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Toxicity assessment and bioremediation of textile effluent by isolated *Pseudomonas Oleovorans* PAMD_1 bacteria

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ABSTRACT

Toxic effluents containing azo dyes are discharged from various industries which adversely affect water resources and ecosystem integrity. The objective of this study was to compare the toxicity of untreated as well as treated textile effluent with newly isolated *Pseudomonas oleovorans* PAMD_1 to Nile Tilapia (*Oreochromis niloticus*) as the test organism. The acute toxicity bioassay experiments were conducted to determine the median lethal dose (LD_{50}) and hematological changes in *O. niloticus*. Hematological profile of *O. niloticus* showed significant elevation ($p < 0.05$) in RBC count (4.10; 5.42), Hb concentration (8.5; 14.3), and heamtocrit (9.0; 12.5) in the treated effluent when compared to the untreated. The textile effluent was also analyzed for physicochemical parameters, including BOD, COD, TDS, and color intensity prior to and after treatment with *P. oleovorans* PAMD_1 which showed a substantial reduction in BOD from 371 to 118 mg l^{-1} (68%), COD from 895 to 220 mg l^{-1} (76%), and TDS from 4400 to 3730 mg l^{-1} (15.2%).

Keywords: *Pseudomonas oleovorans*; *Oreochromis niloticus*; Toxicity assay; Poikilocytosis; BOD/COD

1. Introduction

Increasing industrialization and urbanization have lead to environmental deterioration. The discharges of wastes from various industries adversely affect water resources, soil fertility, aquatic organisms, and ecosystem integrity. Azo dyes are the most versatile class of dyes and account for more than 50% of the dyes produced annually [1]. Effluents from textile industries is the major source of azo dyes, there are more than 10,000 dyes used in textile industry and 280,000 *t* of textile dyes are discharged every year worldwide [2].

The disposal of azo dyes causes severe contamination in river and ground water in the vicinity of dyeing industries [3]. Dyes contributed to overall toxicity at all process stages. Also, dye baths might have a high level of Biological oxygen demand/Chemical oxygen demand (BOD/COD < 0.1) [4], color, toxicity, surfactants, fibers and turbidity, and may contain heavy metals [5]. High BOD levels in the untreated wastewater can cause rapid depletion of dissolved oxygen. Effluents with high COD are toxic to the biological life [6].

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The impact of azo dyes in food industry and their degraded products on human health have caused concern over a number of decades, in spite of legislation controlling their use in several countries. Some of the azo dyes have been linked to human diseases, such as bladder cancer, hepato and splenic sarcomas, hematological abnormalities in experimental animals, etc. Benzidine-based azo dyes are widely used in textile dyeing, paper printing, leather, and cosmetic industries. The experimental studies of these dyes on experimental animals show that administered benzidine-based azo dyes excrete, potentially carcinogenic aromatic amines [7].

Dyeing factory effluents that alter the color and quality of the water bodies have been proved to be hazardous to aquatic organisms. In most of the ecotoxicological studies, fish has been considered as an ideal test organism to examine both acute and chronic toxicity of pollutants. Changes in the water quality cause several physiological and biochemical disturbances in fish. The biological changes in fish that are related to the exposure or to the effects of contaminants are called biomarkers. Toxic compounds from the textile effluent strongly affect the rate of feeding, absorption, and metabolism in fishes. As a result the dye effluent brings, growth reduction, protein content of muscles, liver, and gills [8].

The main aim of the azo dye containing wastewater treatment is to reduce the associated toxicity. Many reports cite textile wastewater as a major contributor to toxic load on aquatic ecosystems [1,9]. There are several methods like chemical precipitation, coagulation, flocculation, and absorption that are currently employed for the removal of colors from the textile waste water, each with its own demerits. Chemical decolorization often requires biogenic reductants. These biogenic compounds are sometimes not effective and often require further treatments [10]. Microbial decolorization and detoxification, is a cheaper and eco-friendly alternative compared to chemical and physical methods [11].

