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Decolourisation of textile-dye-containing effluents using biofilm:A case study

Maihafizah Mohd Zahari*, Zaharah Ibrahim*, Fauziah Othman, Madihah Md Salleh*,Nooraini Abdul Rashid*, Adibah Yahya*, Aishah Husin* and Chan Giek Far***

*Department of Biology, Faculty of Science, Universiti Teknologi Malaysia,81300 Skudai, Johor Darul Takzim, Malaysia

**Microscopy and Microanalysis Unit, Laboratory of Enzyme Technology and Microbial Institute of Bioscience, Universiti Putra Malaysia, Serdang, Selangor Darul Ehsan,Malaysia

Abstract

Bioremediation based on microbial technologies has been extensively used for treating coloured textile wastewater. In this research, the potential application of the indigenous and exogenous bacterial cultures, found as biofilm were investigated for colour removal. Initial study in the treatment of the textile wastewater showed that the mechanism involved in decolourisation was degradation, which was carried out *via* molecular technique involving amplification of the DNA sequence responsible for decolourisation using pure dye; Orange II. In laboratory scale experiment, the bacteria were grown as mixed culture in suspension and biofilm using shake flasks technique. Their abilities to decolourise textile wastewater were studied under semianaerobic conditions. Generally, bacteria in the form of biofilms were found to remove colour at faster rates compared to that of suspended cells. Evidence of biofilm formation during decolourisation of textile wastewater was also examined using SEM.

Introduction

The textile industry uses more than 100 billion gallons of water each year in its preparation and dyeing processes. In Malaysia, textile wastewater accounts for 22% of the total volume of industrial wastewater (Rakmi, 1993). The textile wastewater has strong colour in the form of persistent organics and also variety of the other pollutants including chloride, ammonia, organic nitrogen, nitrate, phosphate and heavy metals such as Fe, Zn, Cu, Cr and Pb (McMullan *et al.,* 2001). Synthetic dyes have been used increasingly in textile and dyeing industries because of its cost effectiveness in synthesis,firmness, and variations in colour compared with natural dyes (Griffiths, 1984). Synthetic dyes also can be classified by their chromophores such as azo, antraquinone and indigo chromophores. Azo dyes used in the textile industry are constituted the largest group of over 10,000 commercial dyestuffs which account for the majority of the synthetic dyes (Villegas-Navarro *et al.*, 2001). Many studies indicated that most of the azo dyes were affected human health as they are highly toxic (McMullan *et al.,* 2001). Since azo dyes are relatively resistant to biological and chemical degradation, therefore, it makes colour removal in particular, a major interest of scientific research (Banat *et al.*, 1996). The presence of very small amount of dyes in water (less than 1 ppm for some dyes) is highly visible and affects the aesthetic merit, water transparency and gas solubility in lakes, rivers and water bodies. The removal of colour from wastewaters is often more important than the removal of the soluble colourless organic substances, which usually contribute the major fraction of the Biochemical Oxygen Demand (BOD). Methods for the removal of BOD from most effluents are fairly well

established. However, dyes are more difficult to treat because of their synthetic origin and mainly complex aromatic molecular structures. Such structures are often synthesized to resist fading on exposure to sweat, soap, water, light or oxidizing agents and this renders them more stable and less amenable to biodegradation (Seshadri *et al.*, 1994). Despite the existence of a variety of chemical and physical treatment processes, bioremediation of textile effluent is still seen as an attractive solution due to its reputation as a low-cost, environmentally friendly, and publicly acceptable treatment technology (Banat *et al.*, 1996). A number of biological processes such as biosorption have been proposed as having potential application in removal of dyes from textile wastewater (Bustard *et al*., 1998). Mechanisms involved in the colour removal of wastewater include adsorption, transformation or metabolisation of coloured textile effluent by bacterial cells. Microbial consortium in the form of biofilm has the ability to decolourise and metabolize dyes since the presence of intracellular mechanisms that will bring about the degradation or biosorption of dyestuffs (Watnick and Kolter, 2000). Biofilm usually composed of a mixed microbial population of cells growing on surfaces surrounded by exopolysaccharides (EPS). Consequently, in most natural environments, biofilm is the prevailing microbial lifestyle. The EPS secreted by various types of bacterial strains serve mainly to protect the bacteria against desiccation and predation, as well as to assist in adhesion to surfaces. The formation of EPS generally occurs in two forms: capsule, which is tightly bound to the surface of bacterium and slime, which is only loosely attached to the bacterium (Abrahamson *et al*., 1996). EPS are also considered as the most immediate interfacial boundary between the bulk aqueous phase and the bacterial cells. EPS generally consist of a wide variety of macromolecular compounds including acidic polysaccharides and proteins, as well as lipids (Fang *et al.,* 2002). This study aimed at investigating the potential application of biofilm consisting of selected indigenous and exogenous bacteria in the bioremediation of colour from textile wastewater.

