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Decolourization and detoxification of textile organic effluents by *Alcaligenes* sp. and application of medicinal plants to investigate acute toxicity of degraded products

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Abstract
The remediation of dye contaminated environment was evaluated using indigenous bacterial strains isolated from wastewaters from an effluent treatment plant of a Textile Industry in Kano, Nigeria. The isolates were identified using 16S rRNA gene sequence analysis as *Alcaligenes* sp. *APO4* and some of the isolates were mutated using UV-irradiation inducement. Both the wild and mutant types of the bacterial strains were separately cultured in thirteen (13) different reaction flasks containing the same concentration of organic effluent. Decolorization efficiencies of the bacteria was measured as a function of the operational parameters (aeration, dye concentration, pH, temperature, total viable count) and the optimal operational conditions obtained for decolorization of the dyes were: pH 6.5 -8.0 at 37 ºC. The mutant strains exhibited higher potential to decolorize and detoxify the dyes effluent up to 92.6% and 83.9% respectively after 24 days of incubation under aerobic condition. This study offers valuable inferences to the practical application of *Alcaligenes* sp. for the treatment of other sources of organic pollutants.

Keywords: *Alcaligenes* sp., decolourization, detoxification, organic dyes, textile effluent, toxicity

1. Introduction

There are several contaminants in wastewater, with organic pollutants playing the major role. Many kinds of organic compounds, such as PCBs, pesticides, herbicides, phenols, polycyclic aromatic hydrocarbons (PAHs), aliphatic and heterocyclic compounds are included in the wastewater (Lindholm-Lehto *et al.,* 2015) [15]. The industrial organic dyes, agricultural activities as well as anthropogenic sources are incidence that contributes to organic wastewater, endangering the safety of the water resource (Saraswathi & Balakumar, 2009; Thomas & Janz, 2015) [27]. It is estimated that approximately 15% of the dyestuffs are lost in the industrial effluents during manufacturing and processing operations (Khaled *et al.,* 2009) [11]. The presence of textile dye even in low concentration in effluent is highly visible and undesirable (AbdurRahman *et al.,* 2013) [1]. There are more than 100,000 commercially available dyes with over 700,000 tons of dyestuff produced annually (Cai *et al.,* 2015) [3]. Synthetic dyes are chemically diverse and divided into azo, triphenylmethane or heterocyclic/polymeric structures (Cheunbarn *et al.,* 2008; Kanmani *et al.,* 2011) [4, 13]. Discharge of textile dyes without proper treatment may lead to bioaccumulation that may incorporate into food chain and affect human health. Azo dyes inhibit the activity of tyrosinase enzyme that leads to inhibition of melanin synthesis and results in hypo pigmentation (Dubey *et al.,* 2007) [6]. Presence of dyes in aqueous ecosystem increases the level of total organic carbon (TOC), salt concentration and pH (Franciscon *et al.,* 2012) [7]. These factors not only diminish the photosynthesis by impeding the light
penetration into deeper layer of water bodies, but also deteriorate the water quality and cause lowering of the gas solubility in water, resulting in acute toxic effect on aquatic flora and fauna, which severely affects the environment (Saratale *et al.,* 2009) [22].

The existing conventional physico-chemical wastewater treatment methods such as coagulation, flocculation, membrane filtration etc. were found to be less effective, since they exhibit drawbacks such as operational complexity, inability to remove the recalcitrant azo dyes and their metabolite and also generate large amount of sludge as secondary pollutants and the whole process is expensive in terms of cost (Maulin *et al.,* 2013; Tahir *et al.,* 2015) [26]. This poses a challenge to researchers in finding alternative biological methods that is efficient and cost effective, to clean the environment. The microbial decolorization and detoxification of azo dyes is of considerable interest and is promising since it is inexpensive, eco-friendly, producing less amount of sludge (Joshi *et al.,* 2008; Lade *et al.,* 2015) [10, 14]. Microbes such as bacteria, fungi, yeasts, actinomycetes and algae are normally used for the decolourization of various organic dyes (Saratale *et al.,* 2015) [23]. The prime objective of this research, is the isolation of organic dyes decolorizing bacterium from a dyes contaminated environment, and test its ability to decolorize organic components of textile dyes to a non-toxic products and to induce mutation in the bacterium by UV- irradiation/ photochemical mutagens and also to compare

the activities of the mutant type with wild type and to assess the toxicity of treated (degraded) sample.

