Available online at www.scholarsresearchlibrary.com

Scholars Research Library

Der Pharmacia Lettre, 2015, 7 (6):234-238
(http://scholarsresearchlibrary.com/archive.html)

Scholars Research Library
ISSN 0975-5071
USA CODEN: DPLEB4

Biodegradation of azo dyes by *Bacillus subtilis* 'RA29'

Arun Kumar[1], Jayata Chopra[1], Santosh Kumar Singh[3], Amir Khan[3] and R. N. Singh[2, 3*]

[1]*Department of Biotechnology, Bhojia Institute of Life Sciences, Bhud, Baddi, District-Solan (H.P.), India*
[2]*S.B.S. (P.G.) Institute of Biomedical Sciences and Research, Balawala, Dehradun, Uttrakhand (India)*
[3]*School of Life Sciences, Glocal University, Mirzapur Pole, Saharanpur, U.P (India)*

ABSTRACT

Azo dyes account for most of all textile dyes stuffs produced and have been the most commonly used synthetic dyes in textile, food, paper making and cosmetic industries. Release of azo dyes into the environment from the effluents of dye- utilizing industries has become a major concern in wastewater treatment. Some azo dyes have been linked to human bladder cancer, spleenic sarcomas, hepato-carcinomas & chromosomal aberrations in mammalian cells. Bacillus subtilis 'RA29' (NCBI accession no: JF901735) was applied on four different azo dyes (Congo red, Amido black, Acid orange & Rhodamine B) for the decolorization. In Yeast Malt broth (YMB), Bacillus subtilis 'RA29' showed 98.23% decolorization of Congo red, 78.32% Amido black, 96.69% Acid orange & 6.69% Rhodamine B at pH 7.0 after 20 hr of incubation at 37^{0}C. During the growth and Congo red decolorization kinetic study, the maximum decolorization (99.7%), cell yield (347.80) with biomass concentration 17.216mg/ml was observed after 16 hr of incubation at temperature 37^{0}C under static aerobic condition. The enzyme activities of laccase, azoreductase, peroxidase enzymes in partially purified extracellular crude protein were detected as 7.34U/ml, 0.168 U/ml, 0.134 U/ml respectively. Liquid chromatography mass spectroscopy (LCMS) studies detected the tentative presence of AcetoAcet-P-ChloroAnilide, 3-(2,2-dichlorovinyl)-2,2-dimethyl cyclopropane-1-carboxylic acid chloride, N-{4-[(4-Bromophenyl) sulfamoyl] phenyl}-N^2-[(4-chlorophenyl) sulfonyl]-N^2 (2 ethoxyphenyl) glycinamide and 2, 2, 2-Trichloroethanol at retention time 1.571 min, 13.68 min, 19.153 min and 18.234 min respectively.

INTRODUCTION

Azo dyes account for most of all textile dyes stuffs produced and have been the most commonly used synthetic dyes in textile, food, papermaking and cosmetic industries [1, 2]. The textile industry is one of the greatest consumer of water which is used for dyeing processes i.e. about 100L of water is used to process about 1kg of textile material as a result generates high amount of effluent [3]. Many of the organic dyes are hazardous and may affect aquatic life and even the food chain [4]. Due to their chemical structures, dyes are resistant to fading on exposure to light, water and many chemicals and, therefore, are difficult to be decolorized once released into the aquatic environment [5].

Release of azo dyes into the environment from the effluents of dye utilizing industries has become a major concern in waste water treatment since some azo dyes or their metabolites may be mutagens or carcinogens [6]. Some azo dyes have been linked to human bladder cancer, splenic sarcomas, hepatocarcinomas & chromosomal aberrations in mammalian cells [7]. Presence of these dyes in aqueous ecosystem diminishes the photosynthesis by impeding the light penetration a deeper layers by it deteriorating water quality & lowering the gas solubility. Further the dyes and/ or their degradation products may be toxic to flora and fauna [8].