A number of micro-organisms have been found to be able to decolorize textile dyes including bacteria, fungi, and yeasts [12]. There are several studies proposed for the utilization of micro-organisms in the degradation of synthetic azo dyes. Sarayu and Sandhya [13] demonstrated the degradation of Remazol orange using *Pseudomonas aeroginosa* bacteria. Similarly, different micro-organisms have been used in the degradation of synthetic dyes like *Arthrobacter*, *Micrococcus glutamicus*, *Staphylococcus arlettae,* and *Pseudomonas putida* mt-2 [4,14–16]. Generally, color removal by bacteria has been linked to oxidoreductase enzymes, such as laccase and azoreductase [17,18].

This case study summarizes the research undertaken to determine the contribution of textile effluent associated with toxicity. The significant contributions of this study are (i) the illustration of the toxic characteristics of textile effluent on water bodies and (ii) the establishment of the potential application of isolated bacteria in bioremediation.

2. Materials and methods

2.1. Chemicals and source of sample

Chemicals purchased from SRL, India, were EDTA (CAS-94108-75-5), $KMNO_4$ (CAS-7722-64-7), Idoine (CAS-7553-56-2), H_2SO_4 (CAS-7664-93-9), and Sodium thiosulphate (CAS-7772-98-7).

The dye house effluent was collected from Textile Industry, Tirupur, Tamil Nadu, India. Effluent sample was collected in a clean plastic container and transported immediately to the laboratory and stored at 4 °C prior to analysis. Physicochemical characteristics of wastewaters were analyzed within 24 h of collection using standard methods [19].

2.2. Biological treatment with bacteria

The pure active isolated microbial strain *Pseudomonas oleovorans* PAMD_1 from the soil contaminated with untreated textile waste effluent [20], inoculum, was developed in 100 ml of nutrient broth from the stock culture and incubated for 24 h at 37 °C under static conditions. Textile industrial effluent was inoculated with 2×10^6 CFU/ml of the strain and kept under static conditions up to 120 h. Effluent without inoculum was kept as control. Physiochemical parameters of the effluent and the hematological parameters of the effluent treated *Oreochromis niloticus* (Nile Tilapia) fish were measured using standard methods.

2.3. Fish bioassay

2.3.1. Test organism and maintenance

O. niloticus were procured from Kelavarapalli Reservoir, Avalapalli Village (via) Hosur, Tamil Nadu, India. This source was selected, since it has a government-approved aquarium and there are no industries nearby. The fish were acclimatized for at least 15 d prior to the experiment in a glass aquarium (15 l) filled with dechlorinated water containing good plankton population to serve as food and *Ceratophyllum demersum* (a submerged hydrophyte) to oxygenate water.

2.3.2. Experimental design

A total of 60 healthy adult Nile Tilapia (*O. niloticus*) of both sexes was used. The average body length and weight of the fish at the beginning of the experiment were 12.5 cm (12–13 cm) and 75 g (60–90 g), respectively. There was no statistical difference between the study groups and controls regarding the size of the fish. This may be important because smaller animals are generally more active than large ones, so metabolism could also be higher in smaller animals [21].

Fish were batch-distributed (6 batches and10 numbers per batch) into six similar-sized glass tanks with volume of 15 l each. Batch 1 was kept without treatment to serve as control. Batches 2–6 were exposed to five different concentrations of the textile effluents (12.5, 25, 50, 75, & 100%) for determining the acute toxicity (96 h).

2.3.3. Determination of LC_{50} and mortality rate

The 96 h LD_{50} value of textile effluent for the fish *O. niloticus* was determined by the graphical method described by Miller and Tainter [22]. The observed percentage mortality was converted into probit referring to the probit table (Table 1). The percentage dead for 0 and 100 are corrected before the determination of probits as under [23]:

Corrected % formula for 0 and 100% mortality: For 0% dead: 100 ($0.25/n$), For 100% dead: 100 (n–$0.25/n$), Where n—No. of animals used.

The probit values are plotted against log-dose and then the dose corresponding to probit 5, which is 50%. The confidence intervals were calculated from the observations.

2.3.4. Hematological examination

After acute exposing at different concentration (12.5–100%) of the untreated and biologically treated textile effluents, two sample fish were taken from respective groups. Variations in blood parameters were monitored by taking approximately 1 ml of blood sample from the caudal vessels using heparinized syringes with a 1.10 × 40 mm injection needle. Blood samples were transferred to tubes containing ethylenediamine tetra acetic acid (EDTA) as an anticoagulant. Hematological parameters including the total number of erythrocytes were carried out using Dacie and Lewis [24]. Blood was diluted to 1:200 with Hayem's fluid and then counted with a Neubauer counting chamber under a light microscope. The MCV gives an indication of the status or size of the red blood cells (RBC). The average size of the RBCs was expressed in femtoliters (ft). MCV was calculated by dividing the hematocrit (as percent) by the RBCs count in millions per micro liter of blood, then multiplying by 10.

