Bioremediation and Biofouling Perspective of Real Batik Effluent by Indigenous Bacteria

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Abstract

A reasonable decrease has been found in almost all pollution indicators, which indicated emblems of natural bioremediation in real batik dye effluent from source (S1) to sink (S3). Among nine bacteria only FS5 exhibited the utmost biofouling potential in a microtiter plate assay with a noteworthy optical density of 0.629 ±0.042 (p<0.05). Furthermore, an endeavor was made to characterize the biofouling bacterial isolate (FS5) evaluating via 16S DNA sequencing. The present research based on the 16S DNA sequencing displayed that the isolate FS5 was similar to *Bacillus* sp. Extracellular polymeric substances (EPS) produced by FS5 were harvested and their dry weight (capsular 25.39 ±1.19, slime 21.73 ±1.40 and total 32.55 ±0.92 μgml\(^{-1}\)) and viscosity (169.24 ± 0.96 mPa) was measured. The total carbohydrates (TC) (70%) concentration in EPS was higher than total protein (TP) (30%) concentration. Thus, identifying an appropriate biological control agent against this biofouling bacterium is suggested as further course of action.

Keywords: Batik textile dye, bioremediation, biofouling potential, microtiter plate assay, EPS

1. Introduction

The textile sector is the third largest alien exchange earner after the electronic and palm oil sectors in Malaysia, adding total earnings of RM 18.0 million (US$5.4 million) from manufactured exports in 2007 [1]. There are about 1500 textile factories in Malaysia, many of which operate as backyard or cottage industries producing the local ‘batik’. Colour has been included in the water quality standards for the discharge of industrial effluents in Malaysia. Under the Environmental Quality (Industrial Effluents) Regulations, 2009, the limits of colour for discharge of effluents according to standards A and B are 100 and 200 Platinum–Cobalt (PtCo) units, respectively [2].

The chemicals employed in textile sector are diverse in chemical composition. In general, textile wastewater is colored (10–200 mg/l), varying in hydraulic flow rate, having high; pH, temperature, biological oxygen demand (BOD), chemical oxygen demand (COD), total dissolved solids (TDS) and total suspended solids (TSS) [3]. In addition to dyes, various salts and chemicals are major sources of heavy metals in wastewater [4]. Sediments, suspended and dissolved solids are important repositories for toxic heavy metals and dyes [5] causing rapid depletion of dissolved oxygen leading to oxygen decline in the receiving water [6]. The metals and contaminants like dyes tend to persist indefinitely, circulating and eventually accumulating through out the food chain [7]. Various reports have mentioned the direct and indirect toxic effects of dyes and metals in the form of tumors, cancers and allergies besides growth inhibitions on different trophic levels like bacteria, protozoans, algae, plants and different animals including human being [8].

The occurrence of dyes and metals leading extreme damage to the aquatic biology. As a result, the self-purification ability of the stream and conventional biological treatment systems is [6]. Numerous researchers have evaluated live microbial systems for bioremediation of metals and dyes contaminated soils and waters [9]. However, there is a need of valued research to sort out the potential of various microbes for the rehabilitation of our natural resources.

Biofouling is instantly related to the occurrence of microorganisms which can damage the membrane by attaching to surfaces. Bacterial cells can encase themselves in the form of a matrix of polysaccharides and proteins and form a slimy layer known as biofilms [10]. However, surface related adhering does not mean static; biofilms are very dynamic systems. Their composition is a progressive process in which colonizing bacteria move...
to or are transported to a surface, attach and via a series of steps, produce a biofilm [11]. The organisms like *Vibrio proteolyticus* [12], *Escherichia coli*, *Pseudomonas aeruginosa*, *Shewanella oneidensis* [13] and *Bacillus subtilis* [14] have been found to be involved in the biofouling process.

Extracellular polymeric substances (EPS), which are excreted by bacteria and composed of a variety of controlling factor of membrane fouling in MBRs [16]. It was reported that filterability of sludge decreased with the increase of bound EPS [17]. Chang and Lee [18] observed that an increase of extracellular polymeric substances was one of the factors leading to flux decline in the membrane-coupled activated sludge system. EPS have complex components and their fouling mechanisms are highly complex. More comprehensive research on the EPS behaviors, their effects on fouling and EPS concentration mitigation is needed for real wastewater in order to better understand the role of EPS in real MBR applications.