2. Materials and methods

2.1 Sample collection

The textile dye effluent samples were collected from Common effluent treatment plant, Kano-Nigeria, in sterile plastic containers. The samples were brought to the laboratory and processed within 48 hrs. This sample was used in isolation of microbes and in treatment trials.

2.2 Pre-treatment of the sample

A filter paper was folded and inserted on the mouth of the soxhlet extractor unto which some quantity of XAD-2 resin was carefully dispensed. 2.5 liters of textile wastewater was measured using graduated cylinder and carefully dispensed in the XAD-2 resin. The organic components of textile waste effluent were retained within the organic resin as residues and the filtered were collected and discarded. The resulting residues as the desired component needed for the analysis, were allowed to dry for 2 hrs within the soxhlet extractor and then 50 cm^3 of ether was passed through the dried residues of XAD-2 resin contained in the soxhlet extractor, and all the organic components in the XAD-2 were miscible with ether as organic solvent which are collected as a filtered and were warm gently in a hot air oven at 180 ^oC to allow the ether to evaporate in 48 minutes. The solid deposit was allowed to cool to 35 ^oC and weighed to 30 grams, which were diluted to 1000 cm^3 with sterile de-ionized water and properly labelled as a stock solution (1000 mg/dm^3).

2.3 Isolation and enumeration of bacterial cultures

Total bacteria were enumerated by spread plate method using 0.1 ml of the dilution 10^{-1} to 10^{-4} onto nutrient agar. All cultures were incubated for 24 hr to 48 hr at 37 ^oC. After incubation morphologically distinct colonies were selected and screened for textile effluent decolourization (Khalid *et al.*, 2008) [12]. The candidate isolates were picked and transferred in 1 ml Luria Bartani (LB) broth and incubated at 25 ^oC for 48hrs. The pure culture was prepared and stored at 4 ^oC for further studies.

2.4 Decolourization of textile dye effluent by isolated bacterial strains

For checking the dye effluent decolorization by bacterial strains, decolorization assay was performed, in which the bacterial colonies from pure culture were transferred to 25 ml Luria bartani (LB) broth and allowed to grow for 48 h at 37 ^oC. The effluent was mixed with the bacterial culture at 1:1 (v/v) and incubated at 30 °C under shaking conditions for 48 hrs un-inoculated effluents with LB broth served as control. Decolorization was determined in 5 ml aliquots from each flask after 48 hrs of incubation. The culture was centrifuged at 10,000 rpm for 15 min to remove the cells. The absorbance of the supernatants was measured at 533 nm using spectrophotometer at regular intervals of 1 hr during the decolourization process.

2.5 Identification and taxonomic characterization of dye decolorizing bacterial strain

Bacterial cells from cultures of isolated strain *AP04* was collected by centrifugation at 10,000 rpm for 10 min. The identification of strain *AP04* was performed by using standard morphological, gram staining, biochemical and molecular characterization methods as described by Franciscon *et al.* (2012) [7]. The sequential digestion was done by lysozyme (2.5 mg/ml, 37 ^oC for 1 h) and proteinase K (200 mg/ml in 1% SDS, 55 ^oC for 1 h), followed by incubation in 1% CTAB and 0.7M NaCl at 65 ^oC for 15 min. Ice cold ethanol was used for the precipitation of DNA after extraction with phenol/ chloroform and then dissolved in dH_2O. The universal 16S rRNA primers 8f (5´-GAG TTT GAT CAT GGC TCA G-30) and 1495r (5´-CTA CGG CTA CCT TGT TAC G-30) were used and PCR amplification was performed in total reaction volume of 20 µl by using Master Mix RED (150mM Tris-HCl, pH 8.5, 40 mM $(NH4)_2SO_4$, 4.0mM $MgCl_2$, 0.2% Tween 20; 0.4 mM dNTPs; 0.05 U/µl Amplicon *Taq* DNA polymerase; inert red dye and a stabilizer).