Most recent advances

Studies carried out at the research laboratories have resulted in the isolation of various mixed bacterial cultures capable of growth on several kinds of azo (Amaranth), diazo (Remazol Red) and reactive dyes (Synazol Blue, Synazol Yellow), both under aerobic and anaerobic conditions (Mohd Zahari *et al.*, 2004, Husin *et al.*, 2003). Furthermore, they also were capable of growing in filter sterilized textile wastewater (fstw) supplemented with glycerol 0.5% (Mohd Zahari *et al.*, 2004). 26 different types of pure cultures were isolated from wastewater and biofilm formed in textile treatment ponds. Most of them were gram-negative bacteria, non-motile and produced exopolymeric substances (EPS). Referring to Figure 1, it was clearly shown that different concentration (mg/L) SF Red 3BS showed maximum absorption peak of 517.5 nm and 542.5 nm in fstw. The selection of SF Red 3BS was based on it being one of the commonest dye used in all steps of dye processing. However, the absorbance value of each concentration for this dye at both wavelengths is not shown. These isolates were further screened for colour removal using synthetic dye; SF Red 3BS (CDM + 100ppm dye) and filter sterilized textile wastewater (fstw) in separate experiments (Table 1).

According to Figure 1, filter sterilized textile wastewater (fstw) brought the maximum absorbance at 517.5 nm and 542.5 nm. It should be noted that, fstw as originated from real wastewater, collected from the textile treatment pond with initial colour concentration of 519.0 ADMI units. In particular, colour intensity of fstw was lesser than the real wastewater by 21.8%; that is 405.9 ADMI units. The wastewater was further filtered using 0.2 µm pores filter membrane

to remove indigenous microorganisms (culturable and unculturable microorganisms) by using of 0.2μm pores filter membrane. However, by doing so, some of the other wastewater parameters such as COD, TSS and colour intensity were generally decreased.

Table 1: Screening on colour removal by potential isolates in SF Red 3BS in a synthetic medium (CDM) and filter sterilized textile wastewater (fstw)

Types of Sample	CDM + 100 ppm dye after 3 days incubation		Fstw after 12 hours incubation	
	Residual Colour (ADMI Unit)	Colour removal (%)	Residual Colour (ADMI Unit)	Colour removal (%)
Control	9320	0	406	0
Bacillus cereus	5840	37	146	64
Aeromonas hydrophila	740	92	28	93
Aeromonas caviae	800	91	51	87
Enterobacter aerogenes	1100	88	27	93
Citrobacter freundii	800	92	84	79
Shigella flexneri	1000	89	112	72

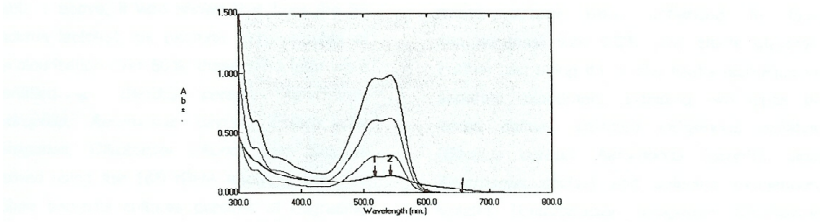


Figure 1: Spectrum of filter sterilized textile wastewater (fstw) and pure dye used for screening (____) Filter sterilized textile wastewater (IX); (.....) SF Red 3BS (100 mg/l); (__) SF Red 2BS (50 mg/l); (___) SF Red 3BS (25mg/l); at two maximum peaks: (1) 517.5 nm;(2) 542.5 nm.