This research focused on the complete degradation of the Congo red dye (Azo dye) by isolated extracellular bacterial enzymes because it is a benzene based dye, known to metabolize to benzedene, a known human carcinogen and its decomposition products include oxides of nitrogen, oxides of sulfur, oxides of carbon. These decomposition products may irritate eyes, skin and mucous membrane. It is also a carcinogenic dye which comes in the effluents of textile industries during dyeing & rinsing processes [9]. As common physical or chemical methods for dye removal are expensive and sometimes generate secondary pollution. On other hand, some cases traditional biological processes are combined with physical and chemical treatments, such as flocculation, chemical coagulation, precipitation, were also achieved for the better decolorization of the waste water [10]. Besides these methods, the biodegradation by the bacterial enzymes appears to be an attractive alternative method. Decolorization of azo dyes by bacterial strains was typically initiated by azoreductase-catalyzed anaerobic reduction or cleavage of azo bonds [11], followed by aerobic [12] or anaerobic [13] degradation of the resulting aromatic amines by a mixed / pure bacterial community [14, 15].

In the present study efforts were made to identify the metabolites/ byproducts involved in the Congo red (Azo dye) decolorization by *Bacillus subtilis* 'RA29'.

MATERIALS AND METHODS

Decolorization studies on different dyes:
50ppm of each azo dye (Congo red, Amido black, Acid Orange and Rhodamine B) were supplemented separately to different flasks containing 100 ml of Yeast Malt broth (YMB) and inoculated with the culture of *Bacillus subtilis* 'RA29'. Control of each dye was also setup which contained YMB but devoid of *Bacillus subtilis* 'RA29'. All the test and control flasks were kept at 37^{o}C for 24hr. Absorbance of all the test and control flasks was measured at regular interval (0-24 hr) by UV-Visible spectrophotometer against YMB blank. Alongside biomass was also measured at 600nm for all the test flasks. Percent (%) decorization and cell yield were calculated by the formula with the help of the standard graph of biomass.

$$\% \text{ decolorization} = \frac{C-T}{C} \times 100$$

Where,
C = absorbance of control flask, T = absorbance of test flask

$$\text{Cell Yield} = \frac{\text{g. of biomass generated}}{\text{g. of substrate decolorized}}$$

Enzymatic studies:
72 hr old 100 ml nutrient cultured broth with Congo red dye was centrifuged at 10,000 rpm for 10 min. Supernatant was removed and mixed with equal amount of 70% ice-cold ammonium sulfate, vortexed well and centrifuged at 14,000 rpm for 20 min at 4^{0}C. Supernatant was discarded and pellet washed twice with phosphate buffer (pH 7.0) and processed for further purification by 0.22µm dialysis membrane for the salting out of the ions and degraded protein extracts. After dialysis 50 µl of protein sample was analyzed by Lowry's method to quantify the amount of protein. For that sample volume was made 1 ml by adding 950 µl of 0.1 N NaOH. Then, 5 ml of alkaline copper sulfate solution was added and incubated at 37^{0}C for 10 min. 500 µl 1N Folin's reagent was added in the reaction mixture, and incubated for 30 min at 37^{0}C. Absorbance was measured at 660 nm against blank (devoid of protein sample). To determine the laccase activities, 0.1 ml of extra cellular protein was added to 4.9 ml sodium acetate buffer (50 mM, pH 4.5), which contains 1 mM guaiacol as substrate and incubated at 37ºC for 15 min. 1U of enzyme activity was defined as the amount of enzyme that elicited an increase in A_{465} of the absorbance unit per ml. Azoreductase activity was assayed by measuring the decrease in optical density at 496.5 nm. The reaction mixture contained 25 mM potassium phosphate buffer (pH 7.0), 20 µM of Congo red dye and enzyme (0.5 µg). The reaction was initiated by the addition of 0.1 mM NADPH. Initial velocity was determined by monitoring the change in the amount of substrate in the first 2 min in a quartz cuvette of 1cm light path.