The PCR conditions used were 94 ^oC for 5 min as initial denaturation, 30 cycles at 94 ^oC for 1 min final denaturation, 54 ^oC for 1 min annealing, 72 ^oC for 3 min extension and final extension was at 72 ^oC for 10 min (Su *et al.*, 2007) [25]. The amplified PCR product was ran on 0.8% agarose gel and nucleotide sequences of purified PCR products were determined by sequencing, using two universal primers 8f and 1495r (Staley *et al.*, 2001) [24]. The length of 16Sr RNA gene sequence was found to be 387 base pairs and this sequence was subjected to BLAST search tool in gene database of NCBI (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) and corresponding sequences were downloaded. Clustal W multiple sequence alignment tools were used for the alignment of sequences and phylogenetic tree was constructed by using MEGA 5.2 by neighbour-joining method (Ooi *et al*., 2007) [19].

2.6 Growth condition, media and irradiation

After 70-80% confluent cells were cultured in mineral salt medium overnight, UV-irradiation was performed on the cells according to the method of Dhan Wada *et al.* (1995). UV-irradiation was carried out in a chamber safe for UV-B exposure. The UV-dose was quantified in joules per square meter with the use of a microvolt ammeter. The bacterial strains were grown in mineral salt medium on Luria Bertani (LB plates). In all experiments, the strains were grown from a single colony in mineral salt medium at 30 ^oC until they reached an optical density at 600 nm (Dhan Wada *et al.*, 1995); For UV-experiments, serial dilutions of bacterial cultures were plated on LB plates and irradiated with a dose of UV (280 nm) light of 10 j/m^2, with a rate of 0.25 $j/m^2/s$. Colonies of survivors were scored after 24 to 48 hrs of incubation at 30 ^oC and later sub-cultured on nutrient agar ready for broth formation (Cheesbrough *et al.*, 2005).

2.7 Mineral salt broth for wild and mutant types of *Alcaligenes* species

Mineral salt medium 99.9 cm^3 was dispensed into 250 ml Erlenmeyer flask and distinct isolates from nutrient agar slants were picked gently using a sterile wire loop and inoculated into the mineral salt medium. The mixture was shaken and incubated at room temperature of 30 ^oC for 24 hrs, the mineral salt broth for bacterial strains were kept near a freezing point in order to have a control of microbial load (inoculums size).

2.8 Growth of bacterial strains in organic effluent

Mineral salt broth of bacterial strains were dispensed in 99.0 cm^3 quantities into 250 ml Erlenmeyer flasks which were arranged sub sequentially thirteen (13) in number, in order of one day interval. To each flask was added 1 cm^3 of the effluent sample. The flask was inoculated at optimum temperature of bacterial strains in an incubator spun at 20 rpm for twenty four (24) days. Controls were run under the same reaction conditions of 99 cm^3 mineral salt broths with 1 cm^3 of effluent but excluding bacterial strains and the experiments were performed in three replicates. The optical density (absorbance) at different wavelengths, the total viable count (TVC), concentration and pH of cultures were determined (Cheesbrough *et al.,* 2005).

2.9 Extraction and analysis of degraded product

The bacterial strains was inoculated in minimal medium containing 100 cm^3 of organic dye effluents and incubated under ambient conditions. After complete decolorization, dye degraded samples were subjected to centrifugation at 10,000 rpm for 10 min. Supernatant obtained was filtered through Whatman #1 filter paper and then extracted thrice with diethyl ether. The extract obtained was evaporated to dryness in rotatory evaporator (Parshetti *et al.,* 2006) [20].

