ISSN : 0974 - 7451 Volume 11 Issue 7

Environmental Science
An Indian Journal
Current Research Paper

ESAIJ, 11(7), 2015 [254-260]

Toxicological assessment of reactive red 35 and its biodegraded products using cytogenotoxicity, oxidative stress and phytotoxicity studies

Rakesh K.Soni[1], P.B.Acharya[1], H.A.Modi[2]*

[1]P.G.Department of Microbiology & Biogas Research Centre, Gujarat Vidyapith, Sadra - 382 320, (INDIA)

[2]Department of Life Sciences, School of Sciences, Gujarat University, Ahmadabad - 380 009, (INDIA)

E-mail: hamodi1954@yahoo.co.in

ABSTRACT

Water-soluble azo dyes are extensively used in textile industries and known to be a major environmental pollutant. Toxicity analysis of the treated dye using *Allium cepa* root cells showed remarkable improvement in cell viability, root length, mitotic index and chromosomal aberrations than that of untreated RR35. Decreased genotoxicity potential as DNA damage in cells was also observed in biologically treated dye. Induced superoxide dismutase (SOD) activity and suppressed catalase (CAT) activity along with increased lipid peroxidation, and protein oxidation indicated oxidative stress in root cells exposed to the dye molecules. These symptoms were abridged in root cells treated with biologically treated dye solution, which indicates diminution of toxicity of the dye after treatment. Phytotoxicity studies further reflected the decreased in toxicity of the dye after biological treatment.

KEYWORDS

Allium cepa;
Cytogenotoxicity;
Oxidative stress;
Phytotoxicity;
Reactive Red 35.

INTRODUCTION

Increasing industrialization lead to biomagnifications of xenobiotic compounds in the environment has become a major concern. Azo dyes and its derivatives are the major cause of environmental pollutions, as these are resistant against degradation. Due to wide structural diversity these dyes are used extensively as substrates in textile, leather, plastics, papers, hair, mineral oils, waxes, food, and cosmetics industries[1]. Textile and dye processing industries are major release of these dyes into the environment. It was estimated that approx. 2,80,000 tons of textile dyes are released into the environment through industrial effluent every year[2].

Azo dyes meet the requirement of life to make colorful. On the other hands it adversely affects the aquatic, soil and atmosphere. Azo dyes and its derivatives, majorly aromatic amine, have shown to be responsible for human bladder cancer and tumorigenic to various animals and mutagenic to aquatic life[2], [3]. Increased irrigation of partially treated industrial waste water adversely affects soil fertility and destruction of important soil flora and fauna. Accumulations of high concentration of solids in the soil restrict the plant seed germination rate as well as inhibit growth of root and shoot. It also decreased the chlorophyll content of plant leaves[3]. In many

cases products formed during the degradation of azo dyes are more toxic than parent molecules and present serious environmental problems[2]. Reactive Red 35 (RR35), a vinyl sulfone based monoazo dye are proved hazardous to human as it is responsible for causing skin, eyes and respiratory irritation. Therefore, it is necessary to investigate the toxic impact of dye after treatment before discharge into the environment.

Higher plants are considered as biosensors to evaluate the toxic effects of environmental pollutants and have been recommended as test organisms by United National Environmental Program, US Environmental Protection Agency, and World Health Organization[4]. Bioassays of *Allium cepa*, *Vicia faba*, *Lactuca sativ* and *Zea mays* are used to evaluate the cytogenotoxicity potential of treated waste[5]. The root tips are first to get exposed to pollutant spread in soil and water[6]. *A. cepa* assay is highly endorsed for assessment of treatment efficiency of waste water[7]. The *A. cepa* assay is widely exploited to study the cytotoxicity as well as genotoxicity of environmental pollutants. Oxidative stress induced in plant cells due to the presence of pollutant like the carcinogenic dye is also extensively studied[8], [9].

The present study describes the toxic effect of untreated RR35 and treated (metabolites obtained after degradation with *Pseudomonas aeruginosa* ARSKS20) dye solution by performing *A. cepa* assay and phytotoxicity assay on *Sorghum vulgare, Triticum aestivum and Phaseolus mungo*. The comparative study determines the significant reduction of toxicity potential in the biotreated dye sample than native dye.

