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Toxicity Assessment of Biologically Degraded Product of Textile Dye Acid Red G

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ABSTRACT

Azo dyes are of environmental concern due to their recalcitrant nature. Several azo dyes and their decolorised and degraded products exert toxic and mutagenic effects on the flora and fauna. The nature and position of the aromatic rings and amino nitrogen atoms play an important role in the toxic properties of azo dyes. Several studies have thus far been emphasised on biodegradation of azo dye pollutants, though the role of their biodegraded product is rarely studied. Given a lack of this understanding, we have analysed the effects of degraded products of a di-azo textile dye Acid Red G by newly isolated bacterial species, *Pseudomonas aeruginosa* PFK10, and *Brevibacillus choshinensis* PFK11. The genotoxicity and cytotoxicity of Acid Red G and their degraded products were tested on the HeLa cell line and Human lymphocyte cell, respectively. The data of MTT assay has been shown that activity of degraded products of the Acid Red G was comparable to their parent dye. But chromosome aberration assay and sister chromatid exchange assay did not show any significant changes in chromosomes as compared to positive control mitomycin.

Keywords: Azo dye; MTT assay; Comet assay; Chromosomal aberrations assay; Sister chromatid; Exchange assay

1. INTRODUCTION

The discharge of untreated textile industry effluents into the environment is detrimental due to its toxic and mutagenic intermediate products formed by the breakdown of dyes present in effluents[1-3]. Textile effluents are rated as a major source of pollution among all the industrial sectors that are directly discharging into drinking water resources, like rivers, wells, lakes, etc. without treating them properly[4]. Textile industries generate effluents having high chemical oxygen demand and biochemical oxygen demand, which make treatment of these effluents very difficult[5-7]. This effluent sometimes affects gas solubility in water bodies and because of reduced light penetration, it decreases photosynthetic activity in aquatic life which may lead to declining of living organisms present in water bodies[8]. Detoxification of dye after biodegradation is of prime concern for maintaining several pollutants in water and soil. Therefore, the treatment of effluent of the textile industry becomes necessary prior to their final discharge to environment.

Azo dyes are one of the most commonly used synthetic dyes in different industries such as in textile dyeing and printing, food and cosmetics as well as paper and leather. The toxicity of effluent is due to the presence of dye or its partially degraded products which may have a detrimental impact on flora and fauna of aquatic life form. Studies revealed that azo dyes are harmful to cells, as they induce the formation of multilobulated and extremely condensed nuclei, micronucleated cells and also induce binucleated as well as endoreplication cells[9]. It is also reported that the cytotoxic effects detected due to azo dyes may be due to the direct action of dyes on the cells or the formation of secondary products resulting from the azo bond reduction[10]. The secondary products can interact with the DNA molecule, damaging both their structure and function[11].

There are several other methods such as flocculation, precipitation, coagulation, adsorption, membrane filtration, irradiation, ozonisation, and Fenton's oxidation methods are being used for the treatment of dye wastewaters. These physical and chemical treatments are more expensive and can generate a large amount of dye contaminated sludge. Due to expensive cost for treating dye by physical, chemical and photochemical approaches, another method significantly attracted attention for the treatment of textile wastewaters which is also a more cost-effective alternative and recognised as a biological method. In biological methods, bacterial or fungi produce a number of different enzymes (azoreductase, tyrosinase, manganese peroxidases, and laccase) which catalyse the oxidation of several phenolic and aromatic compounds of different dye with or without the use of cofactors irrespective of their symmetry. During biodegradation of azo dyes, some secondary products generated that impact on mutagenicity, genotoxicity and carcinogenicity are vaguely reported.

Thus, in the present study, the activity of biodegraded product of di-azo dye Acid Red G against mutagenicity,

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genotoxicity, and carcinogenicity have been assessed. In the present study, the toxicity profile of a textile di-azo dye Acid Red G and their biodegraded products was analysed by performing (i) MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (ii) chromosomal aberrations (iii) sister chromatid exchange (SCE) assay and (iv) comet assay (single cell gel electrophoresis assay).

2. MATERIALS AND METHODS

2.1 Media

During the determination of bacterial activity for decolorisation and degradation of Acid Red G, Bushnell and Haas's medium was used. The pH of the medium was adjusted to 7±0.2. DMEM- Dulbecco's Modified Eagle Medium was used for culturing HeLa cell lines[12]. *Pseudomonas aeruginosa* PFK10 and *Brevibacillus choshinensis* PFK11were cultured in the Luria-Bertani medium at 37 °C.