2.10 Dye decolourization assay

Aliquots of 2 ml of a clear dye solution was aseptically withdrawn after ever 24 hrs and centrifuged at 10,000 rpm for 15 min. the supernatant obtained after centrifuging was read at maximum absorbance (λ_{max}) and using a UV-Visible recording double beam spectrophotometer (Agilent 8453). Biotic and abiotic controls were carried out side by side. Care was taken not to draw out portions of the microbes in the aliquot. All samples from the culture medium had to be diluted prior to measurement in order to keep the change in absorbance values measured below 1.0 absorbance units per centimeter of path length. Because of the low water solubility of the organic dyes, an equal volume of methanol was mixed with the analytical solution to ensure complete solubilization prior to measurement. Decolorization was assessed by monitoring spectrophotometrically the absorbance at the wavelength maximum for each cultured solution and also by the reduction of the major peak area in the visible region for each cultured solution.

This approach was used because color change may occur during biodegradation with a concomitant wavelength shift. Therefore, using the wavelength maximum of the cultured solution alone would not be sufficient to accurately reflect the full degree of decolorization. To obtain additional information regarding the changes, the area under the curve in the visible regions (400-800 nm) was integrated. Dye decolorization activity was calculated by applying the following formula (Saratale *et al.,* 2006) [21].

$$\textbf{Percentage decolourization (\%)} = \frac{X_i - X_f}{X_i} \times 100 \qquad (1)$$

Where, $\mathbf{X_i}$ is the initial absorbance and $\mathbf{X_f}$ is the observed absorbance.

2.11 Total organic carbon measurement

Alcaligenes sp. *AP04* treated organic dyes effluent culture was analysed for change in (Total organic carbon) TOC using a TOC analyser (Shimadzu, Japan). Culture was centrifuged at 10,000 rpm for 10 min and then the supernatant was analysed at interval of 6 days (Table 1).

2.12 Toxicity study on decolorized products

The native organic dyes and its degraded products (after incubation period) were evaluated for their phytotoxic effect (Alabi *et al.,* 2005; Parshetti *et al.,* 2006; Mehta & Bhardwaj, 2012; David & Rajan, 2015) [2, 20, 17, 5]. The studies were carried out using *Moringa oleifera*, *Vernonia amygdalina* and *Monodora myristica* seeds. Five seeds of each were grown in the petri plates (Borosil; 100 9 17 mm) with daily supply of 5 ml of the treated sample of dyes effluent and its degraded products in the concentration range 4,000 ppm and incubated at room temperature. The experiment was carried out in replicate of three. After 7 days growth, length of plumule and radical were measured. Percentage of germination was also recorded (Jadhav *et al.,* 2008) [9] and cytotoxicity was tested by brine shrimp (*Artemia salina*) lethality assay (Meyer *et al.,* 1982) [18].

