Identification of Textile Effluent Decolourizer by Using 16S rDNA Sequencing

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***Abstract*—**Chemically treated textile waste-water released by manufacturers pose a detrimental effect to the environment. This study is to identify microorganism that can degrade dyes present in the waste-water as this technique is more eco-friendly. The dye decolourizer were isolated and identified using 16s RDNA sequencing methods and analyzed using Dot Plot programme and sequences obtained were compared with data from GeneBank in BLAST programme. The bacteria isolated have 99% similarities to *Bacillus sp., Bacillus cereus* and *Bacillus thuringiensis.*

***Keywords*—**Decolourization, azo dyes, 16rDNA sequence, *Bacillus*, textile waste-water.

I. Introduction

TEXTILE waste-water discharged into the aqueous ecosystem cause aesthetic problems, poor light penetration and oxygen transfer into water bodies and impart mutagenic activity to the contaminated water or soil [1]. Manufacturers have opted for chemical treatment because it's fast and easy. This action can cause secondary disposal problems. Some 10,000 dyes are currently manufactured; many of these are azo dyes due to their –N=N– bond structure and it is estimated that at least 15% of them are released into the environment [2].

The use of reactive dye is important for optimum binding with textile. Reactive dyes are coloured compounds that contain one or two functional groups capable of forming covalent bonds with the active sites in fibres. A carbon or phosphorous atom of the dye molecule will bind to the hydroxyl groups in cellulose, amino, thiol, and hydroxyl groups in wool, or amino groups in polyamides [3]. Between 20-50 percent of the reactive dye used by the textile industry is lost in exhaust and wash water [4].

The use of anaerobic digestion to treat dye wastewater is a cost-effective alternative to the physical and chemical treatment. Anaerobic reduction of azo dyes occured with the presences oxygen sensitive azo reductase such as those that secreted by *Clostridum* and *Eubacterium.* Dye decolorization under these conditions requires an organic carbon as energy source. Simple substrates like glucose, starch, acetate, ethanol and more complex ones, such as whey and tapioca, have been used for dye decolorization under methanogenic conditions [5], [6] and [7].

The aim of this study is to identify and characterize the type of microorganisms that can degrade azo dyes isolated from textile waste-water.

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II. Materials and Methods

A. Dyes

Sufimix Supra Blue (SSB), an anionic dye; Sufimix Supra Red (SSR) and Sufimix Supra Yellow (SSY) both were used in this study.

B. Sample

Textile waste-water was obtained from a textile industry in Senawang, Negeri Sembilan, Malaysia.

C. Isolation of dye decolourizer

Samples of wastewater taken from textile treatment plant were used as source to isolate potential dye decolourizing bacteria. Serial dilution via spread plate method was applied to obtain single culture [8]. Plates were grown aerobically and anaerobically at 37 °C. The inoculum was used to screen for azo dyes decolourizer.

D. Screening for dye decolourizer

Screenings of bacterial cultures for decolourization of azo dyes were conducted using liquid media. CDM and minimal media were used. Each medium was added with either one of three reactive azo dyes (SSR, SSY or SSB) at the concentration ranging from 0.01 g/L to 0.1 g/L (w/v). Assessment of dye effluent was assessed by measuring COD and colour removal. The culture was tolerated under lower concentration of azo dye before degree by degree increased until maximum level of 0.1 g/L (w/v) in periodic of time. Experiments were run under aerobic and facultative anaerobic in non-shaking incubation condition at 37 °C. Samples were taken every 24 h to analyze for colour and COD. The best decolorizing strains were further studied for identification.

E. Determination of 16sRDNA sequence

16S rDNA identification is considered as the most reliable tools for identification of microorganism species. The procedures involve several steps including growing the pure culture for overnight. Genomic DNA extraction was done using DNA purification kit (Promega). PCR amplification of 16S rDNA was done by using two universal primers (16S Forward primer 5' AGA GTT TGA TCC TGG CTC AG 3'

and 16S Reverse primer 5' AAG GAG GTG ATC CAG CC 3'). The PCR amplifications of total 30 cycles were done using an denaturation step of 1 min at 94^{0}C, followed by annealing step of 1 min at 58.6^{0}C, extension step at 72^{0}C for 2 min and final extension for 8 min at 50^{0}C and was stored at 4 ^{0}C in a thermocycler (Perkin Elmer GeneAmp PCR System 9700).