2.2 Collection of Bacterial Isolates, Sample Collection, and Analysis

Several bacteria were isolated from textile effluent which was collected from a treatment plant at Ankleshwar, Gujarat, India. The textile di-azo dye Acid Red G was procured from the textile dye manufacturing and dying industry. Initially, textile di-azo dye Acid Red G was treated with different bacterial strains under sequential static – shaking condition for 24 h at 200 rpm. The capability of discoloration of textile di-azo dye Acid Red G by different bacterial strains was initially evaluated. Finally, two bacterial strains were selected on the basis of their higher potential for discoloration of textile di-azo dye Acid Red G. Therefore, supernatants of di-azo dye Acid Red G treated with these bacterial strains were obtained by centrifugation at 10,000 rpm for 15 min. Supernatants were acidified by adding concentrated HCl and then the double amount of ether was added, and the organic phase was collected. Organic phase supposed to have consisted of degraded product of dye Acid Red G. Ether was evaporated and dryness of extraction was brought by adding anhydrous Na_2SO_4 at room temperature. The parent dye and its degraded products by two different bacteria were dissolved in DMSO for further analysis. The partial genome sequence of these two bacterial strains (*P. aeruginosa* PFK10 and *B. choshinensis* PFK11) were determined.

2.3 Cell Cultivation and Seeding of HeLa Cells

The HeLa cells were procured from the National Repository of Animal Cell Culture (NRACC), National Centre for Cell Sciences (NCCS), Pune, India. The cells were cultured in D-MEM[13]. Trypsin solution was used to detach these confluent cells (70% - 80%) in corning flasks. Thereafter, trypsin was removed, and cells were re-suspended in D-MEM growth medium. The counting of cells was performed using a hemocytometer. Thereafter, cell density was adjusted to $1x10^5$ cells per milliliter. The suspension of cells (1000 µl) was transferred into a pre-labeled microcentrifuge tube. Three sets for a replica for each concentration (5 µl - 500 µl) of the compound were made (MTT Assay). Two sets for replica for each concentration (20 µl and 50 µl) of compound used were made with negative control, positive control and internal control (Comet Assay). The tubes were incubated at 37 ^{O}C and 5 % CO_2 for 24 h.

2.4 MTT Assay

The MTT assay is used to quantify cell metabolic activity and proliferation of HeLa cell lines. The cells in its active metabolic state can convert the yellow tetrazolium salt MTT into the purple formazan crystals. The intensity of the purple color is a measure for the activity of the enzyme NAD(P) H-dependent cellular oxidoreductase in the cells which can be determined spectrophotometrically at a wavelength of 540 nm[29]. The cells will not be able to form the formazan crystals from the MTT dye if the dye is toxic to the cells. The MTT assay was conducted using a slightly modified method[14,15]. After seeding the cells in 96- well microplate, it was incubated 37˚C at for 24 h in an incubator. After that, the cells were exposed to the D-MEM growth medium consisting test compound and incubated for a further 24 h at 37 ^{O}C under 5 % CO_2 condition as shown in Table 1. Each compound was tested in 11 different concentrations (5 µg, 10 µg, 20 µg, 40 µg, 50 µg, 80 µg, 100 µg, 150 µg, 200 µg, 250 µg, 500 µg) and positive and negative control were mitomycin (50 µg) and DMSO respectively).

A 100 µl of MTT solution (5 mg/ml) was added to the wells (tissue culture well plate 24, HiMedia)) and incubated for 1h at 37 °C under 5% CO_2 conditions. After incubation, the medium was removed and washed with N-saline to remove excess dye from vials before the addition of DMSO (200 µl) into each well. The purple color was measured at wavelength 540 nm[16].

Table 1. The concentration of dye metabolites PFK10 and PFK11

MTT Analysis	
S. No.	**Concentration (µl)**
1	5
2	10
3	25
4	50
5	75
6	100
7	125
8	175
9	200
10	250
11	500
12	DMSO
13	Mitomycin C (50)
14	Negative control

2.5 Comet Assay

In comet assay, the DNA lysis and electrophoresis are performed under neutral or alkaline environments. The DNA is stained using acridine orange dye. This metaphor from astronomy is visually appropriate as the resultant image obtained with this technique seems like a "comet", the distinct

head consisting of intact DNA and a tail containing damaged or broken pieces of DNA i.e. as the tail intensity is higher than the head, it indicates DNA damage[17]. The Comet assay was used to detect single and double-stranded DNA damage. The assay was carried out as per a slightly modified technique given by Singh[18].