LC-MS identification of the compounds involved:
The degradation products of Congo red dye by the bacterial strain *Bacillus subtilis* 'RA29' were concentrated under reduced pressure with the help of rotatory evaporator and dried to powdered extract. Dried extract was dissolved in 10 ml of methanol and filtered through Whatman filter paper no. 42. Then the sample was passed through Na_2SO_3

column, collected and concentrated to 1ml. Liquid chromatography/mass spectrometry (LC/MS) with a quadruple ion trap MS was done. The column used was Symmetry (Walters) C18 column (250 × 4.6 mm). A 25µl sample volume was injected using the system's auto sampler. Solvent A contained 10mM ammonium acetate (pH 3.0) and solvent B consisted of HPLC-grade methanol. Gradient method was used as: 80/20: 0-5 minutes, 80/20: 5minutes, 40/60: 10 minutes, and 20/80: 25minutes. The flow coming from the LC was split 1:28 and introduced to the ESI (electrospray ionization) turbo spray. A scan rate of 4.000amu/s was performed under negative ionization in the enhanced scan mode. The UV response during LC/MS was monitored at 360 nm. The LC/MS was operated in the positive ion mode using Diode array detector and electro spray ionization (ESI) source: Ion spray voltage 4.500 V, declustering potential 50 V, Entrance potential 10 V, Curtain gas 40 psi, Nebulizer gas 45 psi, Turbo gas 80 psi. Finally compounds identification was corroborated based on the relative retention time and mass fragmentation pattern spectrums with those of standards and the NIST147 library database of the LCMS system.

RESULTS AND DISCUSSION

Bacillus subtilis 'RA29' was applied on four different azo dyes (Congo red, Amido black, Acid orange & Rhodamine B) for the decolorization. In Pre-optimized Yeast Malt broth (YMB), *Bacillus subtilis* 'RA29' showed 98.23% decolorization of Congo red, 78.32% Amido black, 96.69% Acid orange & 6.69% Rhodamine B at pH 7.0 after 20 hr of incubation at 37^0C (figure 1.0).

Decolorization kinetics showed maximum decolorization of (99.7%), cell yield (347.80) with17.216 mg/ml biomass was observed after 16 hours of incubation at temperature 37^0C by the *Bacillus subtilis* 'RA29' (figure 2).

Isik *et al*., 2003 showed the decolorization efficiencies for Congo Red degradation over 9 days of the incubation period inoculated into nutrient broth with *E.coli* under anaerobic conditions. The maximum decolorization was found 98% under anaerobic conditions while 30% and 39% colour removal efficiencies were obtained under aerobic and microaerophilic conditions.

Isolated extracellular proteins were partially purified by ammonium sulfate precipitation followed by salting out through 0.22 µm dialysis membrane and final quantified by Lowry's method (320.89µg/ml). Further crude proteins were tested for any activity of Laccases, azoreductases and peroxidases enzyme and found as 7.34 U/ml, 0.168 U/ml, and 0.134 U/ml respectively. In a similar kind of study done by Suwannawong *et al.*, 2010 who partially purified laccase from the crude extract of a solid state culture of *Lentinus polychrous* Lév. by salt fractionation, Sephacryl S-300 and DEAE-cellulose columns respectively. After the two chromatography steps, the enzyme was purified only about 5.3-fold, with an overall yield of 5.6% and a laccase specific activity of 14.3 U mg^{-1}.

Liquid chromatography (LC) of the metabolite formed Present study reported the presence of AcetoAcet-P-ChloroAnilide, DV Acid Chloride or [3-(2,2-dichlorovinyl), -2,2-dimethyl cyclopropane-1-carboxylic acid chloride, N-{4-[(4-Bromophenyl) sulfamoyl]phenyl}-N^2-[(4-chlorophenyl) sulfonyl]-N^2- (2ethoxyphenyl) glycinamide and 2,2,2-Trichloroethanol at retention time 1.571 min, 13.68 min, 19.153 min and 18.234 min respectively(Figure 3).