Sequencing of 16S rDNA identification was achieved by analyzing the 16S rDNA sequences obtained from both primers by using Dot Plot programme in order to obtain full length sequences of bacteria A, B and C. It was carried out by analyzing the pairwise sequence alignment of forward and reverse partial 16S rDNA. Identification was achieved by comparing the contiguous 16S rRNA sequences obtained with the 16S rRNA sequence data from the reference and type strains available in public databases GenBank using the BLAST. The phylogenetic reconstruction was done using ClustalX version 1.83 [9],[10]. A Treeview programme (version 1.6.6) was used to visualize the phylogenetic tree.

II. RESULTS AND DISCUSSIONS

A. Isolation and screening of dye decolourizing bacteria

Large During initial screening, it was found that only four of the colonies (coded as A, B, C and Y) could reduce colour after 48 h incubation under facultative anaerobic condition at 37 °C. It is known that the azo bond can be reduced by bacteria, facultative anaerobic as well as obligate anaerobes [11], [12] Knapp and Newby, 1994).

B. Decolourization of azo dyes in different media under facultative anaerobic condition

Figure 1 shows the decolourization rate of different media containing effluent applying bacteria cultures after 48 h. Only culture B was able to decolourize azo dyes in all experiment. Decolourization rate of culture B in raw, sterile and non sterile media were 8, 15 and 4 ADMI h-1, respectively. Culture C showed no removal of colour in sterile media and minimal media effluent respectively, but was able to decolourize non sterile media 5 ADMI h-1. Non-sterile media were found to be decolourized by all cultures, with the highest removal were culture A and Y, both 8 ADMI h-1. This probably has to do with the role of indigenous microbe that complemented augmented microbes to achieve unspecific reduction of azo dyes. Secondly, cleavage of variety residues of azo dyes might possibly involved some of co-metabolites being produced within the microbial community [13].

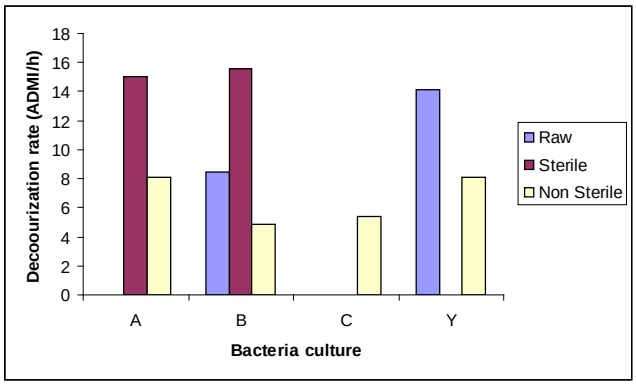

Fig. 1 The decolourization rate of different media containing effluent applying bacteria cultures after 48 h

Figure 2 shows the decolourization rate of CDM media containing pure azo dyes by bacteria cultures after 48 h. All cultures were able to decolourize all media, but at different rate. Cultures A showed highest removal of media containing blue dye (SSB) with removal rate of 12 ADMI h-1, followed by Y, B and C. In media containing red dye (SSR), highest removal was achieved by culture Y with decolourization rate, 52 ADMI h-1. While for yellow dye (SSY), both culture A and Y shares highest decolourization rate that was 37 ADMI h-1. Culture C exhibited lowest decolourization rate for all dye amongst the culture. This was followed by culture B.

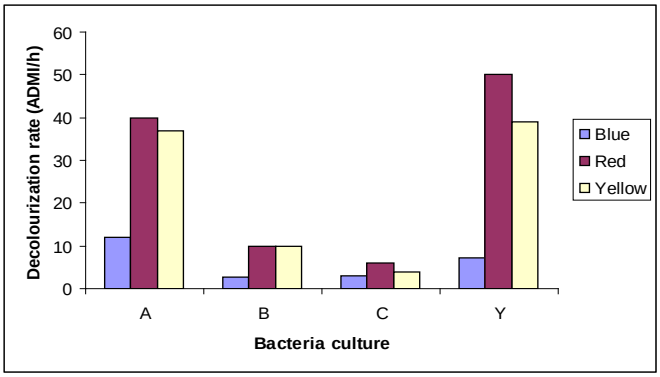

Fig. 2 The decolourization rate of CDM media containing pure azo dyes applying bacteria cultures after 48 h

Similar experiment was repeated using minimal media. The result was shown in Figure 3. Bacterial culture B had reduced colour of SSB, SSR and SSY at the highest rate that were 30, 72 and 32 ADMI h^{-1} respectively. Lower decolourization rate of similar dyes by culture of Y was observed that were 27, 10 and 32 ADMI h^{-1}, respectively. On the other hand, culture A and culture C both exhibited lowest removal for yellow and blue dye media respectively.