Numerous bacteria capable of dye decolourisation have been reported. Referring to Table 1 above, it was shown that from the 26 bacteria isolated, six bacteria were capable of decolourisation over 50%; these were later identified as *Bacillus cereus*, *Aeromonas hydrophila, Aeromonas caviae, Enterobacter aerogenes, Citrobacter freundii* and *Shigella flexneri* using the 16S rRNA analysis. Effort to isolate bacterial cultures capable of degrading azo dyes started in 1970's with reports of a *Bacillus subtilis*, then *Aeromonas hydrophila* followed by the *Bacillus cereus* (Banat *et al*., 1996). Similar observation achieved by Husin *et al.* (2003) where *Aeromonas hyrophila* and *Bacillus cereus* in the form of biofilm had shown the same ability to treat coloured textile wastewater. Banat *et al.* (1996) had also reported that

isolation of such microorganisms proved to be a difficult task. Extended periods of adaptation in chemostat cultures are needed to maintain the performance of the isolates. As an example, a gram-positive bacteria; *Bacillus cereus*, showed a decrease of colour removal from 96% (Husin *et al.*, 2003) to only 64% after 12 h of incubation. Stock cultures were preserved at -80°C with addition of 12.5% glycerol into fstw. Even though they were isolated from enrichment cultures previously, they were maintained in fstw as sole carbon sources in semi-anaerobic conditions for over a year (Husin *et al.*, 2003). This might cause in disruption of intracellular function due to cell dwarfing in very limited nutrient condition (Yu *et al.*, 2001).

An investigation into the efficiency of growth and decolourisation for these cultures, exogenous and selected indigenous, concluded they were facultative, with an ability to grow under both aerobic and anaerobic conditions in fstw, but with highest growth rate and decolourisation under semi-anaerobic conditions in fstw; 37°C, with shaking (100 rpm), with initial pH of 7.4. Growth and decolourisation of two mixed culture were enhanced in fstw supplemented with 0.5% (v/v) sterile glycerol. Further test using the shake flasks technique in separate experiment, including two types of mixed culture; selected indigenous isolates (*Bacillus cereus, Aeromonas hyrophila and Aeromonas caviae*) and selected exogenous isolates (*Enterobacter aerogenes, Citrobacter freundii* and *Shigella flexneri*) were compared to see any differences in their efficiencies to decolourise filter sterilized textile wastewater(fstw).

Mechanism of decolourisation

Following screening study for dye decolourisers,6 of the isolates (ANB1, ANB2, ANB3, Cb01,Cb02 and Cb15) were selected to further determine their mechanism of decolourisation via molecular probing. This involved amplification of two genes (I*sfA* and *ssu*D) for enzyme synthesis of aromatic sulphonates degradation (Quadroni *et al.,* 1999). For this purpose, genomic DNA of the most potential bacteria, *Aeromonas caviae* was extracted using Promega Wizard Genomic DNA Purification Kit. The genomic DNA and suitable primers were used for the amplification of the desired gene. The sample of genomic DNA (1 µg) was mixed with 10 pmol of forward and reverse primers and 2x PCR Master Mix (25 µL). Four sets of different primers were used to amplify the genes of interest (Table 2). Polymerase Chain Reaction (PCR) was performed for 25 cycles 94°C for 1 min, 50°C for 1 min and 72°C for 2 min. PCR products were observed by agarose gel electrophoresis.

Bacterial decolourisation of azo dyes could either be due to azo reduction and/or desulphonation. The disappearance of colour is due to the reductive cleavage of azo bond(s), which is catalysed by enzymes such as flavin reductase and quinone reductase (Russ *et al.,* 2000). For decolourisation of sulphonated azo dyes, the release of the sulphonic group may be required to decolourise the azo dye since desulphonation results in the destabilization of the benzene ring structure (Kertersz and Wietek, 2001). Since decolourisation of azo dyes by the all six selected isolates above may involve desulphonation and / or azo reduction, primers of genes for desulphonation (Table 2) were used to amplify the I*sfA* gene and *ssu*D gene. The amplification of the I*sfA* gene and *ssu*D gene yielded fragments of ~700 bp and ~1400 bp respectively from bacterium *Aeromonas caviae*. This might signify the possibility of desulphonation by this bacterium, which is related to decolourisation of the sulphonated azo dyes (result not shown).