A 180 µl of 1 % normal melting point agarose (HiMedia) was gelled on a fully frosted slide (75 mm x 25 mm). 100 µl of 0.5 % low melting point agarose containing 20 µl cell suspension as shown in Table 2 was layered on the top of the NMP agarose. Once the cell suspension layer forms, again 100 µl of LMP agarose was added to fill the residual hole to form an additional layer to increase the distance between the gel surface and HeLa cells. Once the agarose gel solidified, the slides were placed in freshly prepared lysis solution [2.5M NaCl, 100 mM EDTA and 10 mM Tris-HCl (pH 10) and 1% Triton X-100 and 10% DMSO] for 24 h at 4 °C. Thereafter, slides were incubated in an alkaline electrophoresis buffer (300mM NaOH/1mM EDTA, pH >13) for 40 min and then run electrophoresis for 35 min. Followed by alkaline slides gel was neutralised by washing three time with 0.4 M Tris buffer (pH 7.5). The slides were then stained with the fluorescent dye Ethidium Bromide (EtBr) and the comet images were captured under the fluorescence microscope. The portion of DNA damaged was examined by scoring 100 comets for each group (Tri-Tek Comet-Score™ V1.5 software, Germany)[19].

Table 2. Details of samples used for chromosome aberrations study and sister chromatid exchange assay

Test sample	Concentrations (µl)	
	Chromosome aberration study	Sister chromatid exchange assay
PFK10 [A]	20	20
PFK10 [B]	50	50
PFK11 [A]	20	20
PFK11 [B]	50	50
Acid Red G [A]	10	10
Acid Red G [B]	20	20
DMSO	50	50
Mitomycin C	50	50
Negative control	50	50

2.6 Chromosomal Aberrations Assay

Agents causing structural chromosome aberrations in cultured mammalian cells can be identified by the *in vitro* chromosome aberration test. If any chemical has the potential to induce aberration, it is indicated by an increase in polyploidy. Cell strains, cultures of established cell lines as well as primary cell cultures may be utilised in this assay[20]. In the present study, we have used human lymphocyte cells to check the toxic effect of the dye (Acid Red G) and their degraded products. The following experiments were carried out to understand the impact of parent dye and their degraded product on human lymphocyte cells.

2.6.1 Lymphocyte Culture

To know the effect of dye and their degraded products on normal healthy human lymphocyte cells, blood was collected from healthy young normal unexposed and non-addict individuals after signing of his/her informed consent and after giving knowledge of the study.

Lymphocyte cultures were set up by the Hungerford method with slight modifications[21]. A 0.5 ml whole blood was supplemented to a mixture containing 5ml of culture medium of Roswell Park Memorial Institute1640 (RPMI), 0.1ml phytohemagglutinin (Lectin) and 0.05 ml heparin. After that, the culture vials were incubated at 37 °C for 72 h where different doses of dye Acid Red G treated bacterial samples (PFK10: 20,50 µg, PFK11: 20,50 µg), parent dye Acid Red G (20 µg and 50 µg), positive control (mitomycin 50 µg) and negative control (DMSO 50 µg) were added at 48th h of incubation. A 0.1ml demecolcine solution was added at 70th hour of incubation period aiming to arrest cells in metaphase stage. The cells were collected by centrifugation (10000rpm, 10mins) and re-suspended in pre-warmed hypotonic KCL solution (0.075 M) for 15 min and then fixed in chilled methanol/acetic acid (3:1 v/v) (Carnoy's fixative) solution. Suspensions of cells were prepared after several changes of Carnoy's fixative washes and then slides were prepared. These slides were dried on a hot plate (50 °C - 60 °C). The slides were blind coded and labeled after assuring about well spread chromosome. Further, slides were stained using 2 % Giemsa's stain for 10 min. During observation, from each slide, 100 well spread chromosomes in metaphase were scored for chromosomal aberrations study.

2.6.2 Sister Chromatid Exchange Assay

Sister Chromatid Exchange Assay (SCEs) test detects the reciprocal exchanges of DNA between two sister chromatids in duplicating chromosomes. SCEs describe the interchange of DNA replication products at apparently homologous loci. The exchange process presumably engages DNA breakage and its reunion[22].