3. Results and discussion

In biological treatment, the record of degradation and decolorization of organic effluents involves a sequence of steps including mass transfer, operations like absorption and biochemical enzymatic reactions. The activated sludge process is that which involves the development of an activated mass of microorganisms, capable of stabilizing waste aerobically. The textile dye effluent has yielded four effluent decolorizing and morphologically distinct bacterial strains namely *AP01*, *AP02*, *AP03*, *AP04* and *AP05*. They were isolated and screened for their textile dye effluent decolorization ability with inoculum suspension of 1:1 (v/v) ratio (Fig.1). *AP04* was found to be the most efficient effluent decolorizing strain. It showed 72.8% decolorization in 24 hrs, while other strains showed less decolorization abilities. Therefore, strain *AP04* was selected for further study. The wild strains of *Alcaligene* specie demonstrated a remarkable role to decolorize and detoxify organic components of textile effluent to 42.23% and 51.09% respectively as shown in Fig. (2A). Also the visible portion of the spectrum of organic dyes effluent showed a major peak at 575 nm after 24 days period. This peak shifted to 328 nm with an increased in total viable count from 1.7 x 10^6 cfu/ml to stationary phase 4.1 x 10^6 cfu/ml. While the mutant type of *Alcaligenes* sp. exhibited maximum decolorization and detoxification of organic dyes effluents by 92.64% and 83.9% respectively with the shift in major peak from the visible region of the spectrum from 547 nm to 319 nm after 24 days period of treatment and also with an increased in total viable count from 1.2 x 10^6 cfu/ml to a stationary phase 6.9 x 10^6 cfu/ml and 1.7 x 10^6 cfu/ml (Fig. 2B). Changes in pH were observed due to degradation and decolorization (redox reaction) that took place in the reaction flask, which seems to appear in a straight line as indicated in Fig. 2. Significant reduction in concentration of dye metabolites was observed, indicating detoxification and decolorization by *Alcaligenes* strain. This result recorded corroborate with the findings of Saraswathi & Balakumar, (2009).

The reduction of the major peak in the visible region indicates substantial decolorization and this was indicate by the

decrease in absorption at the maximum wavelength and the decrease in the area under the absorption curve in the visible region. No new peak appeared in the UV-vis region after decolorization and usually decolorizing activity was found to increase with increasing in dye concentration. The remarkable results shown by the mutant types of *Alcaligenes* sp. demonstrate that the mutant forms are potentially more active textile dyes degraders than wild type which means that the mutant types, when altered using UV irradiation carries special genetically codes (enzymes) responsible for textile dyes degradation. Hence the result above solely agrees with the finding of Hai *et al*. (2007a) [8].

The absorptivities are intrinsic properties of a colorant. Measuring the spectral absorption curves of dyes during biodegradation provide a means to determine the reaction rate and give evidence of the structural change of dyes during biodegradation. Previous experience showed that fading of the original colour of the dyes is sometimes accompanied by a color change (wavelength shift) in the solution. Because of this, both the ultraviolet and visible regions were monitored by a UV-Vis spectrophotometer to provide more information about the biodegradation. The decrease of the maximum absorbance of the dyes occurred primarily in the first several days of treatment, with no substantial additional decrease in absorbance as the treatment time increased. The monitoring of the UV-Vis spectra supplied information on the nature of the decolourization and detoxification, particularly with the shifts in the absorption maxima. These could only be the result of chemical change in the conjugated double bond structure, rather than just removal of the unchanged dye molecules. Biosorption shown by *Alcaligenes* sp. demonstrated a very promising result for decolorizing organic components of textile effluents. Spectrophotometric analysis indicated that dye decolorization and detoxification occurred due to the breakdown of dye molecules into colourless end products. The total organic carbon (TOC**)** of the medium containing the dye (100 mg/ml) was initially 1,950 mg/l. It was decreased to 65% and 92% in 24 hrs for wild and mutant strains of *Alcaligenes* sp. contained in the decolorized samples respectively (Table 1).