MATERIALS AND METHODS

Allium cepa assay

Test organism

Equal sized healthy *A. cepa* bulbs (18-20 cm diameter) were used as test plant, procured from local farmers of Sadra, Gandhinagar, India. Before use, the loose outer scales were removed and dry base was scraped. The bulbs were set on test tube filled to top with distilled water for 48 h in the dark for root development in moist condition. After development of 2-3 cm roots, it was washed under running tap water for 5 min and subjected to dye treatment. The experiment was done at room temperature (27 ± 2°C) under 12 h light/dark cycles.

Dye treatment

Textile dye RR35 was obtained from Meghmani Dyes and Pigment, Vatva, Ahmedabad, Gujarat (India). RR35 at 500 mg/L concentration and its metabolites obtained after degradation with *P. aeruginosa* ARSKS20 were used as test solutions. The exposures for 5 d were administrated by placing *A. cepa* bulbs on glass test tubes with roots dipped in experimental solutions *viz*., distilled water as a control (set 1), 500 mg/L of RR35 (set 2), and metabolites obtained after biodegradation of RR35 dye (set 3). Each set consisted of 5 bulbs and the exposure experiments was done at room temperature (27 ± 2°C) under 12 h light/dark cycles. After the exposure, the bulbs were removed and thoroughly washed in running tap water for 5 min. Roots were then excised and proceed for measurements and biochemical analysis.

Root length and cell viability

The root length (longest five roots per bulb) was recorded[10]. Percentage cell viability from roots of *A. cepa* was measured by Evan's BLUE staining method[11].

Cytotoxicity

Cytotoxicity study was performed by method reported earlier[12] with modification i.e., using acetocarmine instead of (2%) propano-orcein as staining solution. Root meristems were chosen randomly from each set and excised for fixation in Carnoy solution (1:3 acetic acid:ethanol) followed by hydrolysis with 1N HCl at 60°C and washing with distilled water. The root tips were stained with acetocarmine and crushed by pressing and excess stain was removed by washing with the distilled water. Slide was prepared by adding a drop of 45% acetic acid and covered with a cover slip and observed under the compound microscope at 40X magnification (BA 210, Inkarp, India; 3 MP Motic Cam-

era). The mean value of root length, mitotic index, and chromosomal aberrations in cells at various phases of cell division were examined[10].

Comet assay (single cell gel electrophoresis)

To assess the DNA damage in *A. cepa* root cells by RR35 and its metabolites, comet assay was carried out by the methods described by single cell gel electrophoresis method[13], [8]. Randomly chosen 25 nuclei were analyzed in the assay. To measure Tail length (TL) and %DNA damage (%T) computerized image analysis system (Comet version 1.5) was used.

Lipid peroxidation, protein oxidation and antioxidant enzyme status

Lipid peroxidation, protein oxidation and antioxidant enzymes, namely superoxide dismutase (SOD, E.C. 1.15.1.1) and catalase (CAT, E.C. 1.11.1.6), were measured by spectrophotometrically[8].

Antioxidant enzyme assays were initiated by adding aliquots of enzyme extracts containing 50 µg proteins to 3 mL reaction mixture. One unit of SOD activity was defined as the amount of the enzyme that inhibited NBT reduction by 50% and expressed in per mg protein per minute. CAT activity was expressed in nmol of H_2O_2 utilized per mg protein per min.

Lipid peroxidation was measured as the amount of malondialdehyde (MDA) produced by the thiobirbuteric acid (TBA) reaction. The concentration of MDA was calculated using an extinction coefficient (ε = 155 mM/cm) and expressed in nmol per gram fresh weight (FW) of the root. Measurement of protein oxidation was done by estimating the amount of carbonyl content formed from the modification of 2, 4-dinitrophenyl hydrazine (DNPH) by free radical. Carbonyl concentration was calculated from the difference in absorbance at 380nm for DNPH-treated (test) and HCl-treated (blank) samples (ε = 22 mM/cm) and expressed in nmol of DNPH incorporated per mg of protein.

Phytotoxicity assay

Test plants

The plant seeds of *S. vulgare, T. aestivum and P. mungo* were acquired from the Seed Collection Centre, Gandhinagar, Gujarat (India).

Dye treatment

RR35 at 2500 mg/L concentration and its metabolites obtained after degradation with *P. aeruginosa* ARSKS20 were used as test solutions to perform phytotoxicity assay.