The present study is focused on the natural remediation and biofouling potential of real batik textile dye effluent by indigenous bacteria. The systematic investigation on EPS in real batik wastewater effluent would provide valuable insights.

## 2. Materials and method

### 2.1 Sampling and Analysis of Effluent

Sampling of effluent was carried out from “Natural Batik Village” Kuantan, Malaysia. Sampling of batik textile dye effluent was carried out at the time of dyeing and washing process at three different sites [source (S1), middle point (S2) and sink (S3)] having a distance of 200 m. Standard procedures (Spot or Grap) were followed during sampling. Temperature and pH of the wastewater were determined at the spots, whereas, rest of the parameters were determined instantly after bringing the samples in the Environmental laboratory, University Malaysia Pahang, Malaysia. Table 1 show the methods followed during physicochemical and microbial analysis of batik dye effluent.

### 2.2 Isolation of Bacterial Strains from Effluent

The wastewater samples were serially diluted and plated over Zobell agar plates. The inoculated plates were incubated at 28 ± 2°C for 24-48 h. The selected bacterial colonies were purified and subcultured in nutrient agar medium for further evaluation [20].

### 2.3 Screening of Biofouling Potential of Bacterial Isolates

The bacterial isolates were grown over night in nutrient broth at 37 °C. Aliquots of 3 μL were inoculated in six parallel wells of a 96 well microtiter plate. Then the plate was incubated at 37 °C for 72 h., the wells were rinsed after the incubation period with physiological saline to remove the adhered cells and fixed with 2 μL of 99.99 % ethanol for 10 min. The adhered bacterial material was then stained adding 2 μL of crystal violet (2 %) for 20 min. The plate was rinsed with tap water and the adhered cells were quantified via an ELISA reader at 570 nm [21].

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH</td>
<td>Orion 976 pH meter model 64 Direct at spot</td>
</tr>
<tr>
<td>EC</td>
<td>PW9526 digital</td>
</tr>
<tr>
<td>TSS</td>
<td>Hot air oven Memmert</td>
</tr>
<tr>
<td>TDS</td>
<td>Hot air oven Memmert</td>
</tr>
<tr>
<td>Metals</td>
<td>Atomic Absorption Spectrophotometer</td>
</tr>
<tr>
<td>COD</td>
<td>COD reactor, Spectrophotometer</td>
</tr>
<tr>
<td>BOD</td>
<td>COD reactor, Spectrophotometer</td>
</tr>
<tr>
<td>CFU</td>
<td>Quebec Colony counter</td>
</tr>
</tbody>
</table>

### 2.4 Molecular Characterization

The DNA was extracted [22] from the FS5 isolate by alkali lysis method and subsequently amplified by PCR using the universal forward and reverse primers 5’AGAGTTTGATCCTTGGCTCAG3’ and 5’ACGGCTACCTTGTTACGACTT3’, respectively. The following parameters were used for PCR amplification: 30 cycles of initial denaturation step at 94 °C for 2 min, followed by denaturation step at 94 °C for 45 sec, annealing at 52 °C for 30 sec, extension at 72 °C for 1 min and final extension at 72 °C for 2 min. The PCR product bromide and the amplicon was sent for sequencing to First-Base, Malaysia. Specific primer 5’GTATTACCCGCGCGTGCCTGG 3’ was provided along with the sample for sequencing. The nucleotide sequence obtained was compared with the known bacterial sequences available in the NCBI database using the bioinformatics tool BLAST and a phylogenetic tree was constructed using the PHYLIP program [23].

### 2.5 EPS Production and Harvesting

To produce EPS, isolated FS5 strain was grown as pure culture in a reported mineral medium. The composition of mineral medium utilized for the biopolymer production was as follows; 25 gl⁻¹ glucose, 0.2 gl⁻¹ MgSO₄·7H₂O, 2 gl⁻¹ K₂HPO₄, 1 gl⁻¹ KH₂PO₄, 1 gl⁻¹ NH₄Cl and 0.01 gl⁻¹ yeast extract. The initial pH of medium was adjusted to 7.0. Glucose and MgSO₄ were sterilized separately and mixed aseptically with other ingredients before inoculation. Bacterial strain was inoculated in the mineral medium from the slants, and it was incubated in an orbital shaker at 250 rpm for 3 days at 25 °C. After 3 days, the broth changed into highly viscous. After incubation (3 days), the medium was centrifuged at 6000g for 15 min at 4 °C to obtain slime EPS (in centrifuged supernatant) and capsular EPS (in bacterial pellet or microorganisms). The supernatant was precipitated with 2.2 volumes of absolute chilled ethanol by incubating the mixture at -20 °C for

Table 1 Methods used to analyze different physicochemical parameters (Eaton et al [19])
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1 h. Precipitated EPS was collected by centrifugation at 6000g for 15 min at 4 °C. The supernatant was removed and the pellet containing slime EPS was dried at room temperature.