SCE assay was performed using Wolff and Perry method[23]. The lymphocyte culture was set up accordingly to the Hungerford method and after 24 h of incubation, 50 µl 5-Bromo-2-deoxyuridine (5 mg/ml; Sigma) was added in SCE labeled culture vials. Then the vials were incubated in dark for 48 h at 37˚C. Different doses of dye Acid Red G treated bacterial samples (PFK10: 20,50µg, PFK11: 20,50 µg), parent dye Acid Red G (20 µg and 50 µg), positive control (mitomycin 50 µg) and negative control (DMSO 50 µg) were exposed/ added after 48 h of incubation. After exposure, the vials were kept in an incubator for further completion of 72 h. Metaphases were blocked during the last 2 h by adding 0.1ml demecolcine. The slide preparation method was similar to that used for the lymphocyte culture. After 2 days of preparation, slides were stained in Hoechst 33258 (100 µl /ml) for 20 min. After rinsing in tap water, the slides were mounted in Sorensen's buffer (pH6.8) under a cover glass and exposed to UV light from a Black ray lamp for 1 h - 2 h on a slide warmer at 60 °C. Finally, slides were rinsed in ice-cold Sorensen's buffer followed by tap water and stained in 4 % Giemsa's stain. Minimum 30 well spread second division M2 metaphases were scored for

calculating sister chromatid exchange frequencies.

The following equation was used to calculate the replicative index.

$$Relicative\ Index\ (RI) = \frac{1(M1) + 2(M2) + 3(M3)}{100}$$

where *M*1: first division metaphase; *M*2: second division metaphases; *M*3: third division metaphase

2.7 Statistical Analysis

For chromosomal aberrations analysis and sister chromatid exchanges, the t-test was applied to differentiate the impact of different treatments.

3. RESULT AND DISCUSSION

3.1 MTT Assay

The cytotoxicity study was performed to analyse the toxic nature of parent dye Acid Red G and its degraded metabolites on HeLa cell lines. The assay was performed with varying concentrations of degraded products ranging from 5 µg/ml - 500 µg/ml. The result of the MTT cytotoxicity assay after exposure of azo dyes and its metabolites are as shown in Fig. 1. Mitomycin was used as a positive control. Percentage cell viability was observed in the range of 52.9 % - 99.8 % in the case of parent dye Acid Red G and 55.4 % - 97.6 % in the case of metabolites as shown in Fig. 1. As the concentration of dye and its metabolites were increased it showed slight toxicity.

Previously, MTT assay was performed with two different dyes Reactive Red 141 (RR141), Reactive Red 2 (RR2) and its metabolites by Oturkar[24]. In his study, we found very less cell line density in the presence of the parent dyes at 0 h. The di-azo dye RR141, during its degradation (0 h to 8 h) demonstrated a decreasing effect of toxicity. Whereas, after 2 h, the periodic decrease in cell density during mono-azo dye RR2 was observed, however after 6 h, a uniform decrease in the toxicity of dye metabolites were seen, unlike diazo dyes. The change in toxicity was due to different types of cell interaction with RR2 and RR141 and their biotransformation.

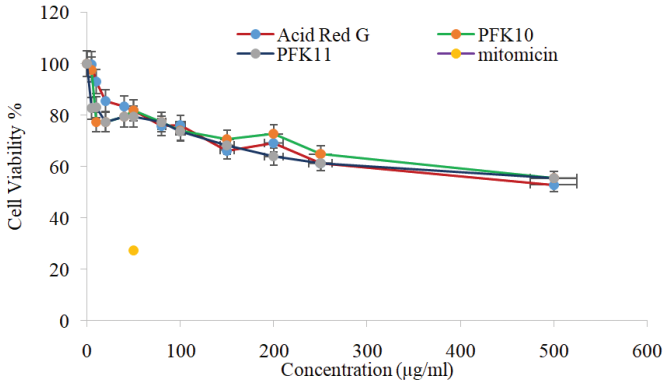

Figure 1. Cytotoxicity of Acid G before and after degradation.