Phytotoxicity test

Healthy seeds of *S. vulgare*, *T. aestivum* and *P. mungo* were screened (10 seeds of each plant), and irrigated daily with 10 mL of distilled water as a control (set 1), 2500 mg/L solution of RR35 solution (set 2), and its extracted metabolites solution obtained after its biodegradation (set 3). Percent germination and lengths of plumule and radical were measured after 7 days[7].

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using Tukey–Kramer multiple comparison test.

RESULTS AND DISCUSSION

Allium cepa assay

A. cepa assay is the most validated and reliable test for *in situ* monitoring of the presence of genotoxic and cytotoxic substances in the environment[13]. Therefore cytotoxicity, comet assay, antioxidant enzyme status of RR35 and its biodegraded products (metabolites) were evaluated at 500 mg/L concentration.

Root length and percentage cell viability

Root length is considered as a macroscopic parameter for testing cytotoxicity of the textile dye[6]. In the present study, the root length was retarded by 23% in RR35 at 500 mg/L (TABLE 1). Only 3% reduction in root length was observed in the treated dye sample when compared to control. The toxicity of RR35 stunted the growth, as far as the root length is considered. The toxicity was fairly reduced after treatment with the biodegraded dye, as observed in terms of the root length. Root length reported to decrease by 15% when exposed to untreated Red

TABLE 1 : Root length, percent cell viability and cytogenotoxicity towards *A. cepa* root cells exposed to RR35 dye and its degraded products

Analysis	Control	RR35 [a]	RR35 metabolites [a]
RL (cm)	6.7 ± 0.1643	5.18 ±0.1356	6.5 ± 0.1844
% Cell viability	94.33 ± 0.8819	53.66 ± 2.028**	84.33 ± 1.764$^{\$\$}$
MI[#]	7.83 ± 0.2066	11.28 ± 0.2556**	8.56 ± 0.1643$^{\$\$}$
MN	0	2	0
CB	0	3	1
TA	2	16	6
Frequency of TA	0.0646 ± 0.0161	0.7579 ± 0.0409	0.2807 ± 0.0270
TCA	2060	2155	2137
TMO	161	243	183

a 500 mg/L, RL: root length; MI: mitotic index; MN: micronuclei; CB: chromosomal breaks; TA: total number of alteration; TCA: total number of cells analyzed; TMO: total number of mitotic cells observed. #Values are mean of three experiments ± SEM and significantly differ from treatment with control (root germinated in water) at ** P < 0.001 and with RR35at $$ P < 0.001 by one-way analysis of variance with Tukey- Kramer comparison test.

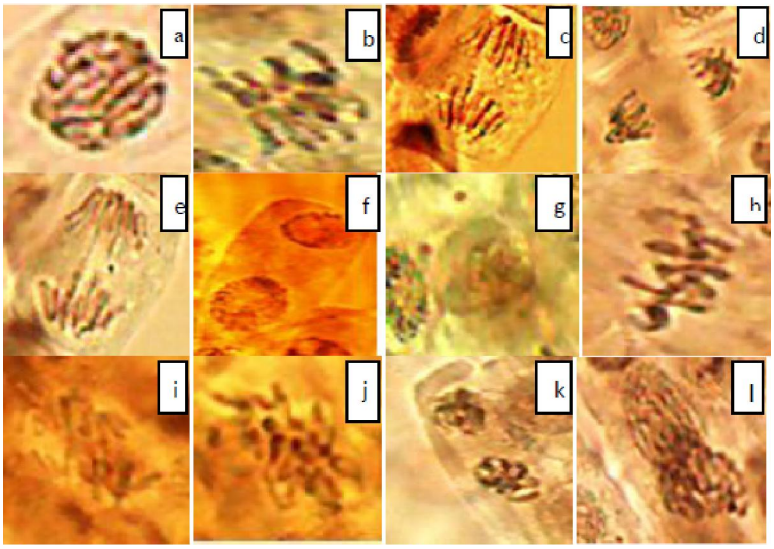

Figure 1 : Different types of aberration induced by Reactive Red 35 in *A. cepa* root tips; (a) normal prophase, (b) normal metaphase, (c) normal anaphase, (d) normal telophase, (e) anaphase bridge, (f) binucleated cell, (g) micronuclei, (h) sticky metaphase, (i) multi polararity, (j) fragmentation, (k) telophase clumping, (l) vagrant chromosome

HE3B, which was only 1% after treatment with consortium of *Providencia* sp. SDS and *P. aeruginosa* BCH[14].