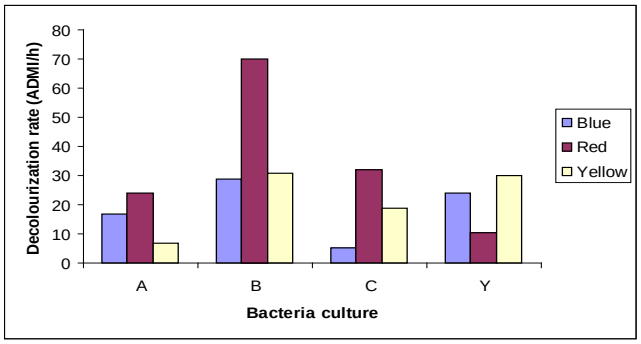

Fig.3 The decolourization rate of minimal media containing pure azo dyes applying bacteria cultures after 48 h

C. Identification of dye decolourizing bacteria

Genomic DNA extraction

Identification of the selected isolates was conducted using 16S rDNA gene sequence analysis technique. Genomic DNA extracted from bacteria A, B, C and Y were electrophoresed on 1 % (w/v) agarose gel at 100V for 1 h. Result indicated that the genomic DNA (1 kb) was successfully extracted from bacteria A , B and C.

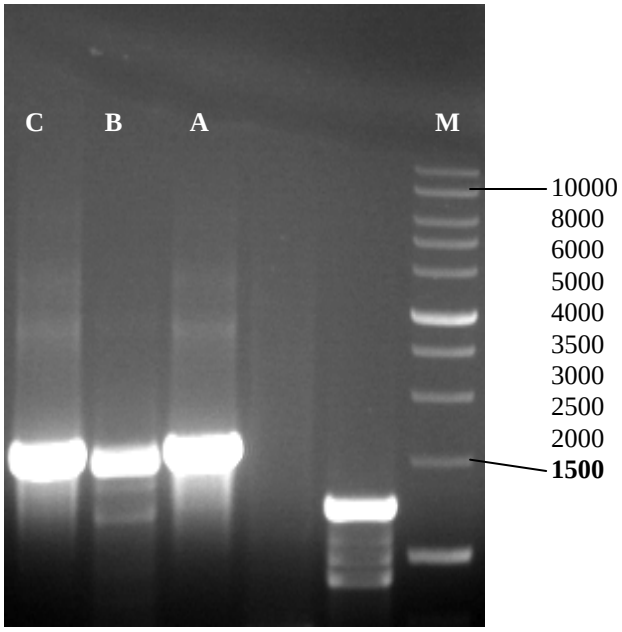

Fig. 4 Agarose gel electrophoresis analysis of the 16S rDNA gene fragment after purification amplified using universal primers. Lane M: Gene Ruler Ladder Mix Marker. Lane A: The amplified 16S rDNA from bacterium A after purification. Lane B: The amplified 16S rDNA from bacterium B after purification. Lane C: The amplified 16S rDNA from bacterium C after purification

Referring to Figure 4 no other bands were observed on the agarose gel indicated to a suitable condition employed during PCR thus avoiding non specific reaction from occurring. The 1.5 kb size fragment obtained was the exact target size of the 16S rDNA gene required from the PCR. From visual observation of the gel, amplified gene of 16S rDNA from lane A, lane B and lane C should high and intact yield. Purification of the PCR product would enhance its concentration and quality respectively. Another factor that ensured the successful of PCR is the quality of DNA template (genomic DNA) obtained from each bacterial isolates. A highly concentrated and intact DNA template resulted to a highly specific reaction of Taq Polymerase and primers to anneal the 16 S rRNA fragment.