3.2 Comet Assay

Single-cell gel electrophoresis DNA damage studies were carried out using HeLa cell lines *in vitro* using two different concentrations of Acid Red G Dye and its degraded products by

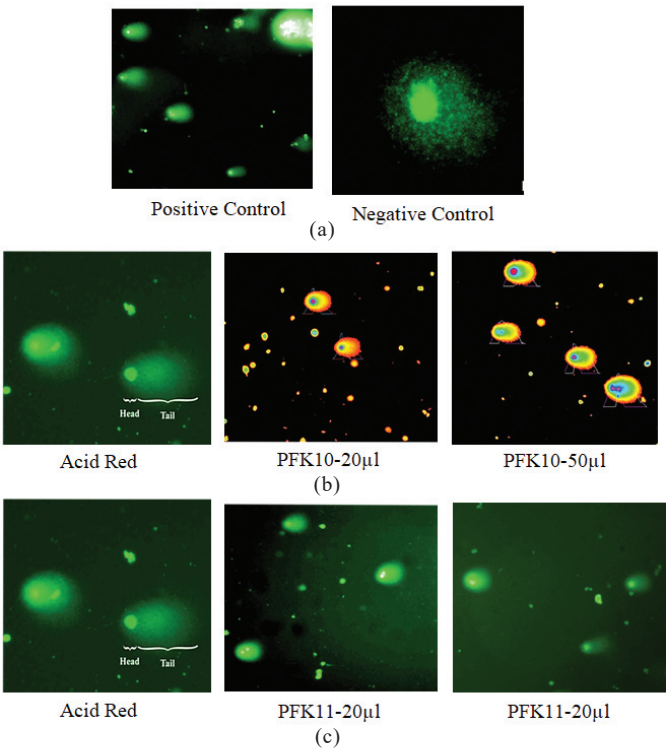

Figure 2. Fluorescence microscopic analysis of comet assay: (A) Control sample (Positive (mitomycin) and Negative), (B) Dye and treated sample PFK10 (20 µg, 50 µg) , (C) Dye and treated sample PFK11 (20 µg, 50 µg).

PFK10, PFK11 as shown in Table 3 and Fig. 2. It was observed that DNA damage was slightly higher in cells exposed under metabolites of the dye degraded with PFK10 (20 µg and 50 µg) as compared to the metabolites of the dye degraded with PFK11 (20 µg and 50 µg). The untreated dye showed 36.92 per cent DNA in tail (20 µg). The mean % DNA damage in exposed cells showed a slight increase ($p < 0.05$) than the negative control (not exposed to any chemicals), but the values were found lesser than positive control.

Comet assay was previously performed by Karsli-Ceppioglu[25]. In his study, he used four different dyes: indigotin, 6-bromo indigotin, indirubin, and 6-bromo indirubin. The results of this study showed a dose-dependent increase in DNA migration in the presence of indigotin and 6-bromo indigotin. Cells incubated with 50 µg/ml indigotin and 6-bromo indigotin ($p<0.05$) for 30 min showed higher DNA damage. Indiruin and 6-bromo indirubin did not induce DNA damage

3.3 Chromosomal Aberration Assay

For chromosome aberration study, the cell line was exposed with two different concentrations of PFK10, PFK11 and Acid Red G as shown in Table 4 and Fig. 3. The total number of altered cells was higher in the dye acid red G treated sample(17) compared to metabolite treated samples PFK10 (11) and PFK11 (5). As positive control mitomycin C was used which showed highest number of cells with alteration. Result of chromosome numerical and structural (chromosome and chromatid) were not revealed statistically increased as compared to Positive control/Mutagen. ($P>0.05$)

After chemical exposure, we can observe the chromosome

Table 3. Comet assay of dye Acid Red G and its degradation products by bacterial isolates PFK10 and PFK11

Different concentration	Comet length (px)	Comet height (px)	Comet area (px)	Head diameter (px)	Tail length (px)	Tail area (px)	% DNA in tail	Tail moment	Olive moment
PFK10 [A_{20}]	233	246	46069	220	13	5969	20.207819	2.627017	13.076704
PFK10 [B_{50}]	264	193	29110	196	68	9614	31.153679	21.184502	21.04809
PFK11 [A_{20}]	302	197	29061	218	84	297	15.871269	13.331865	6.827095
PFK11 [B_{50}]	233	254	46956	180	53	4195	26.612461	14.104604	18.977659
Acid Red G [A_{10}]	265	253	50452	226	39	6130	22.014479	8.585647	17.867706
Acid Red G [B_{20}]	189	221	39084	136	53	2548	36.92078	19.568014	28.779057
Internal Control	147	137	14250	134	13	2243	11.412549	1.483631	7.078419
Negative Control	287	252	52066	266	21	6434	10.225803	2.147418	8.296177
Positive Control	88	36	1454	18	70	1200	80.500174	56.350121	26.306009