2.6 Dry Weight of Crude EPS

Dry weight of the capsular EPS, crude slime EPS, and bacterial broth (combined slime and capsular EPS) were quantified by drying at 105 °C to a constant weight [24].

2.7 Viscosity

Viscosity was carried out only for the EPS produced bacterial broth after 3 days of fermentation. Viscosity of EPS produced culture (bacterial) broth was measured using viscometer (DV-II+PRO, Brookfield), 18 mL of culture broth was used to quantify the viscosity using ULA S34 spindle, at 60 rpm and room temperature.

2.8 Chemical Characterization of EPS

The total carbohydrates (TC) content of extracted slime EPS was determined by the phenol-sulphuric acid method [25]. The total protein (TP) content of the extracted EPS was investigated by the Bradford [26] method with bovine serum albumin as a standard.

2.9 Statistical Analysis

The results obtained in experiments were expressed in terms of means (average) and standard error (S.E.) using SPSS software. Probability (p-value) less than 0.05 and 0.01 was considered significant and highly significant, respectively.

3 Results and Discussion:

Apparently, the effluent samples collected from batik dye industry during dyeing and washing conditions were dark (S1) to grey (S3) in color, with fishy (S1 and S2) to pungent (S3) smell and high in temperature (30–50 °C) (average = 40.00 °C)]. Closed to source (S1), the pH of the effluent was highly alkaline (11.32) but it reduced towards neutrality (S3 = 7.50) at sink. Electric conductivity (EC) of the effluent was quite low (ave. = 3.79 µS cm⁻¹) and it declined (1.10 µS cm⁻¹) away from the source of emission (Table 2). In sample 3 of the effluent TSS was quite high (18525.60 mgL⁻¹) compared to the first two samples (S1 = 516.27 mgL⁻¹, S2 = 734.00 mgL⁻¹) but TDS decreased in sample 3 (2636.00 mgL⁻¹), however, increased from to S1 to S2. Overall, there was observed a significant high load of COD (average = 2334.00 mgL⁻¹) than BOD (average = 878.00 mgL⁻¹) though both followed same decreasing trend towards sink (S3) after considerable increase at S2 (Table 2). Heavy metals analysis of the effluent released from textile industry showed their high amounts that decreased down the stream. The average amount (mgL⁻¹) of metals ions like Cu²⁺ (5.54), Fe³⁺ (4.83), Mn²⁺ (3.57) and Zn²⁺ (2.69), was considerably higher, while it kept <2mgL⁻¹ in case of Cd²⁺ and Cr³⁺ in the effluent. The bacterial count (CFU) was significantly higher (10.6 x 10⁵) at S3 (sink) compared to S1 (0.20 x 10²) and S2 (0.87 x 10³) (Table 2). Physicochemical status of batik effluent revealed a reasonably high load of pollution indicators (Table 2). Color is imparted to a water body by dissolved constituents (dyes and pigments) that absorb white light and emit light at specific wavelengths.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>S1</th>
<th>S2</th>
<th>S3 (sink)</th>
<th>Avg.</th>
<th>S.E. (±)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smell</td>
<td>Fishy</td>
<td>Fishy</td>
<td>Pungent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temp.</td>
<td>50.00</td>
<td>40.00</td>
<td>30.00</td>
<td>40.00</td>
<td>5.62</td>
</tr>
<tr>
<td>Color</td>
<td>Black</td>
<td>D. grey</td>
<td>grey</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>11.32</td>
<td>9.00</td>
<td>7.50</td>
<td>9.20</td>
<td>2.34</td>
</tr>
<tr>
<td>EC</td>
<td>5.71</td>
<td>4.56</td>
<td>1.10</td>
<td>3.79</td>
<td>1.45</td>
</tr>
<tr>
<td>TDS mg L⁻¹</td>
<td>1341.00</td>
<td>3676.7</td>
<td>2636.00</td>
<td>2512.5</td>
<td>756.46</td>
</tr>
<tr>
<td>TSS mg L⁻¹</td>
<td>516.27</td>
<td>734.00</td>
<td>18525.60</td>
<td>6591.9</td>
<td>4976.2</td>
</tr>
<tr>
<td>COD mg L⁻¹</td>
<td>2232.00</td>
<td>3450.0</td>
<td>1321.00</td>
<td>2334.0</td>
<td>290.36</td>
</tr>
<tr>
<td>BOD mg L⁻¹</td>
<td>935.00</td>
<td>1048.0</td>
<td>651.00</td>
<td>878.00</td>
<td>174.67</td>
</tr>
<tr>
<td>Cu²⁺ mg L⁻¹</td>
<td>3.84 x 10⁻³</td>
<td>4.31</td>
<td>5.54</td>
<td>1.73</td>
<td></td>
</tr>
<tr>
<td>Cd²⁺ mg L⁻¹</td>
<td>0.65</td>
<td>0.60</td>
<td>0.31</td>
<td>0.52</td>
<td>0.15</td>
</tr>
<tr>
<td>Zn²⁺ mg L⁻¹</td>
<td>6.27</td>
<td>1.12</td>
<td>0.69</td>
<td>2.69</td>
<td>2.31</td>
</tr>
<tr>
<td>Fe³⁺ mg L⁻¹</td>
<td>6.00</td>
<td>5.20</td>
<td>3.31</td>
<td>4.83</td>
<td>0.90</td>
</tr>
<tr>
<td>Cr³⁺ mg L⁻¹</td>
<td>2.51</td>
<td>1.34</td>
<td>1.10</td>
<td>1.65</td>
<td>0.27</td>
</tr>
<tr>
<td>Mn²⁺ mg L⁻¹</td>
<td>6.21</td>
<td>0.29</td>
<td>4.23</td>
<td>3.57</td>
<td>1.10</td>
</tr>
<tr>
<td>Bacterial</td>
<td>0.20 x 10</td>
<td>0.87 x 10</td>
<td>10.6 x 10</td>
<td>3.89 x 10</td>
<td>3.78</td>
</tr>
<tr>
<td>Count</td>
<td>10⁴</td>
<td>10³</td>
<td>10⁴</td>
<td>10⁴</td>
<td></td>
</tr>
</tbody>
</table>