The use of treated and untreated dyeing effluents has a direct impact on the environment and therefore a source of concern and the need to assess the phytotoxicity and cytotoxicity of the dyes before and after decolorization and degradation become necessary. The result of relative sensitivity test of *Moringa oleifera*, *Vernonia amygdalina* and *Monodora myristica* were also studied (Table 2). In this study of phytotoxicity, the mean length of plumule and radical of *M.oleifera* were 20.4±0.72 cm and 9.4±0.61 cm, and *V. amygdalina* 18.3±1.44 and 8.94±0.25 cm respectively. While *M. myristica*, 19.57±0.83 and 8.27±0.42 cm. Distilled water was used as a control to achieve 100% germination of 5 seeds. In contrast, plumule and radical length was 18.2±0.34 cm and 8.29±0.67 cm for *M. Oleifera*, 15.1±0.16 cm and 7.62±0.58 cm for *Vernonia amygdalina* and 16.25±0.24 and 7.19±0.2 cm for *M. myristica* respectively. *M. oleifera*, *V. amygdalina* and *M. myristica* attained 100% germination when treated with 4,000 ppm sample of decolorized products (Table 2). In the phytotoxic study, the 4,000 ppm of organic effluents has caused inhibition of germination of *M. oleifera*, *V. amygdalina* and *M. myristica* seeds by 70, 55 and 60% respectively and the length of radical and plumule were decreased in comparison to control (distilled water) respectively. The dye degraded sample did not show any inhibitory effect on seed germination and the length of radical and plumule were decreased (Table 2). These results suggest that dye degraded products are less toxic than original dye causing almost negligible inhibitory effect on seed germination, radical and plumule length. The possible reason for this is the inhibition of the seed germination pathway or its regulators by the dye. The phytotoxicity studies revealed that biodegradation of organic components of textile effluents with *Alcaligenes* sp. *AP04*, also led to detoxification of pollutant. Cytotoxicity assay revealed a decrease in toxicity of organic dyes effluent after treatment with the control dye samples, and the percentage mortality of brine shrimp larvae was 30%. In the case of treated sample containing wild strains of *Alcaligenes* sp. *AP04*, the percentage was 10%, whereas no cell death was observed in treated sample containing mutant strains of *Alcaligenes* sp. *AP04* (Table 3).

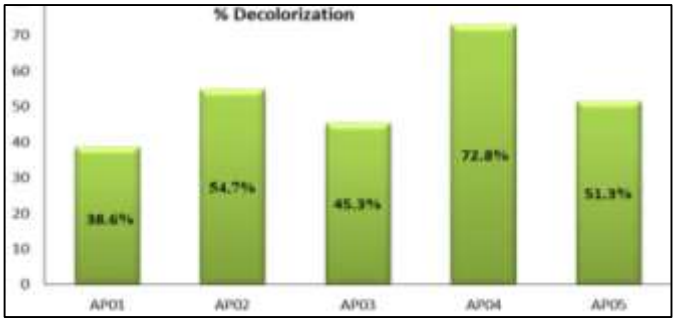

Fig 1: Shown are the dyes decolorizing efficacies of the five isolated strains as a measure of their percentages

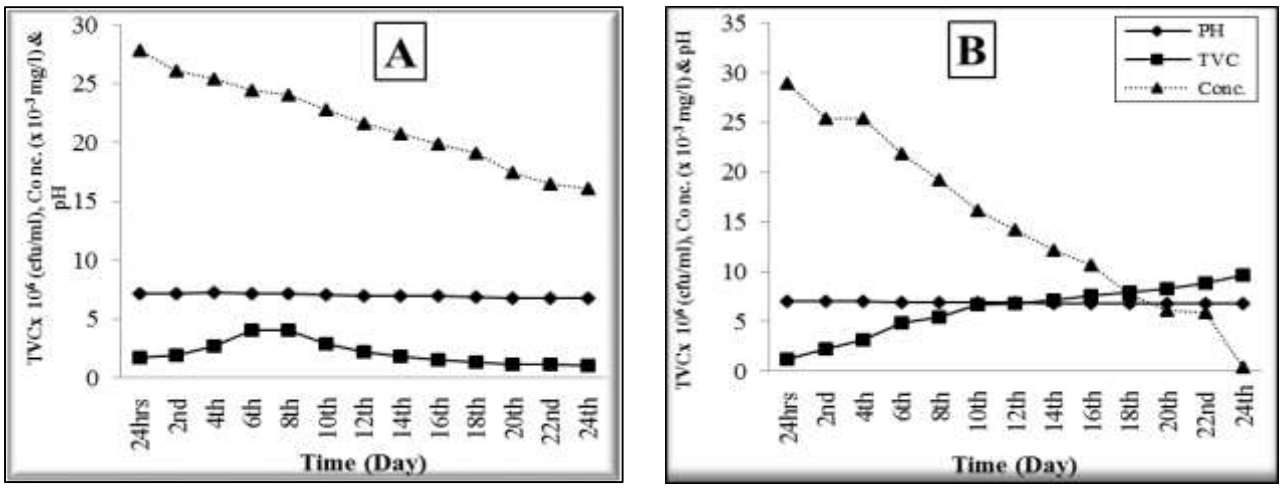

Fig 2: Growth profile of wild (A) and mutant (B) types of *Alcaligenes specie* in mineral salt medium