Toxicity profiles of RR35 and its degraded metabolites to cells of *A. cepa* root shows 94.33%, 53.66%, and 84.33% cells viability in the roots exposed to distilled water, RR35, and degradation metabolites, respectively (TABLE 1). Significant reduction in the cell viability was observed when exposed to RR35 dye, and when the dye was treated with *P. aeruginosa* ARSKS20 the degree of toxicity was significantly decreased compared to the untreated dye. Cell viability of the root cells in A. cepa reported 86.5% when exposed to 500 mg/L Remazol Red dye, which was increased to 94.69% with exposure to the metabolites obtained after degradation by *P. aeruginosa* BCH[11].

Cytogenotoxicity

A method of measuring the degree of cytotoxicity of pollutant is depending on levels of mitotic index (MI). MI serves as an important parameter of cytotoxicity studies in environmental monitoring[7]. Decrease in the mitotic index (MI) could be used to

assess the level of cytotoxicity of a test compound[9]. The increase in MI was observed due to the uncontrolled proliferation of meristematic cells of *A. cepa*, causing tumor formation and ultimately cell damage[14]. In the present study, higher MI (11.28) was observed in root cells exposed to 500mg/L RR35, which was reduced to 8.56 after dye degradation (TABLE 1). The results indicate that the cytotoxicity level of dye was brought down to a level very close to control (7.83) after biodegradation. Increased MI value in presence Reactive Blue 160 was also reported[12]. Along with the MI, chromosomal aberration was also accepted as an indicator of genotoxic damage induced by carcinogenic compounds[14].

In the present study, frequencies of chromosomal aberrations like laggard formation, chromosomal breaks, anaphase bridges, stickiness, binucleated cells and micronuclei were significantly reduced after biodegradation compared to the untreated dye at 500 mg/L (Figure 1). These results indicate the reduction of the genotoxic potential of the dye upon biodegradation by *P. aeruginosa* ARSKS20. Chromosomal aberrations, nuclear abnormalities, induction of micronuclei, fragmentation in the DNA, binucleated cells, multipolarity, anaphase bridge and sticky metaphase were observed earlier in course of the toxicity studies of different azo dyes[10], [14].

Comet assay

Comet assay, a gel electrophoresis-based method that measures migration of negatively charged broken DNA of the microbial cells. It is a versatile, relatively simple and sensitive method to measure DNA damage for *in vitro* and *in vivo* biomonitoring of environmental pollutants[14]. Genotoxicity potentials of RR35 (500 mg/L) and its biodegraded metabolites (TABLE 2; Figure 2) were studied by measuring percent tailing of DNA (%T) and tail length (TL), which were analyzed by ANOVA test. The considerable DNA damage was observed in the cells exposed to RR35, whereas those exposed to the biodegraded metabolites of the dye showed lesser DNA damage i.e., nearly equivalent to control. Similar detoxification study of textile effluent by a consortium of *Providencia* sp. SDS and *P. aeruginosa* BCH was reported previously[7].

Oxidative stress: antioxidant enzymes status, protein oxidation and lipid peroxidation

Oxidative stress due to imbalance of reactive oxygen species (ROS) resulting from the presence of environmental pollutants within an organism can cause damage to tissues and cellular components, thus the stress can be used as biomarker of environmental pollutants. Protective enzymatic and non-enzymatic mechanisms are present in plant cells, which scavenge ROS and reduce their deleterious effects[11]. Results of antioxidant enzymes indicate (TABLE 3)

TABLE 2 : Genotoxicity analyses of RR35 and its metabolites obtained after degradation

Analysis	Control	Untreated	Treated
TL (μm)	26.33 ± 1.764	92.33 ± 1.202	33.67 ± 1.453*
%T	32.27 ± 1.092	60.49 ± 1.901	40.88 ± 1.908*

a 500 mg/L. Values are mean of three experiments, SD (±), and significantly differ from the control (distilled water),* P < 0.001, by one- way analysis with Tukey- kramer comparison test