There was a gradual change in the color from black to grey of the effluent from source to sink indicating sign of decolorization. The decreasing color intensity of the effluent has been related to adsorption/chemical transformation of dyes (including metal complex) by biotic and abiotic component of the effluent [27]. The increase of bacterial count at sink might have been responsible for color change.

Initially the temperature of the effluent was considerably high (50 °C), however, declined to mesophilic status (30 °C) at sink (S3), which ultimately could have favored biologically mediated remediation of effluent. High temperature reduces solubility of gases in water that ultimately express as high BOD/COD. COD and BOD levels recorded in effluent samples decreased down the stream. However, high values of BOD/COD as observed in this study case demand significant amount of dissolved oxygen (DO) for enhanced intrinsic remediation of dye wastewater. In General, alkaline pH of dye wastewater is coupled with the process of bleaching [28] and it is enormously objectionable in water ecology. Both chemically and biologically mediated adsorption/reduction of dyes are initiated with decreasing pH level under redox-mediating compounds [29]. Decline in pH i.e., 11.32 to 7.50 of effluent down the stream significantly improved bacterial count and thereby coupled remediation. Conductivity is calculated to set up a pollution zone around an effluent discharge. It is
susceptible to variation in dissolved ions and mineral salts. Electric conductivity noted in effluent was noticeably low and it gradually decreased and paralleled with decreasing metal ions concentrations ([S1 to S3]) thereby signifying their biotic [30] and abiotic elimination [31]. Divalent cations are considered to be vital bridging agents between negatively charged expolymers and bacterial surfaces [32]. In addition, the diminishing metal ions concentrations in effluent could also be correlated to their leaching into the soil bordering effluent channel [33].

TSS and TDS in effluents correspond to filterable and nonfilterable residues, respectively. There was observed an increase in TSS in effluent of batik from source to sink. Though, decrease in TDS at S3 with an increase in bacterial count and declining pH and EC suggested course of flocculation (as TSS). Microbial community (both aerobic and anaerobic) establishes itself in granulated floc as activated sludge plays a important role in biodecolorization/bioremediation of wastewater [34].