The percentage of morphological abnormalities, such as shape and size of RBC was calculated from the blood smear prepared according to Lee et al. [25] by observing approximately 100 RBCs in 10 microscopic fields (10x × 100x) using an oil immersion. Morphometric measurements of RBCs were made with an occulometer, standardized with micrometer scale as parallel magnification. Similar experiments were also carried out in biologically treated effluent-exposed *O. niloticus* fishes of same experiment design.

The data observed in the experiments were statistically analyzed for the calculation of standard error of mean. The experiments were performed in triplicates for each sample. Results are reported as mean ± standard error. Significant testing was performed at 5%

Table 1
Probit table (abridged from table IX of fisher and yates: *Statistical Tables of Biological, Agricultural, and Medical Research*, Oliver and Boyd, Edinburgh)

%	0	1	2	3	4	5	6	7	8	9
0	–	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33

level using SPSS statistical software tool and significant results had $p < 0.05$.

2.4. Physicochemical parameters assay

2.4.1. Determination of total dissolved solids

A clean dish of suitable size was dried at 102–105 ℃ in an oven. 100–250 ml of thoroughly mixed effluent sample was accurately pipetted into a dish, weighed, and evaporated to dryness on a steam bath. The residue was dried in an oven for about 1 h at 103–105 ℃ and re-weighed after cooling to room temperature. The total dissolved solid of untreated and biologically treated samples were calculated as per the standard procedure [19].

2.4.2. Determination of BOD and COD

Biological Oxygen Demand analysis of the untreated sample of the effluent was first analyzed for BOD within 24 h followed by analysis of biologically treated sample for BOD as per the procedure described by Aneja [19]. Similarly COD of untreated sample (immediate analysis) and biologically treated sample were performed as per the standard procedure [19].

3. Results and discussion

3.1. Fish toxicity assay

3.1.1. Mortality

Fish mortality was mainly dose dependent during acute exposure, the percentage of mortality was calculated from Table 1 (probit table). Table 2a showed the increasing and corrected percentage of mortality for different exposure concentrations of textile effluent to the untreated *O. niloticus* (control). Similarly, Table 2b depicts the probit analysis of biologically treated *O. niloticus*. From Fig. 1, the 96 h LD_{50} value of raw textile effluent for the *O. niloticus* was found to be around 68%, whereas it increased significantly to 93% for biologically treated textile effluent. The desired level of confidence was set by 95% confidence interval reflects a significance level of 5%. The confidence intervals in terms of dose are 14.69–75%. The data clearly showed the concentration of the toxic effluent and the percentage of mortality. Fish exposed to higher concentrations underwent rapid death. The increasing LD_{50} value clearly shows the reduction in toxicity to a greater extent by the potential isolate *P. oleovorans* PAMD_1

Table 2b
Corrected probit analysis LD_{50} of *P. oleovorans PAMD_1* treated *O. niloticus*

Group	Dose	Log dose	Probit*
1	0	0	3.04 ± 0.02
2	12.5	1.1	3.04 ± 0.05
3	25	1.4	3.04 ± 0.05
4	50	1.7	3.04 ± 0.03
5	75	1.88	4.16 ± 0.02
6	100	2.0	5.44 ± 0.02

*Average of triplicate, mean ± SE of 3 observations.