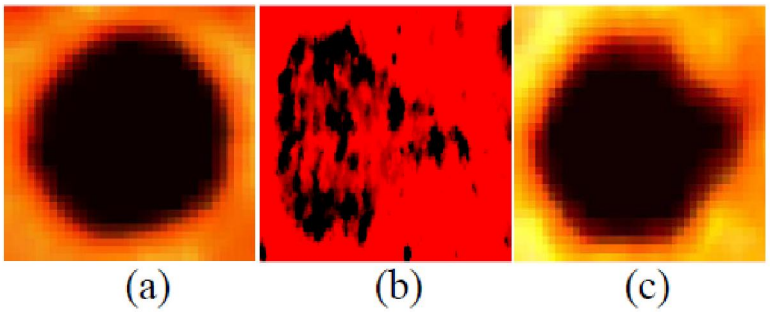

Figure 2 : Comet observed; the sample analyzed as control (a), dye Reactive Red 35 (500 mg/L) (b), metabolites obtained after degradation (c)

TABLE 3 : Analysis of antioxidant enzyme activity, lipid peroxidation and protein oxidation from root cells of *A. cepa* exposed to RR35 and its metabolites

Analysis	Water	RR 35[a]	RR35 metabolites
SOD	4.2021 ± 0.0627	12.9767 ± 0.1710**	5.1600 ± 0.2215$$
Catalase	31.2970 ± 1.067	11.6873 ± 0.7566**	25.9602 ± 0.605$$
Lipid peroxidation	0.7677 ± 0.0194	1.5333 ± 0.0895**	0.8839 ± 0.0465$$
Protein oxidation	7.0549 ± 0.0723	13.6693 ± 0.1188**	8.7963 ± 0.0759$$

a 500 mg/L, Values are mean of three experiments ± SEM and significantly differ from treatment with control (water) at, ** P < 0.001 and with RR35 at $$ P < 0.001 by one- way analysis of variance with Tukey- kramer comparison test.

TABLE 4 : Phytotoxicity studies of RR35 and its metabolites formed after degradation

Parameter	Control	RR35[a]	RR35 metabolites
Sorghum vulgare			
Germination (%)	90	60	90
Shoot (cm)	16.40 ± 0.3408	5.00 ± 0.3633**	14.40 ± 0.4259$$
Root (cm)	10.55 ± 0.5083	1.45 ± 0.2446**	9.94 ± 0.3432$$
Triticum aestivum			
Germination (%)	100	60	90
Shoot (cm)	12.79 ± 0.2588	0.90 ± 0.6600**	11.56 ± 0.4729$$
Root (cm)	9.23 ± 0.33	2.71 ± 0.2762**	6.41 ± 0.2383$$
Phaseolus mungo			
Germination (%)	100	70	90
Shoot (cm)	14.14 ± 0.5344	6.68 ± 0.7592**	10.98 ± 0.9540$$
Root (cm)	5.23 ± 0.2345	2.50 ± 0.4791**	4.91 ± 0.2779$$

a 2500 mg/L, Value are mean of three experiments ± SEM. significant different from the control (water) by **P < 0.001, significant different from the RR 35 by $$P < 0.001

a significant increase in SOD, and a suppressed CAT activity was observed in *A. cepa* root cells when exposed to RR35 (500 mg/L). After treatment of the dye with *P. aeruginosa* ARSKS20 increase in SOD levels and CAT suppression was significantly reduced, indicating reduction of toxicity in the biodegraded dye. Similar results were reported during toxicity studies of Remazol Red dye and Amaranth dye[11], [15]. Protein carbonyl and MDA are the end products of protein oxidation and lipid peroxidation, respectively. These end products are considered as biomarkers of oxidative stress and cellular damage[9]. Increased protein oxidation (93%) and lipid peroxidation (99%) were observed with RR35 (500 mg/L), whereas the results were nearly equivalent to control in case of biodegraded dye. Results were under closed agreement those reported with Red HE3B and Rubine GFL dyes, and their metabolites[14], [1].

Phytotoxicity assay

Phytotoxicity assessment becomes more dominantly included to monitor pollution as to be a less expensive and easier than other methods of toxicity evaluation. Untreated textile dye-containing effluents can detrimentally affect the agricultural crops if discharged onto the land[2]. Thus, assessment of toxicity prior to discharge is of utmost importance. In the present study, it was observed that degraded RR35 dye was less toxic for germination of *S. vulgare, T. aestivum* and *P. mungo* seeds than the untreated dye (TABLE 4). RR35 inhibited seed germination of *S. vulgare, T. aestivum* and *P. mungo* by 40, 40 and 30%, respectively. On the other hand, the treatment of the seeds with degraded dye inhibited the germination only by 10% in all cases. When compared to control, decrease in shoot and root lengths of *S. vulgare, T. aestivum* and *P. mungo* seeds were 69.51 and 86.25%, 92.96 and 70.63%, and 52.75 and 52.19% with untreated dye whereas 12.19 and 5.78%, 9.61 and 30.55%, and 22.34 and 6.11% when

exposed to metabolite obtained after degradation of dye, respectively. Similar findings were reported with Direct Brown MR, Malachite Green and its subsequent degradation products[16], [17].