Table 1: Total organic carbon reduction of wild and mutant strains of *Alcaligenes* sp. *AOP4* treated culture of the decolorized samples containing organic components of textile effluents

Time (day)	TOC Reduction (%)	
	Wild strains decolorized sample	Mutant strains decolorized sample
24 hrs	28 ± 0.57	26 ± 0.97
6th day	37 ± 1.23	39 ± 0.64
12th day	41 ± 0.81	49 ± 0.76
18th day	54 ± 1.01	72 ± 1.23
24th day	65 ± 0.92	92 ± 0.77

Table 2: Comparison of phytotoxicity of organic dyes decolorized samples for wild and mutant strains of *Alcaligenes* sp. *AOP4* after incubation period

Parameter studied	*M. oleifera*	Wild strains decol. sample	Mutant strains decol. sample	*V. amygdalina*	Wild strains decol. sample	Mutant strains decol. sample	*M. myristica*	Wild strains decol. sample	Mutant strains decol. sample
	Control			Control			Control		
Radical length (cm^3)	9.4±0.61	4.7±0.86	8.29±0.67	8.94 ±0.25	4.51±0.63	7.62±0.58	8.27±0.42	5.19±0.27	7.19±0.20
Plumule length (cm^3)	20.4±0.72	13.1±0.27	18.2 ± 0.34	18.3±1.44	11.3±0.33	15.1±0.16	19.57±0.83	12.2±0.11	16.25±0.24
% Seed germination	100	70	100	100	55	100	100	60	100

Table 3: Cytotoxicity of the treated samples

Samples	No. of Nauplii added	Nauplii alive (n)	Nauplii death (n)	% Mortality
Positive control	10	7	3	30
Negative control	10	10	0	0
Decolorized sample containing wild strains of *Alcaligenes sp. AP04*	10	8	2	10
Decolorized sample containing mutant strains of *Alcaligenes sp. AP04*	10	10	0	0

4. Conclusion

Most water treatment plants are faced with increasing organic contaminants particularly due to increased production pressure and discharge restrictions. Often the existing microbiological treatment cannot handle these operational changes. Ever increasing organics, metals and solids loading, many of which are constantly prone to upsets and carryover in biological treatment process. System upgrades or expansion are one answer to keep the plant in compliance, but a major capital investment. Biological stimulation of *Alcaligenes* sp. is a cost effective alternative. It enable plants to meet discharge limits with existing treatment facilities, while minimizing capital expenditure by reducing or eliminating surcharges and fines (Mahbub *et al.*, 2011) [16], and this could also minimize the disruption of the natural habitat of the ecosystem (Cheunbarn, 2008) [4].

A potential organic dyes decolorizing strain *Alcaligenes* sp. *AP04* was isolated from an effluent treatment plant and showed great decolourization potentials on textile dye effluent. Complete biotransformation of organic components of textile waste effluent was confirmed by UV-Vis analysis and decolourization of dye was confirmed with phytotoxicity studies. This study further indicates that the organic dye effluents can be degraded using this bacterial culture and the selected plants may be used for the removal of some dye effluents from wastewater (bio-coagulants). Therefore, further

research is needed to monitor and quantify these substrates interactions in the decolourization and degradation of organic components of textile waste and its derivatives. The optimization of the degraded fragments produced is of great interest and concern as this might increase or lead to the toxicity of the overall aquatic and terrestrial environment.

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