Table 2: Different types of primer for amplifying genes involved in dye degradation

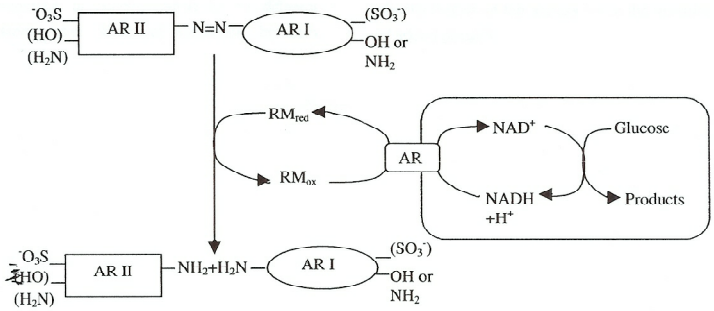
Primer	Tm(°C)	Expected size (bp)	References
lsfA gene			
LsfAfor 5'-CAA GCT CAA GGA CCA GTT CG	59.4	639	Quadroni *et al.* (1999)
LsfArev 5'-C AG CGA AGG CAC GAT TAC C	58.8		
ssuoperon			
Ssuprofor2 5'- AAG AGC TCC CCA AAG GTT ATC GCG	64.4	5300	Kahnert *etal.* (2000)
Ssuprorev1 5'-TCG TGC AAG CGC TCT TCC	58.2		
ssuF gene			
SsuFfor 5'-AGC CAT CAA CGT TCG TAA CC	57.3	216	Quadroni *et al.* (1999)
SsuFrev 5'-AGC TTG GCG ATG GAG ACC	58.2		
ssuDgene			
EE24for 5'-CAT CTG GAA GCT TAC TCA ACT G	58.4	1220	Kahnert *etal.* (2000)
EE27rev 5'- ATA ACC AAG CTT TCA CTG GCG	57.9		

Proposed mechanism on decolourisation

Several researchers from the University of Stuttgart, Germany had experimentally proven that azo dye reduction *in vivo* may involve the role of bacterial cytoplasmic and extracellular 'azoreductases'. Stolz *et al.* (2000) reported on gram-negative isolate *Sphingomonas sp.* BN6, which has recently, through molecular analysis, been shown to represent a novel species named *Sphingomonas xenophaga* after its ability to "eat foreign compound". It was demonstrated that an extracellular mechanism of dye reduction existed in addition to the nonspecific cytoplasmic enzymes which functioned as azoreductases by transferring electrons via reduced flavin groups to the dye molecule and thus bringing about a purely chemical reduction. Refering to Figure 2, Keck *et al.* (1997) demonstrated that certain quinone-based compound generated during metabolism of specific substrate acted as mediators shuttling redox equivalents to azo dye molecules from the bacterial membrane. Russ *et al.* (2000) had investigated if cytoplasmic enzymes played any role in dye decolourisation *in vivo*, a flavin reductase [NAD(P)H: flavin oxidoreductase] was cloned and overexpressed in both *E. coli* and *S. xenophaga*. In cell extracts, the strains with overexpressed flavin reductase demonstrated elevated azoreductase activity; however in whole cell studies these strains showed little improvement in their dye decolourising capabilities. Russ *et al*. (2000) also provided evidence that reports of aerobic azoreductases could be explained by the isolates in which such a phenomenon was described having elevated flavin reductase activities.

Figure 2: Proposed mechanism for the redox-mediator-dependent reduction of azo dyes by *Sphingomonas xenophaga*

BN6. (AR: Azoreductase, RM: Redox mediator). Reproduced from Keck *et al*. (1997).