CONCLUSION

A. cepa test is a classical biomarker of environmental pollutants. Cytogenotoxicity and oxidative stress studies with *A. cepa* confirmed decreased in toxicity potential of RR35 after treatment with *P. aeruginosa* ARSKS20. Phytotoxicity studies also prevails the decreased in toxicity of RR35 after treatment, which promote the potential of biological treatment as a cost-effective and ecofriendly management of environmental pollution caused by textile dyes.

ACKNOWLEDGEMENT

Rakesh K. Soni would like to thank to Dr. J. P. Jadhav, Professor & HOD, Department of Biotechnology, Shivaji University, Kolhapur - 416 004, India for permission to work in their department laboratory and for guidance to my works. Author is also thankful to Dr. Asmita Patil for her support to learn techniques of toxicity studies. Author also acknowledges the Management of Gujarat Vidyapith, Ahmedabad, India for giving permission of this works.

REFERENCES

[1] T.R.Waghmode, M.B.Kurade, H.S.Lade, S.P.Govindwar; Appl.Biochem.Biotechnol., **167**, 1578-1594 **(2012)**.

[2] M.Solis, A.Solis, H.I.Perez, N.Manjarrez, M.Flores; Process Biochem., **47**, 1723-1748 **(2012)**.

[3] N.Puvaneswari, J.Muthukrishnan, P.Gunasekaran; Indian J.Exp.Biol., **44**, 618-626 **(2006)**.

[4] W.F.Grant; Mutat.Res., **310**, 175-185 **(1994)**.

[5] S.M.Sousa, S.V.Pamela, M.S.C.Jose, F.V.Lyderson; Caryologia, **62(4)**, 326-333 **(2009)**.

[6] O.B.Samuel, F.I.Osuala, P.G.C.Odeigah; African J.Environ.Sci.Technol., **4(1)**, 21-27 **(2010)**.

[7] S.S.Phugare, D.C.Dayanand, S.N.Surwase, J.P.Jadhav; Ecotoxicol.Environ.Saf., **74(5)**, 1288-1296 **(2011)**.

[8] V.M.M.Achary, S.Jena, K.K.Panda, B.B.Panda; Ecotoxicol.Environ.Saf., **70**, 300-310 **(2008)**.

[9] S.B.Jadhav, S.N.Surwase, D.C.Kalyani, R.G.Gurav, J.P.Jadhav; Appl.Biochem.Biotechnol., **168**, 1319-1334 **(2012)**.

[10] J.P.Jadhav, D.C.Kalyani, A.A.Telke, S.S.Phugare, S.P.Govindwar; Bioresour. Technol., **101**, 165-173 **(2010)**.

[11] S.B.Jadhav, S.S.Phugare, S.P.Pratibha, J.P.Jadhav; Int. Biodeterior. Biodegrad., **65**, 733-743 **(2011)**.

[12] A.V.Patil, J.P.Jadhav; Chemosphere, **92**, 225-232 **(2013)**.

[13] R.Chakraborty, A.Mukherjee, A.Mukherjee; Environ.Monit.Assess., **153**, 351-357 **(2009)**.

[14] S.S.Phugare, D.C.Kalyani, A.V.Patil, J.P.Jadhav; J. Hazard. Mater., **186(1)**, 713-723 **(2011)**.

[15] S.B.Jadhav, N.S.Patil, A.D.Watharkar, O.A.Apine, J.P.Jadhav; Environ.Sci.Pollut.Res., **20(5)**, 2854-2866 **(2013)**.

[16] G.Ghodake, S.Jadhav, V.Dawkar, S.Govindwar; Int.Biodeterior.Biodegrad., **63**, 433-139 **(2009)**.

[17] G.Parshetti, S.Kalme, G.Saratale, S.Govindwar; Acta Chim.Slov., **53**, 492-498 **(2006)**.