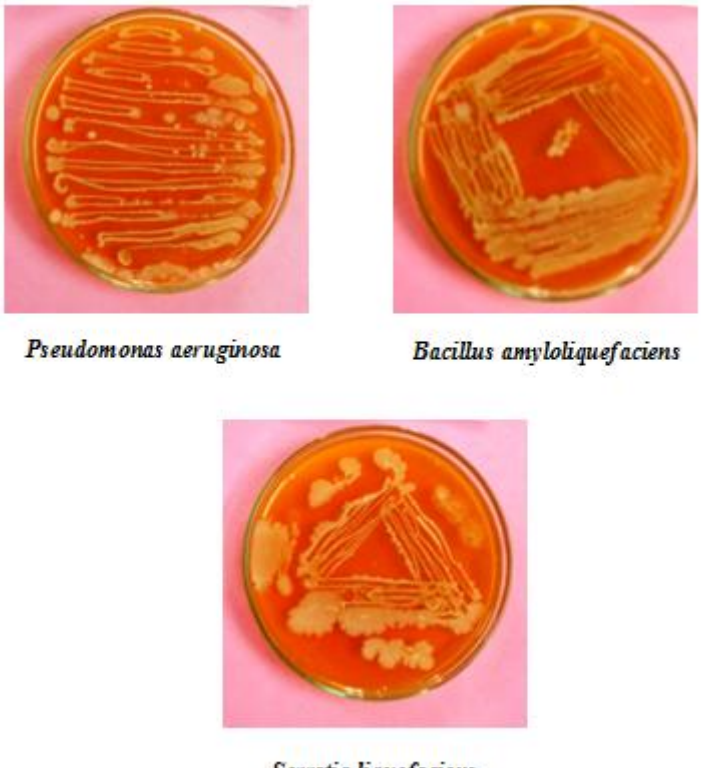

Figure 2: Bacteria culture on Petri disc

16S rRNA sequencing

The traditional identification of bacteria on the basis of phenotypic characteristics is generally not as accurate as identification based on genotypic methods. After the determination of colony colour, the morphological, physiological biochemical and enzymatic characteristics, the bacterial isolate selected by the PCR analysis was approximately identified genus, species and it was confirmed by the 16S rRNA sequencing with help of universal bacterial primers, 16s-UP-F and 16s-UP-R. The universal primers were used for the amplification of 16S rRNA ribosomal smaller subunit as this plays an important role in the protein synthesis.

In the present study nucleotides of first bacterial colony isolates were which was submitted to the Gene bank of NCBI. 1430 nucleotides were compared with bacterial genome for molecular identification. Bacterial sequences 100% resemble with *P. aeruginosa* and received an accession number KU041528. Further, Phylogenetic tree was constructed from species of *P. aeruginosa* using Neighbour-joining method. Construction of phlogenetic tree was used for close resemblance isolated bacteria to other. Damodaran and Jayaseelan (2015) [31] isolated the bacteria from textile effluent. The isolate selected by the PCR analysis was tentatively identified as *P. aeruginosa* and it was confirmed by the 16S r DNA sequencing with help of universal bacterial primers, 16s-UP-F and 16s-UP-R. Sequences of the *P.aeruginosa* isolate showed partial 16S r DNA sequences, consisting of 1258 nucleotides which were submitted to the Gene bank and an Accession Number (KT175510) was obtained. Mohammed EL Amine Bendaha (2015) [32] isolated these strains from soil polluted by hydrocarbons. The 16S rRNA sequence (810 bp) of strain S7PS5 has been deposited in the GenBank data library and has been assigned the accession number KR349493 and allowed the link S7PS5 to the genus *Pseudomonas*. Alignment of this sequence by the blast showed 98.84% of similarity with *Pseudomonas aeruginosa* LMG 1242T/Z76651 as the closest species.