Decolourisation with bacterial biofilms- Anaerobic-Aerobic biodegradation of dyes

Experiments on the use of selected bacteria for colour removal were conducted using shake flasks technique. Real wastewater (Non-fstw) and filter sterilized textile wastewater (fstw) with initial concentration 405.9 ADMI units and 519.0 ADMI Units respectively, were inoculated with either mixed culture of the exogenous or selected indigenous bacteria. For Set (A) experiment, the fstw consisted of *Bacillus cereus* : *Aeromonas caviae* : *Aeromonas hydrophila*, at the ratio of 1:1:1 (v/v). However, for Set (B) experiment, the bacteria involved were *Enterobacter aerogenes* : *Citrobacter freundii* : *Shigella flexneri* also at the ratio of 1:1:1 (v/v). Both sets of experiments were carried out under varying conditions to compare the effectiveness of suspended cells and biofilm in the treatment of textile wastewater. The percentage of decolourisation by 11 different systems after 12 h of incubation by both exogenous and indigenous biofilm is presented in Table 3.

After 12 hours of incubation under optimized condition (37°C, 100 rpm, pH 7.4, supplemented with 0.5% glycerol), both the exogenous (Set B; Systems 4 and 10) and indigenous biofilms (Set A; System 10) achieved almost complete decolourisation (~97%) compared to suspended cells (Systems 5 and 11) which below 80%. From the results obtained, it is important to note that decolourisation of the wastewater containing indigenous microbes (System 6), that containing culturable and unculturable microorganisms; showed only about 1.8% of decolourization compared to the wastewater containing selected bacteria (System 8 and 9) in suspension (29% for Set A; 19% for Set B) and in the form of biofilm (46% for Set A; 48% for Set B). This strongly indicated that the use of selected bacteria capable of colour removal played a significant role in enhancing decolourisation of the wastewater in the absence of added glycerol.

Table 3: Rate of decolourization (% h^{-1}) in shake flasks experiment

Number of system	System components	Rate of decolourisation per hour (0-12h)		Rate of decolourisation per hour (0-12h)	
		A	B	A	B
1	fstw	0.00		0.00	
2	fstw + biofilm	3.74	2.28	2.52	4.25
3	fstw + suspended cell	1.58	0.90	1.92	0.92
4	fstw + biofilm + glycerol	4.15	8.19	2.22	0.08
5	fstw + suspended cell + glycerol	4.52	5.09	1.01	2.75
6	non-fstw	0.15		0.01	
7	non-fstw + glycerol	1.46		0.58	
8	non-fstw + biofilm	3.87	4.04	2.59	4.42
9	non-fstw + suspended cell	2.40	1.62	3.75	1.84
10	non-fstw + biofilm + glycerol	8.12	8.08	0.23	0.22
11	non-fstw + suspendedcell + glycerol	6.56	5.98	0.33	0.35

Note:
Non-fstw : Wastewater contained indigenous bacteria (Initial concentration: 519.0 ADMI Unit)
fstw : filter sterilized textile wastewater (Initial concentration: 405.9 ADMI Unit)
A : Mixed indigenous culture *(Bacillus cereus : Aeromonas caviae : Aeromonas hydrophila)*
B : Mixed exogenous culture *(Enterobacter aerogenes : Citrobacter freundii : Shigella flexneri)*

During the decolourisation study, the rate of colour removal was monitored with time over a period of 24 h. In general, it was found that there were two distinct linear regions that corresponded to two different rates of decolourisation (data not shown, calculated rates shown in Table 3). During the first 12 h of incubation, the rate of decolourisation was higher of biofilm bacteria; System 10 (Set A & B) and System 4 (Set B) was higher (1.2-1.6 times) than those in suspension (System 11). This may be attributed to the presence of extracellular polymers (EPS), which provide diffusion barrier against high inhibitory concentrations of toxic substances, allowing better survival of the bacteria in the form of biofilm. In addition, structural feature of biofilm provide both aerobic and anaerobic zones which were both required for complete mineralisation of the dye. Besides that, biofilms also offer higher solids retention times necessary to prevent washout of adapted microorganism (Jiang and Bishop, 1994). Accordingly, control experiment showed the addition of a meager amount of glycerol (0.5%) contributed a medial effect on decolourisation; rate of decolourisation form System 7 (1.46% h-1) which incorporates the glycerol, was higher than System 6 (0.15% h-1).