Total 9 bacterial isolates (FS1, FS2, FS3, FS4, FS5, FS6, FS7, FS8, and FS9) were obtained: 2 isolates from sample I, 3 isolates from sample II and 4 isolates from sample III. Among the 9 isolates, only FS5 depicted maximum biofouling activity in the microtiter plate assay with a significant optical density (OD) of 0.629 (±0.042) (p>0.05). The other isolates did not show significant biofouling activity (Fig. 1). A similar study was also done by Dhanasekaran et al [35], in which bacillus sp. showed maximum biofouling activity in microtiter plat assay with a significant optical density of 0.596. A similar biofilm screening has been done by Bhosale et al [36] using floating glass cover slips on the sterilized filtered sea water to prove the attachment of Bacillus spp., biofilm and then staining the cover slips with gentian violet.

A jagged band of genomic DNA was found in the gel and 50 ng of DNA was used as template. The amplification of 16S rDNA gene of the bacterial isolate FS5 was performed by PCR technique via universal primers. A sharp band was observed in 1 % agarose gel which confirmed the presence of 16S rDNA. The amplified 16S rDNA sample was sent to First-Base, Malaysia, for sequencing. The nucleotide 16S rDNA sequence for the bacterial isolate FS5 was used for the basic local alignment search tool (BLAST) and the sequence showed resemblance with the Bacillus sp. A phylogenetic tree of the isolate FS5 was constructed using its 16S rDNA sequence with that of the other Bacillus sp. obtained from the NCBI database. The resultant phylogenetic tree showed similarity of the isolate FS5 only at the genus level, whereas it did not show any similarity at the species level, hence FS5 was found in a separate branch (Fig. 2). A related kind of results has been reported for Bacillus sp. isolated from marine biofilms [35].

In view of the fact that, EPS gives a greatly hydrated gel matrix in which microorganisms are fixed, they provide a considerable obstacle to permeate flux in the MBRs. Microbial biofilms play a vital role in biofouling and biodeterioration [37]. The isolated strain FS5 was screened to study its potential of EPS production. In general, slime and capsular EPS are produced by bacterial cells to protect them against unfavorable environmental conditions such as: desiccation, presence of toxic compounds, low temperature or high osmotic pressures, and may contribute to the uptake of metal ions [38].

EPS concentration, produced by Bacillus strain FS5, at the end of 3 days fermentation is presented in the Fig. 3. The quantity of capsular and slime EPS produced by Bacillus strain FS5 was 25.39 (±1.19) and 21.73 (±1.40) μgmL⁻¹ respectively. Similarly the concentration of total EPS produced in bacterial broth (slime and capsular EPS) was 32.55 (±0.92) μgmL⁻¹ (Fig. 3).
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Fig. 2 Phylogenetic tree of Bacillus sp. FS5 with other Bacillus spp. based on 16S rDNA via neighbor joining method.

The total carbohydrates (TC) concentration (70%) in EPS is higher than total protein (TP) concentration (30%). Several authors agree that especially carbohydrates exhibit a high fouling potential [39]. Thus, it is obvious that carbohydrates fraction play a dominant role than proteins fraction in biofouling of membrane.

Fig. 3 EPS characterization of batik dye wastewater produced by Bacillus strain FS5

The viscosity of effluent had remarkable influence on the membrane fouling resistance. Viscosity measurement was carried out, only for bacterial broth, after 3 days of fermentation. The viscosity of the bacterial culture broth amplified, from 101 mPa to the medium before growth to 169.24 (± 0.96) mPa after growth due to bacterial growth during the incubation period and production of EPS. Nagaoka [40] found that there was a strong dependency of the filtration resistance on the dynamic viscosity. They also found that the accumulation of EPS in bioreactor caused the increase of dynamic viscosity leading to severe decline of membrane permeate flux. More polymers and small particles esult of the higher dynamic viscosity. An increase of biopolymers in the sludge suspension will increase its viscosity, and hence reduce the MBRs permeate flux [18].

Conclusions

Batik effluent clearly imparting a high load of chemicals in the form of different pollution indicators. However, the phenomenon of innate bioremediation seemed to be happening on-site. The current research has noticeably exposed the existence of biofouling bacteria Bacillus sp in the batik dye effluent. It was identified using molecular characters. Increase in viscosity of the bacterial broth, after 3 days fermentation, confirmed EPS production. The total carbohydrates (TC) concentration in EPS was higher than total protein (TP) concentration. Identifying an appropriate biological control agent against this biofouling bacterium is thus suggested as further course of action.

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