Note: In [A_{20}], [B_{50}], [A_{10}], [B_{20}], the digits, 20, 50, 10 and 20 indicate the amount concentration of a substance

Table 4. Chromosome aberrations study of dye Acid Red G and its degradation products by bacterial isolates PFK10 and PFK11

Chromosome aberration study			
Exposed dose (µl)	Chromosomal aberration per 100 metaphase		
	Chromosomal aberration (G, B, I, D)	Chromatid (B, G)	Total
PFK10 [A_{20}]	01	10	11
PFK10 [B_{50}]	01	15	16
PFK11 [A_{20}]	00	05	05
PFK11 [B_{50}]	00	06	06
Acid Red G[A_{10}]	01	09	10
Acid Red G[B_{20}]	01	16	17
$DMSO_{50}$	01	03	04
Mitomycin C_{50}	49	67	116
Negative control	00	02	02

Note: In [A_{20}], [B_{50}], [A_{10}], [B_{20}], the digits, 20, 50, 10 and 20 indicate the amount concentration of a substance

numerical and/or structural aberrations, if any. Chromosome aberration includes break, gap, ring, deletion, interchange, inversion, and translocation, whereas chromatid type includes breaking, gap, deletion, double minutes, and exchange. Chromosome type aberration is more severe than chromatid type aberration. But chromatid type aberrations act as a useful tool to diagnose genotoxicity of unknown chemicals.

As reported on toxicological studies on azo dye Red HE3B, it was found that before and after bacterial treatments the effects caused by its metabolites were compared and found that the dye was able to induce oxidative stress as well as a high frequency of chromosome aberrations and micronuclei in root cells of *Allium cepa*[26]. Jadhav[27], *et al.* carried out a chromosome aberration test for genotoxicity analysis. In his study, it was found that in dye Remazol red treated sample (23) the total number of cells with alterations was significantly higher than sample treated with water and metabolite (4 and 6 respectively) which indicate genotoxic nature of Remazol red.

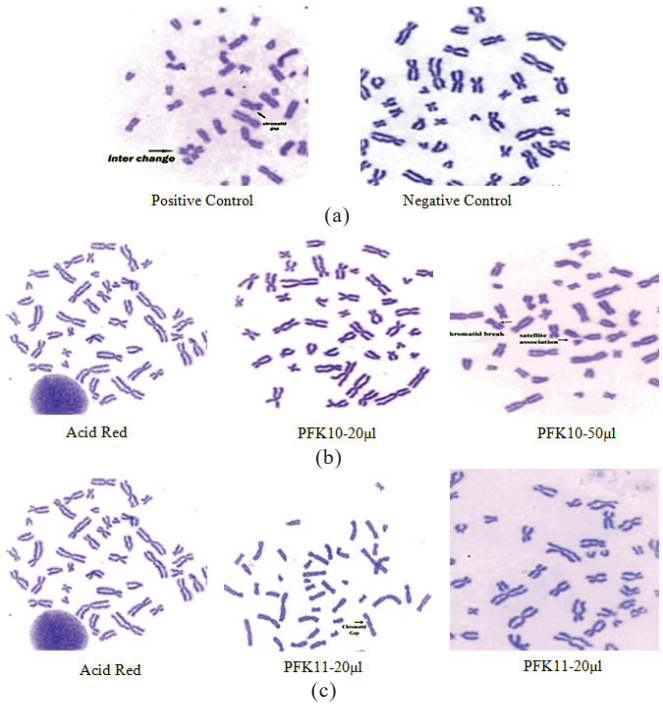

Figure 3. Microscopic examination of chromosome: (A) Control sample (Positive and Negative), (B) Dye and treated sample PFK10 (20 and 50 µg) and (C) Dye and treated sample PFK11 (20 and 50 µg).