As for the suspended cells in the presence of indigenous (System 11), the addition of glycerol showed increased rate of decolourisation to about 1.5-1.7 times more than that without glycerol (System 9) during the first 12h of incubation. Glycerol may be used as an alternative carbon source for growth and may also act as an electron donor for the colour reduction (Nigam *et al.,* 1996 b) during the first 12h of incubation.

Experiments using selected indigenous and exogenous microbes in fstw, showed a similar profile on decolourisation. Rates of colour removal by potential microbes in the formed of biofilm (System 2) were much higher (about 2.5- 4.6 times) compared to suspended cells (System 3) during the first 12 h of incubation. The rate of decolourisation remained unchanged after 12 h. The low efficiency of potential microbes in decolourisation may be related to the low population of cells present and low tolerance towards the toxic elements in the wastewater. Furthermore, dye chromophore act as the sole source of nutrient (carbon and energy source), bacterial growth is dependent on dye degradation, which in turn may contribute to a longer adaptation period and slower rate of decolourisation. The presence of bacteria in the form of biofilm is advantageous since the biofilm provides aerobic and anaerobic zones, which may facilitate complete mineralisation of dyes (Jiang and Bishop, 1994).

Fig. 3(a): Biofilm form by the potential indigenous; *Bacillus cereus, Aeromonas caviae* and *Aeromonas hydrophila* in fstw

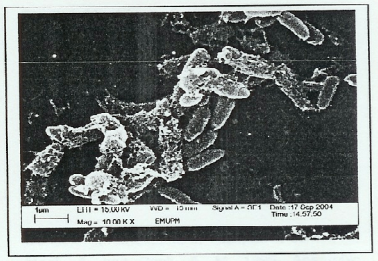


Fig. 3(b): Biofilm form by the potential exogenous; *Enterobacter aerogenes, Citrobacter freundii* and *Shigella flexneri* in fstw

From the work that has been carried out, the best system for dye removal of the textile wastewater is System 10, which indigenous microorganisms contained selected bacteria in real wastewater (or non-fstw) in the form of biofilm. Closer examination of the biofilm clusters using SEM revealed that most of the bacteria were attached to the support matrices by means of extracellular strands. The morphology of biofilm formed by the indigenous and exogenous during the treatment of textile wastewater is shown in Figure 3(a) and 3(b) respectively. At some stage of growth, weblike structure termed as "bioweb" comprising of EPS was found as part of biofilm (Paulsen *et al.*, 1997). This morphology was reported to facilitate quicker plugging of pore throats by trapping floating biofilm fragments and other detritus. Meanwhile, both figures also illustrated the population of cells growing on surface of support matrix enveloped in a matrix of exopolymers. Electron microscopy studies demonstrated that the biofilm is not found as simple layers of bacteria, but are enveloped in the exopolymers. In this manner, biofilm enhances the removal of toxic organic compounds such as those found in textile wastewater (Fang *et al*., 2002).

Bioremediation of textile-dye-containing effluent using biofilm had worked effectively either in the presence of great potential indigenous microbes such as *Bacillus cereus, Aeromonas caviae, Aeromonas hydrophila* or selected exogenous bacteria *Enterobacter aerogenes, Citrobacter freundii* and *Shigella flexneri* in the wastewater. From the work had been carried out, it was also suggested that a great potential of decolouriser bacteria in the form of biofilm system provide a complete decolourisation with only hours of exposure.Biofilm structure and spatial distribution influence all biofilm properties, including fixed cell activity. Biofilm structure and formation can be observed by electron microscopy techniques, enhanced in some cases by image analysis. Mechanism study on decolourisation may take advantage of biofilm processes in textile wastewater treatment system. Therefore, further studies are needed to develop simpler and more precise analytical methods for bacterial biomass correlated with enzyme activity estimation and a better knowledge of the composition and function of the biofilm.

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