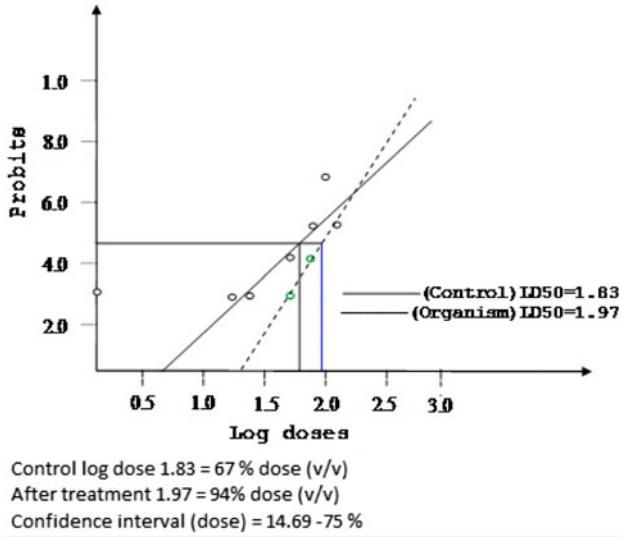

Fig. 1. Calculation of LD_{50} from raw and biologically treated textile effluent exposed *O. niloticus*.

Table 2a
Determination of LD_{50} after textile effluent treated in *O. niloticus*

Group	Dose	Log dose	% Dead	Corrected %	Probit*
1	0	0	0	2.5	3.04 ± 0.06
2	12.5	1.1	0	2.5	3.04 ± 0.04
3	25	1.4	0	2.5	3.04 ± 0.04
4	50	1.7	20	20	4.16 ± 0.01
5	75	1.88	60	60	5.25 ± 0.02
6	100	2.0	100	97.5	6.96 ± 0.02

*Average of triplicate, mean ± SE of 3 observations.

bacteria. Therefore, determining the toxic index, i.e. ratio between the lethal dose and the toxicologically effective dose in the same species, with confidence limits is the prime study to evaluate the toxicity.

3.1.2. Hematological profile

In recent years, hematological variables have been used widely to determine the sublethal concentrations of pollutants. Studies have shown that when the water quality is affected by toxicants, any physiological change will be reflected in the values of one or more of the hematological parameters [26]. In the present study, RBC counts and hemoglobin concentration were decreased in fish exposed to textile effluent. The erythrocyte of healthy control showed the mean value of $8.22 \times 10^6\,mm^{-3}$. The fishes exposed to different concentration of textile effluent show mean value of RBCs as 7.53, 6.28, 5.53, 4.60, and $4.10 \times 10^6\,mm^{-3}$ for 12.5, 25, 50, 75, and 100%, respectively. The results showed a drastic reduction in the total count of RBCs (Table 3), similar results with significant reduction of RBC in fishes exposed to different concentration of textile effluent and toxicants have been reported previously by Soni et al. [27] and Banu et al. [28]. The damaging effect of toxicant on erythrocytes may be secondary, the primary action of toxicant may be on erythropoietic tissues which causes a failure in red cell production, and/or may be due to increase in the erythrocyte destruction [29].

Poikilocytosis and anisocytosis assessments are the most sensitive parameters for examining toxicity of untreated and biologically treated wastewater [27,30]. The illustrative figures (Figs. 2a, 2b, and 2c) depict the physiological changes in hematological cells. RBCs in control fish were ovoid (Fig. 2a), whereas in textile effluent treatments they were in various shapes (poikilocytosis) such as beaked, tear-drop, spherical, elliptical, and pentagonal (Fig. 2b). During acute exposure, the percentage of poikilocytosis increased, with an

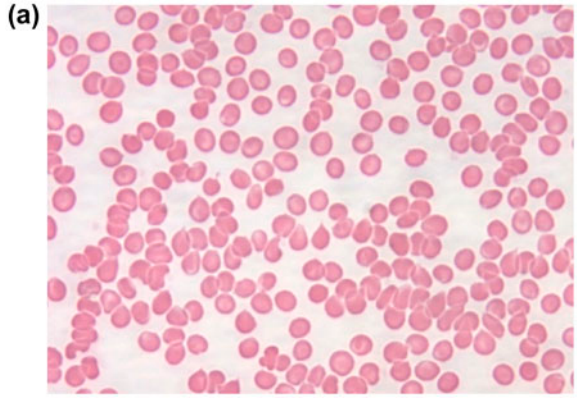

Fig. 2a. Normal RBC structure of untreated (control) *O. niloticus*.

