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Isolation and characterization of *Aeromonas aquariorum* from a dye effluent and its effect in bioaugmentation

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Abstract: *Trypan Blue, a diazo dye and synthetic organic colourant, is commonly used in several textile and printing industries. A substantial portion of such dyes are recalcitrant and carcinogenic which are disposed of untreated in the environment. In this investigation, water sample in which such textile effluents are disposed of was used as a source of isolating a unique bacterium in vitro which was characterized and 16S rDNA gene sequencing was carried out. It was identified to be similar to Aeromonas aquariorum strain N2 based on nucleotide homology and phylogenetic analysis. This Gram negative short rod bacterium was seen to be degrading the dye to non-hazardous compounds including simpler hydrocarbons. Spectrophotometric analysis and SDS-PAGE techniques were performed to give conclusive evidence for the presence of the enzymes responsible for this. Characterization of the effluent with Gas Chromatographic technique indicated the degradation of the active component. The enzymatic profile revealed the enhanced expression of Azoreductase, Alkane Hydroxylase and Catechol 1, 2-dioxygenase in the presence of the dye. This paper narrates the detailed action of the above mentioned bacterium responsible for enzymatic degradation of a foreign pollutant which paves way for safe treatment of colouring agents.*

Keywords: *Aeromonas aquariorum, azodye, dye degradation, enzymatic profile, Trypan Blue.*

I. Introduction

Trypan Blue is a vital stain used to selectively colour non-viable or damaged cells. It is used as an organic colourant in various industries like printing, textile. Trypan Blue is derived from toluidine and its property to kill trypanosomes [1], which is a parasite causing sleeping sickness, lends it the name.

Trypan Blue, apart from causing certain birth defects like encephalocele, is a potential carcinogen to humans and other animals. Thus the degradation of this dye is a step towards bioremediation by reducing health hazards. Conventional and municipal waste water treatment processes involving light, chemicals or activated sludge are unable to degrade such recalcitrant dyes [2]. Such dyes are thus ejected into the aquatic environment causing severe environmental concerns and public health deteriorations. Chemical degradation deals further damage to the environment and hence it paves the way for biological treatment. The reported enzymes, Azoreductase, Alkane Hydroxylase and Catechol 1, 2-dioxygenase express themselves in the presence of the mentioned dye and are responsible for safe degradation to simpler, harmless compounds.

The International Agency for Research on Cancer (IARC), classifies aromatic amines like Benzidine, a component present in several azodyes (mainly amino- substituted) as Class- I known human carcinogen. Hence neutralization of such compounds in vivo in the environment is absolutely mandatory. Biodegradation thus provides a much environment friendly alternative to laborious, expensive and hazardous chemical degradation processes.

II. Materials And Methods

2.1 Sources and Collection

The textile effluent was acquired from Jayram Textile, Barrackpore, West Bengal. It was found to have Trypan Blue dye, partially ultramarine blue, along with diazo and inorganic pigments and salts.

2.2 Characterization of sample

1. Colour- Ultramarine Blue
2. Colour Index- Azo21723, Inorganic Pigments75128
3. Odour- No characteristic odour
4. Temperature- 25°C
5. pH- 6.9
6. Electrical Conductivity- 1.585 µS
7. TDS (Total Dissolved Solid) - 799.2 ppm.
8. TSS (Total Suspended Solid) -10,800 mg/L

2.3 Isolation and Purification

The effluent dye sample was inoculated on Nutrient Agar plates and incubated at 37° C for 24 hours. Bacterial growth was observed. Pure cultures of the bacterial colonies were isolated on Nutrient Agar (NA) plates.

2.4 Characterization of Isolated Organism

On the basis of Gram staining, the organism was found to be a Gram negative short rod (Refer to Fig.1). 16S rDNA gene sequencing was done and it was found to be similar to *Aeromonas aquariorum* strain N2.

2.5 Quantitative estimation of dye degradation

2.5.1 Percentage of dye degradation at different bacteria: dye ratio

In order to test the activity of the bacterium pertaining to the degradation of the dye in the sample concerned, spectrophotometric assay at 575 nm was carried out in different bacteria: dye ratios and the percentage degradation calculated. The reaction mixtures, in separate test tubes containing 10 ml of the bacteria: dye ratios were incubated at 37°C for 48 and 96 hours.

2.5.2 Effect of $FeCl_3$ and H_2O_2 in dye degradation

In order to determine the capability of the isolated organism to degrade the Trypan Blue dye and other inorganic pigments, different sets of reaction mixtures were prepared in presence of $FeCl_3$ and H_2O_2, keeping the reaction volume 4 ml, and incubated at 37°C for 24, 48 and 72 hours. Spectrophotometric assay was then carried out at 590 nm and percentage degradation was calculated.

2.6 Enzyme Assay

Four bacterial enzymes were identified – Peroxidase, Azoreductase, Alkane Hydroxylase and Catechol 1, 2- dioxygenase. Assays for the following enzymes were carried out.

2.6.1 Peroxidase (EC 1.11.1.7) Assay

3 ml of pyrogallol solution and 0.1 ml of the prepared enzyme extract were taken in a cuvette. The optical density (O.D) of the mixture was measured at 430 nm and was taken as the control. This mixture was taken as the blank and later 0.5 ml of 1 % H_2O_2 was added and mixed thoroughly. The O.D was measured at regular time intervals at 430 nm.