Rajendran, [16] studied the chromatogram of the treated effluent sample showed 3 peaks in it with 1 major peak and 2 minor peaks. The retention time (1.460, 2.120, 2.317) and the % of area (5.6, 14.1, 80.4) obtained for the three compounds (peaks) in the chromatogram were not that of the aromatic amines and as the retention time of all the amines were more than 3 min. It was thus confirmed the textiles effluent sample treated with the combination A13 under shaking condition was not found to produce any toxic aromatic amines at the end of five days of incubation. Shobana, [17] studied that during the degradation of reactive orange 16 there is asymmetric cleavage of azo bond, resulting in formation of 1-amino-1-napthalene sulfonic acid, which was confirmed by the standard NIST library data, this is further, converted to aniline. While the naphthalene part of the dye was further biodegraded with opening of one ring, the formation of aldehyde as one of the intermediate is confirmed from the IR data. Usha, [18] studied LC-MS analysis of the dyes and their degraded product confirmed degradation of Reactive Black 5 by *Aeromonas punctata* and Reactive Red 120 dye by *Pseudomonas aeruginosa*. The peak present in the spectrums of the dyes were absent in the degraded products, indicating the entire dye has been decomposed to colorless low molecular fragments by respective cultures. LC-MS analysis of the Reactive Black 5 dye degraded sample demonstrated the presence of a compound with molecular weight of 173 (retention time 3.12 min) which were interpreted as 2-nitroso-1-napthol. Whereas LC-MS analysis of Reactive Red dye degraded product showed a peak of unidentified compound with a molecular weight of 747 (retention time of 2.83 min). Zhao, X., (2004), reported to

identify several degradation products from purified Direct Orange 3 by *Pleurotus ostreatus* with GC-MS. The results showed four compounds, nitrobenzene, 4-nitrophenol, 4-nitroaniline, and 4-nitroanisole, as degradation products. One of these products, 4-nitroaniline, had also been detected by thin layer chromatography. Zhao, [19] also carried out study on Disperse Yellow 3 and the kinetic profile of the degradation product, acetanilide, was determined by HPLC. The calibration curve was constructed by regressing the peak area against concentrations of the standard solutions in the range of 1-10 ppm. The concentration of products in the samples was calculated using linear regression equations from the calibration curves. HPLC quantification was carried out with the detection wavelength set at 254 nm. The results for this product show that it is difficult to be further degraded in the biodegradation. The yield of acetanilide was determined by the ratio of the molar amount of product to molar amount of dye degradation (decolorization minus absorption) and was estimated to be about 25 percent.