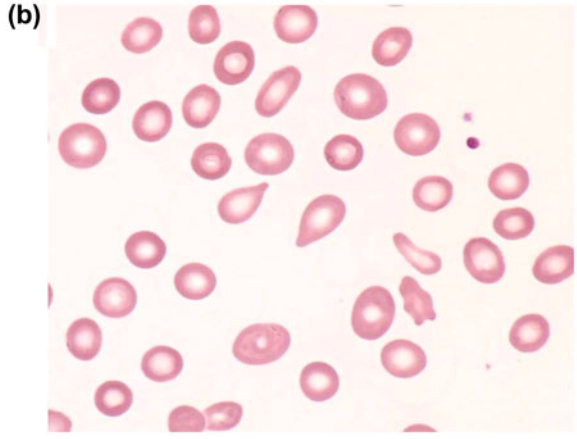

Fig. 2b. Morphologically abnormal RBCs (Poikilocytic) appearance in the raw textile effluent treated *O. niloticus*.

increase in concentration of textile effluent. The abnormal shape may associate with an altered surface membrane area to volume ratio and attributed to disorder of lipid metabolism [31]. Azo dyes may also cause hemolysis due to oxidative stress [32]. The cytotoxicity of dyes and textile dye wastewater to

Table 3
Hematological profile of the control and raw textile effluent treated *O. niloticus*

Group	% Dose (v/v)	No of RBCs ($10^6\,mm^{-3}$)	Hb conc. (g/dl)	Hematocrit (%)	MCV (ft)
1	0	8.22 ± 0.04	18.5 ± 0.03	28 ± 0.05*	34.06
2	12.5	7.53 ± 0.02*	14.96 ± 0.04*	24 ± 0.08	31.87
3	25	6.80 ± 0.02*	12.5 ± 0.03*	21 ± 0.08	30.88
4	50	5.53 ± 0.03*	11.0 ± 0.02*	13 ± 0.04*	23.50
5	75	4.60 ± 0.03	9.10 ± 0.03*	10.5 ± 0.04*	22.82
6	100	4.10 ± 0.01*	8.5 ± 0.26*	9.0 ± 0.02*	21.95

*Significant ($p < 0.05$), each value is a mean ± SE of three observations.

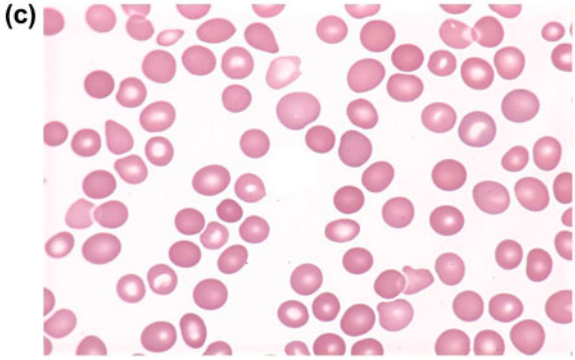

Fig. 2c. Morphologically abnormal RBCs (Anisocytic) appearance in the raw textile effluent treated *O. niloticus*.

erythrocytes in terms of poikilocytosis is well documented by Murugesan et al. [33] and Sharma et al. [30].

Anisocytosis is a variation in cell size (Fig. 2c). The discrepancy in red cell size is due to the presence of larger than normal RBCs (macrocytic) or smaller (microcytic) than normal RBCs. Thus, variations in RBC size were found to be the maximum, thereby establishing it to be the most sensitive index for expressing textile effluent toxicity. The MCV was significantly decreased from 34.14 ± 1.05 femtoliter (Ft) in control fish as shown in Table 3, which reflects an abnormal cell division during erythropoiesis. The decrease in MCV indicates that the RBC has shrunk during exposure to textile effluent [34]. MCV values are completely dependent on the factors of PCV (packed cell volume), RBC count, and hemoglobin concentration. In the present study, the MCV, RBC, and hemoglobin concentration decreased significantly ($p < 0.05$) at higher concentrations of untreated textile effluent. Similar reductions have been reported by Drastichova et al. [35] and by a number of research works in different fish [36]. Thus, the present study has shown relevance of data on fish hematological profile for measuring acute toxicity of pollutants.

The hematological parameter results of *O. niloticus* exposed to five days of biologically treated textile effluent show greater reduction in toxicity when compared with raw textile effluent treatment. The RBC count was greatly increased from 4.1 to 5.42×10^6 mm^3, the hemoglobin concentration from 8.5 to 14.3 gdl^{-1}, and so on (Table 4) in biologically treated effluent-treated fishes when compared to raw effluent treated fishes. Results obtained after biological treatment showed a remarkable reduction in toxicity of the textile industrial effluent.