3.4 Sister Chromatid Exchange Assay

Microscopic scoring to determine the frequency of Sister Chromatid Exchange Assay was performed in 30 second-division metaphases per dose. The replicative index was established in 100 cells per dose, scoring the rate of first (M1), second (M2) and third (M3) cellular division as shown in Table 5 and Fig. 4. Here 30 cells per M2 metaphase was taken into consideration. Analysis of sister chromatid exchanges revealed slightly elevated but not statistically increased exchanges than normal control ($P>0.05$). Similarly, the replicative index (RI) of each was not affected by the exposed chemicals significantly ($P>0.05$).

Table 5. Sister chromatid exchange study of dye Acid Red G and its degradation products by bacterial isolates PFK10 and PFK11

Sister chromatid exchange study		
Concentration (µl)	**Mean exchanges M2 metaphase (per 30 metaphases)**	**Replicative index**
PFK10 [A_{20}]	5	2.2
PFK10 [B_{50}]	6	1.9
PFK11 [A_{20}]	3	2.38
PFK11[B_{50}]	4	2.21
Acid Red G [A_{10}]	6	2.44
Acid Red G [B_{20}]	8	2.3
$DMSO_{50}$	4	2.4
Mitomycin C_{50}	38	0.95
Negative control	4	2.5

Note: In [A_{20}], [B_{50}], [A_{10}], [B_{20}], the digits, 20, 50, 10 and 20 indicate the amount concentration of a substance

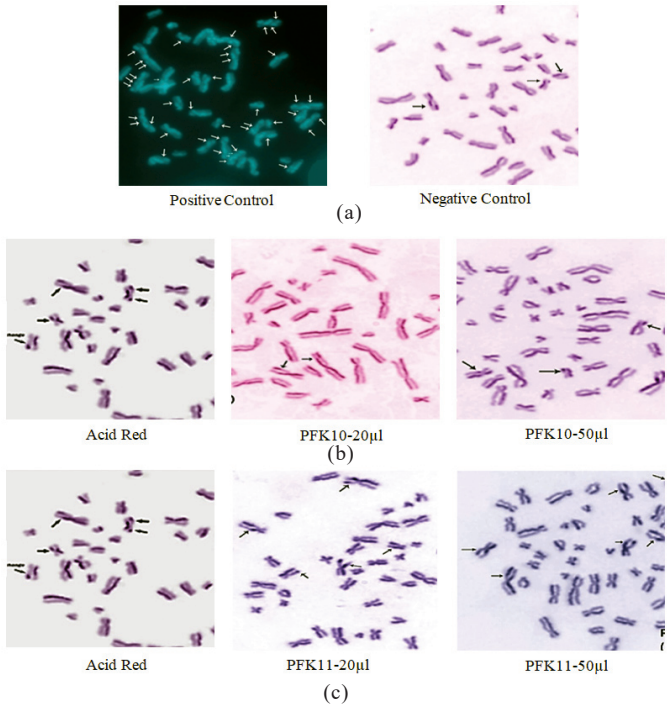

Figure 4. Microscopic examination of chromatids: (A) Control sample (Positive and Negative), (B) Dye and treatedsample PFK10 (20 and 50 µg) and (C) Dye and treated sample PFK11 (20 and 50 µg).

Due to semi-conservative DNA replication, we can visualise the exchange of sister chromatids after special treatment. Few or no exchange of SCE indicates no genotoxic effect. If the SCE frequency is found higher than control, it indicates there is a risk of genotoxicity. Using SCE technique we can also know the growth of the cells. Using the replicative index equation, we can judge the inhibition as compared to control.

4. CONCLUSIONS

The effluents from the textile industry is a mixture of heterogeneous chemicals. Several textile dyes and their decolorisation and degradation products affect biological systems in various ways. Reports have shown toxic effects of several dyes commonly used for clothing materials[28]. To decrease the harmful effects of textile dyes and its products, stringent monitoring of water quality are essential. As these dyes and its decolorised, as well as degraded products, continuously undergo chemical transformations, the precise quantitative measurement of these pollutants is not possible in the environment. The practical and most reliable way to assess their impact is to study the toxicity of water. Results of MTT assay, Chromosome aberration study, SCE study, and Comet assay indicate that Acid Red G Dye and its degraded products by *Pseudomonas aeruginosa* PFK10 and *Brevibacillus choshinensis* PFK11 have no any mutagenic nature or cytogenotoxic activity at lower exposure concentration. When concentration of each chemical is increased and exposed to cell lines, the growth is slightly inhibited, whereas, the elevated DNA damage in Comet study may be due to necrosis of cells after exposure of chemicals.

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