The Polymerase Chain Reaction has successfully amplified the 16S rDNA gene from all cultures tested. Universal primers were used to amplify the 1.5 kb gene fragment encoding the 16S rDNA. Results from the agarose gel electrophoresis showed that the PCR products (16S rDNA gene fragment) obtained were intact and in good purity. Concentration of PCR product was shown in Table I.

TABLE I
CONCENTRATION OF PCR PRODUCT

Sample	Absorbance at 260 nm	DNA concentration (µg/µl)
A	0.0026	0.130
B	0.0061	0.305
C	0.0042	0.210

DNA concentration: WL (260 nm) x 50 ng/µl x dilution Factor (1000x)

16S rDNA analysis

The amplified 16S rDNA gene was sequenced. Each sequencing reaction produces about 1.5 kb nucleotides representing nearly full length sequence of 16S rDNA gene. These sequences were used as unknown for homology search using Basic Local Alignment Search Tool (BLASTn). The result after BLASTn showed bacteria A have 16S rDNA gene sequences closely related to *Bacillus cereus* (99 %) while B, 99 % similarities to *Bacillus thuringiensis* and C 99 % to *Bacillus sp.*

Bacillus sp., *Bacillus cereus* and *Bacillus thuringiensis*

Bacterial strains A, B and C which belong to *Bacillus sp.* with at least 99 % homology similarities. *Bacillus sp*. in particular has shown potential in degrading azo dyes over the years. The earliest evidence was from Wuhrhmann in 1980, when he reported the isolation of *Bacillus cereus* capable of degrading azo dyes successfully. A dye-decolorizing alkali-thermophilic microorganisms namely as *Bacillus sp.* strain SF from wastewater drain of a textile company was successfully isolated [14]. This bacteria was found to be NADH-dependent azoreductase type after studies by using synthesized model substrates based on di-sodium-(*R*)-benzyl-azo-2,7-dihydroxy-3,6-disulfonyl-naphthaline. Dyes with NO_2 substituents, especially in the *ortho* position, were degraded fastest, while analogues with a methyl substitution showed the lowest degradation rates. *Bacillus cereus* group have distinctive

relationships with eukaryotes, some harmful and some beneficial to humans. While *B. thuringiensis* falls typically on the beneficial side of the ledger because of its potent insecticidal activity. *B. cereus* is usually placed in the harmful category. *B. cereus* species is described in literature as "colorful and cosmopolitan lifestyle." *B. cereus* known to cause food poisoning and is an abundant inhabitant of the soil and colonizes plant roots.

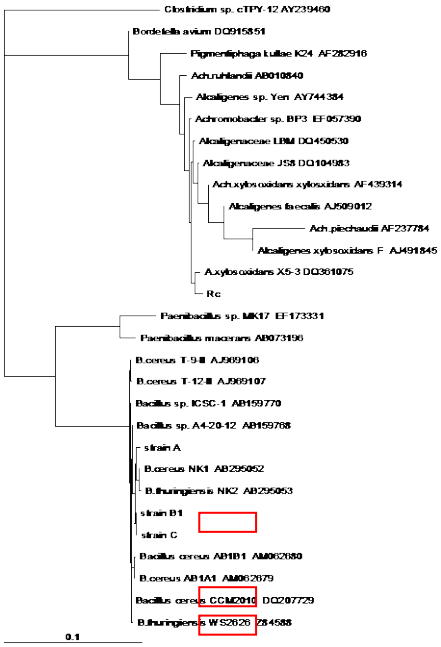

Fig. 5 The phylogram illustrating the relationship between bacterial strains A, B1 and C to other known azo-dye degrading bacteria. The score bar, 0.1, represents 10 nucleotide substitutions per 100 nucleotides. The tree was rooted using complete 16S rRNA sequence of *Clostridium* sp. cTPY-12 (AY239460) as an outgroup.

III. Conclusion

Four pure colonies (A, B, C and Y) consist of single cultures were shown to decolourize the azo dyes in different types media under facultative anaerobic condition at 37 °C. 16S rDNA identification revealed that all strain A, B and C shares 99 % similarities to *Bacillus cereus, Bacillus thuringiensis and Bacillus sp.* The biological techniques of treating textile waste-water needs to be implemented by textile manufacturers in Malaysia since they are eco-friendly and economical. Bacteria such as *Bacillus cereus, Bacillus thuringiensis and Bacillus sp.* are good source for biological treatment of textile waste-water.

Acknowledgment

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