Blood parameters are considered as pathophysiological indicators of the whole body, and therefore, are important in diagnosing the structural and functional status of fish to expose to toxicants. Hence, the present investigation shows the impact of effluent toxicity and the bioremediation using isolated bacterial *P. oleovorans* PAMD_1.

3.2. BOD/COD assay

Table 5, represents the physiochemical properties obtained from the raw textile wastewater sample analysis. The most fearsome values are for COD, BOD, and TDS which may cause a real threat to the environment. BOD and COD levels decreased from the initial concentration of 371 to 118 mg l^{-1} and 895 to 220 mg l^{-1}, respectively, after the biological treatment as shown in Table 6. BOD/COD ratio increased from the initial level 0.41 to 0.54 after 120 h of incubation with *P. oleovorans* PAMD_1. Thus the results obtained after biological treatment showed a very good relationship with other methods which had been reported to reduce the COD load of effluents below the upper limit of 250 mg l^{-1} [37]. The TDS value of the untreated effluent was initially high and in the biological treated effluent the overall reduction was also observed about 15.2%. The

Table 4
Hematological profile of the biologically treated textile effluent on *O. niloticus*

Group	% Dose (v/v)	No of RBCs (10^6 mm^{-3})	Hb conc. (g/dl)	Hematocrit (%)	MCV (ft)
1	0	8.34 ± 0.04*	18.3 ± 0.03*	29 ± 0.05*	34.77
2	12.5	7.90 ± 0.05*	17.9 ± 0.06	25 ± 0.03*	31.64
3	25	7.10 ± 0.02*	17.6 ± 0.03*	22 ± 0.08	31.00
4	50	6.20 ± 0.07	17.1 ± 0.02*	16 ± 0.07	25.09
5	75	5.50 ± 0.06	15.4 ± 0.03*	13 ± 0.01*	23.63
6	100	5.42 ± 0.01*	14.3 ± 0.26*	12.5 ± 0.02*	23.06

*Significant ($p < 0.05$), each value is mean ± SE of 3 observations.

Table 5
Physicochemical parameters of raw textile effluent

Sl. No	Parameters	Raw effluent	NEQS standard*
1	pH	5.49	6–10
2	Color (A-absorbance) 480 nm	>2.0	–
3	Temperature	32 ℃	40 ℃
4	Total Dissolved Solid (mg l^{-1})	4,400	3,500
5	BOD (20 ℃) (mg l^{-1})	371	80
6	COD (mg l^{-1})	895	150

*National Environmental Quality Standards, 1999.

Table 6
Physicochemical characterization of biologically treated effluent at different time interval

		Incubation time (hrs)*				
Parameters	Control	24	48	72	96	120
BOD (mg l^{-1})	371	234	162	148	134	118
COD (mg l^{-1})	895	524	336	268	244	220
BOD/COD ratio	0.41	0.44	0.48	0.55	0.55	0.54
TDS (mg l^{-1})	4,400	4,300	4,050	3,950	3,850	3,730
(A-absorbance) 480 nm	>2.0	>2.0	1.6	1.3	0.85	0.83

*Average of triplicates.

results indicated that biological treatment is more effective for reducing the toxic nature of textile effluent, which is in agreement with previous results reported by Ong et al. [38] and Pourbabaee et al. [37]. The COD level of original effluent was lowered significantly, while the BOD/COD ratio was greatly elevated, which indicates that some non-biodegradable organic materials are also removed in this biodegradation process. Thus the application of isolated bacteria *P. oleovorans* PAMD_1 is more effective process in the bioremediation of textile effluent.

4. Conclusion

The present study infers hematological parameters to be the best for quantifying acute and chronictoxicity of azo dyes. The removal efficiency level of physicochemical parameters has paved way for the adoption of the bacteria which were used. Results obtained after biological treatment showed substantial changes in physicochemical parameters and toxicity reduction. Therefore, the present study established the application of *P. oleovorans* PAMD_1 newly isolated bacteria to make a satisfactory system to tackle the textile industry waste water pollution. Hence, it seems to be a good strain for further research in large-scale treatment of textile industry effluent that may be toxic to the environment.

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