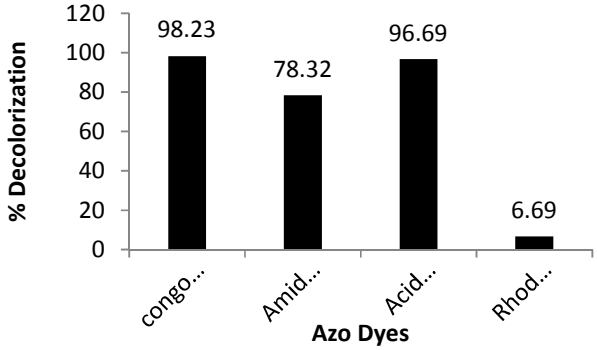

Figure 1.0: Decolorization percentages of four different azo dyes

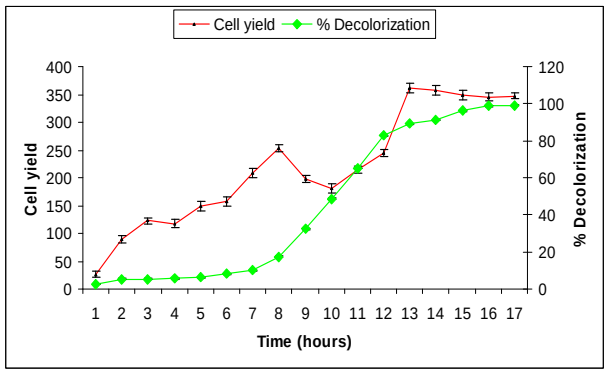

Figure 2: Graph showing % decolorization with cell yield during time

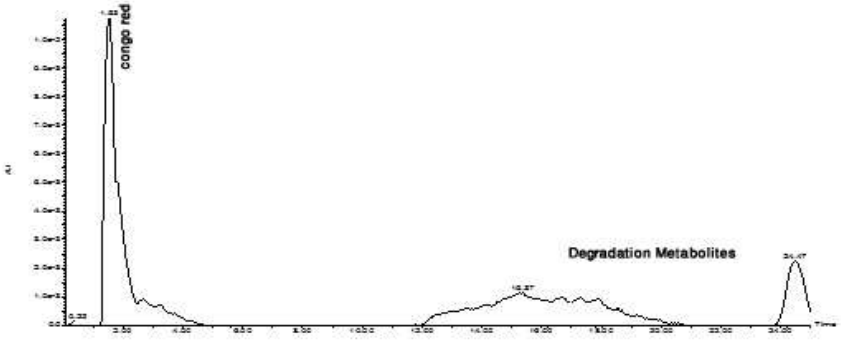

Figure 3: Liquid chromatograh of Congo red dye decolorized byproducts

CONCLUSION

In conclusion *Bacillus subtilis* 'RA29' strain used in this study for the biodegradation of four different azo dyes. During the growth and decolorization kinetic study the maximum decolorization of Congo red azo dye (99.7%), cell yield (347.80) with biomass concentration 17.216mg/ml was observed after 16 hours of incubation at temperature 37^0C under aerobic condition. The enzyme activities of laccase, azoreductase, peroxidase enzymes in partially purified extracellular crude protein were detected as 7.34U/ml, 0.168 U/ml, 0.134 U/ml respectively.

Acknowledgment
The authors gratefully acknowledge the financial support provided by Bhojia Institute of Life Sciences, Budh, Baddi, and District-Solan (H.P.) -173205 and grateful to the Sophisticated Analytical Instrumentation Facility Laboratory of Punjab University for providing the GCMS.

REFERENCES

[1] K T Chung and S E Stevens, *Environmental Toxicology and Chemistry*, **1993**, 12: 2121–2132.
[2] C M Carliell, S J Barclay *et al*., *Water Soil Air Pollution*, **1995**, 21: 61-69.
[3] H Ali, *Water Air Soil Pollution*, **2010**, 213: 251–273.
[4] A Sheikh, K S Rana, *Biology and Medicine.* **2009**,1 (2): 134-138.
[5] J Sharma and B Janveja, *Rasayan Journal of Chemistry* , **2008**, 4: 936-942.
[6] G S Heiss, B Gowan and E R Dabbs, *FEMS Microbiology Letters*, **1992**, 99: 221–226.
[7] J L Bragger, et al., *International Journal of Pharmaceuticals*, **1997**, 157: 61
[8] A M Talarposhti, T Donnelly, G K Anderson, *Water Research* , **2001**, 35: 425–432.
[9] J Sharmaand, B Janveja, *Rasayan Journal of Chemistry*, **2008**, 4: 936-942.
[10] P C Vandevivere, R. Bianchi, W Verstraete, *Journal on Chemical Technology and Biotechnology*, **1998**, 72: 289–302.
[11] T Zimmermann, H G Kulla, T Leisinger, *European Journal of Biochemistry*, **1982**, 129: 197–203.
[12] S Seshadri, P L Bishop, A M Agha, *Waste Manage*, **1994**, 14:127–137.
[13] E R Flores *et al*., *Environmental Science Technology*, **1997**, 31: 2098–2103.
[14] W Haug *et al*., *Applied and Environmental Microbiology*, **1991**, 57: 3144.
[15] M F Coughlin, B K Kinkle, A Tepper, P L Bishop, *Water Science Technology* **1991**, 36: 215–220.
[16] R Rajendran, S K Sundaram, K U Maheswari, *Journal of Environmental Sciences and Technology*, **2011**, 4: 575-576.
[17] S Shobana, E B Thangam, *Journal of Bioremediation & Biodegradation*, **2012**, 3: 5-6.
[18] M S Usha et al., *Journal of Chemical and Pharmaceutical Research*, **2011**, 3: 455.
[19] X Zhao, Analysis of fungal degradation products of azo dyes. *Applied Microbiology and Biotechnology*, **2004**, 1: 33.