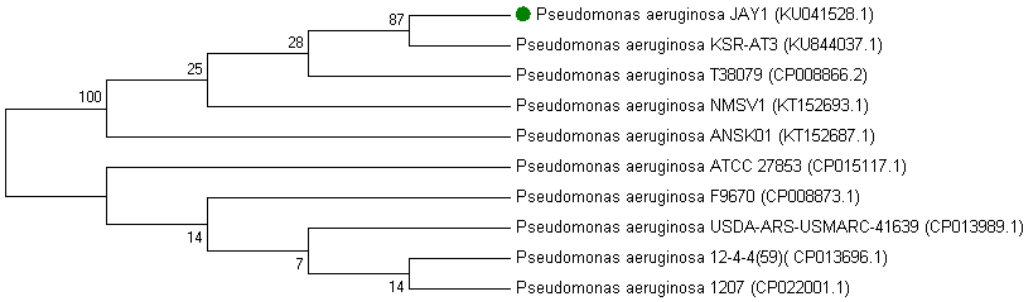

Figure 3: Phylogentic tree of *Pseudomonas aeruginosa*

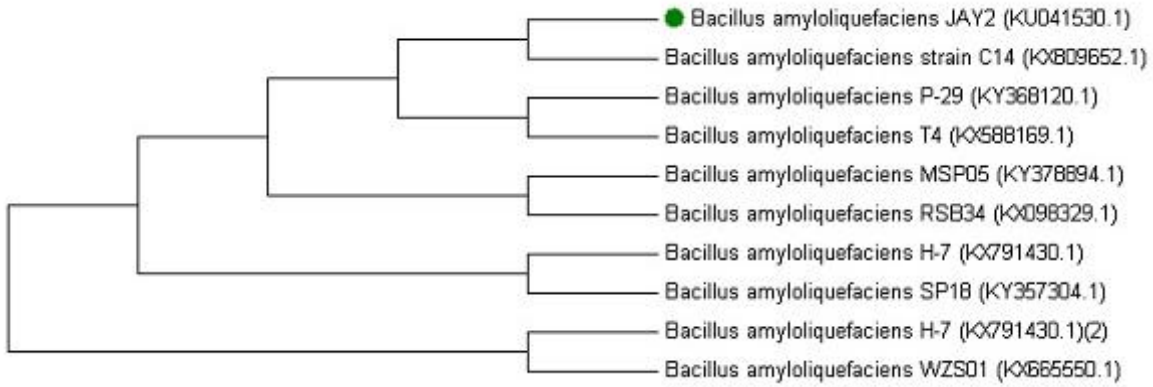

Figure 4: Phylogentic tree of *Bacillus amyloliquefaciens*

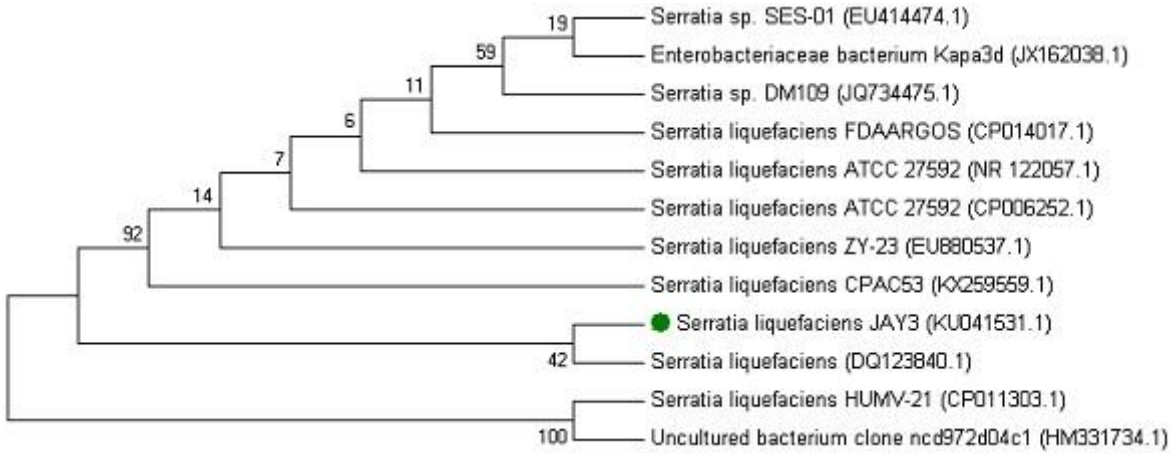

Figure 5: Phylogentic tree of *Serratia liquefaciens*

In the present investigation basepairs of second bacterial have 1320 nucleotides were compare with already well known all bacterial genomes. In these sequences showed 100% similarity with the already established the species *B. amyloliquefaciens* and accepted an accession number KU041530. Further, Phylogenetic tree was constructed from species of *B. amyloliquefaciens* using Neighbour-joining method. Phlogenetic tree analysis was used for similarities between isolated bacteria to other bacteria. Similar finding was revealed by Singh *et al.,* (2013) [33] for *B. amyloliquefaciens* isolate SS35 and Kadaikunnan *et al.,* (2015) [34] for *B. amyloliquefaciens* VJ-1. Although 16S rRNA gene is a widely practiced technique for this purpose, it has limitations regarding the very closely related taxa [35].

In the present study sequences of the third isolates isolate showed partial 16S rRNA sequences, consisting of 1060 nucleotides which were submitted to the Gene bank of NCBI (National Center for Biotechnology Information, USA). Nucleotides were analyzed with already known bacterial genetic code. Genes were mostly similar to that of *S. liquefaciens* and was obtained an accession Number (KU041531). Further, Phylogenetic tree was constructed from species of *S. liquefaciens* using Neighbour-joining method. Phlogenetic tree was carried out for relationship from isolated bacteria to various bacteria. Liu *et al.,* (2010) [36] reported the 16S rRNA sequence similarity between strain TS26-8 and the 9 described *Serratia* species ranged between 99.82% and 95.63%, with the highest similarity to *S. grimesii* (99.82%) and *S. proteamaculans* (99.18%) and the lowest to *S. rubidaea* (95.79%) and *S. marcescens* (95.63%). In the present investigation we analyzed the usefulness of 16S rRNA sequencing for the identification of selected bacterial isolates.

CONCLUSION

The textile industry plays an important role in the world economy as well as in our daily life, but at the same time, it consumes large quantities of water and generates large amounts of waste water. Textile dyeing industries is one of the fastest growing fields and a major export oriented industrial sector, especially in Tirupur. The industrial effluents like textile effluents, tannery effluent, sugar mill effluents and paper mill effluents are enriched media to grow and spread microbial population. The isolates were identified by morphological and biochemical tests in preliminary stage. But identification of bacteria using morphological and biochemical tests were very complicated. The time for microbial identification was reduced by molecular-based PCR techniques when compared to conventional methods of detection. Sequence analysis of highly conserved regions of the bacterial genome, such as the small subunit rRNA gene; now provide us with a universal method of estimating the evolutionary relationships among all organisms. First isolate was *P. aeruginosa* and accession number was KU041528. Second isolate was *B. amyloliquefaciens* and accession number KU041530. Third isolate was *S. liquefaciens* and accession number KU041531. Significant water pollution is due to the discharge of textile dyeing effluent directly to the environment or drainage system is also major concern by the people. With this aim this study aimed to isolate bacterial strains from the textile dyeing effluent collected from Tirupur town with special reference to textile dye degradation through bioremediation. In future, this 3 bacterial isolates will be used for the Decolouration and degradation process of Textile Dye effluents. Further the

isolated and identified bacterial consortium will use for bioremediation of textile effluent.

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