2.6.2 Azoreductase (EC 1.7.1.6) Assay

The assay was carried out in a total reaction volume of 1 ml. The reaction mixture contained 25 mM potassium phosphate buffer (pH 7.0), 20 µM of azo dye and enzyme extract (water source). The reaction was initiated by the addition of 0.1 mM NAD(P)H. The extinction coefficient for Methyl Red at 430 nm was 23,360 $M^{-1}cm^{-1}$. The enzyme activity was measured by a decrease in absorbance over a 2 minute period using an Ultraviolet- visible spectrophotometer. The enzyme activity was defined as the decrease in azodye concentration (µM) per minute. For the control, the enzyme was denatured by boiling it for 15 minutes, followed by the addition of few drops of hydrochloric acid.

2.6.3 Alkane Hydroxylase (EC 1.14.15.3) Assay

320 µl of extract was taken in 0.1M NADH. Also, 5 µl of n-octane solution was taken. O.D. was measured at 340 nm.

2.6.4 Catechol 1, 2-dioxygenase (EC 1.13.11.1) Assay

Catechol 1, 2-dioxygenase activity was assayed using a spectrophotometer. The standard assay of enzyme activity was performed by making an assay mixture containing 500 µl crude extract as source of Catechol 1, 2 dioxygenase, 200 µl of 10 mM Catechol as a substrate and final volume adjusted to1 mL with 50 mM sodium phosphate buffer (pH 7.0). The enzyme activity was monitored by measuring the formation of cis, cis-muconic acid at 260 nm.
(€= 16.8 mM/cm). [3]

2.7 Protein estimation of the isolated enzymes and their characterization

SDS-PAGE was carried out by the Laemmli method. The 12 % Resolving gel and the Stacking gel were prepared and inserted into the gaps between the glass plates. The running buffer was poured into the chamber similar to the above mentioned method. The prepared samples were loaded into the wells and the gel was allowed to run at a fixed voltage for a fixed time (depending on the time taken by the resolving gel). The gel

was then taken out of the glass plates, stained with the Coomassie Brilliant Blue staining solution and kept on a rocker overnight. The following day it was destained thrice at regular intervals for obtaining clear bands.

2.8 Degraded Product Determination

Gas Chromatographic Technique

Column−30-m x 0.25-mm I.D. SE-54 fused silica capillary column.

Detector: Flame ionization (FID). Micro-syringe: 10- µl.

Stock standard solutions at a concentration of 1 µg/ml each were prepared by dissolving 0.0100 g of assayed reference material in iso-octane and diluting to volume in a 10-mL volumetric flask. The stock standard solutions were transferred into Teflon-sealed screw cap bottles and stored at 4°C. Sample recording: The sample volume injected and the resulting peak sizes (in area units or peak heights) were recorded.

2.9 Identification of organism

16S rDNA sequencing

DNA was isolated from the culture. Quality was evaluated on 1.2 % Agarose Gel where a single band of high-molecular weight DNA was observed. Fragment of 16S rDNA gene was amplified by PCR from the above isolated DNA. A single discrete PCR amplicon band of 1500 bp was observed (Refer to gel Fig 5). The PCR amplicon was purified and further processed for the sequencing. Forward and Reverse DNA sequencing reaction of PCR amplicon were carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 1412 bp 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST alignment search tool of NCBI GenBank database.

III. Results

3.1 Percentage Dye Degradation

It is seen that with the progress in time, the percentage dye degradation increases in each of the reaction mixtures. With the increase in concentration of the bacterial culture the dye degradation is enhanced to up to 92.39 % in case of 2:1 ratio. Quantitative estimation was carried out by:

$$\text{Percentage degradation} = \frac{\text{Initial O.D.} - \text{Final O.D.}}{\text{Initial O.D.}} \quad X \quad 100 \qquad (1)$$

This result is illustrated in Table 1 and Fig. 2.

3.2 Effect of $FeCl_3$ and H_2O_2 in the Dye Degradation

In order to observe the activity of the enzyme in the presence of Fenton's reagent, an iron salt in the form of ferric chloride and hydrogen peroxide were used. Spectrophotometric analysis and the respective dye degradation (expressed in %) was carried out for several combinations to study the effect in general. Results are depicted in Table 2. The sample contained the dye in solution and minute quantities of bacteria which express the mentioned enzymes in the presence of this dye. It was observed that when pure culture of the bacterium in discussion was inoculated with the sample, 73.15 % degradation was observed.

In the presence of hydrogen peroxide suitable results were not observed since it had a toxic effect on the bacterial population, causing death. The addition of ferric chloride to the sample brought about upto 97.8% degradation since it caused precipitation of water insoluble matter, and almost their decolourization.

In the presence of $FeCl_3$ and the sample, the bacterium produces the enzymes concerned but the percentage degradation reduces which may be due to the Fe^{3+} blocking the active site of the enzyme repressing their necessary expression after a certain time span. In the presence of $FeCl_3$, hydrogen peroxide and the sample, the bacterium is able to produce the mentioned enzymes and a staggering 91.23 % degradation is observed. The dye degradation is enhanced due to the Fenton's reaction [4] which takes place due to the presence of the iron salt and hydrogen peroxide together which liberates OH^- ions which is a powerful, non- selective oxidant and is majorly responsible for the degradation of the bonds involved in the azodye, Trypan Blue.

3.3 Enzyme Assay

3.1.1.1 Peroxidase Assay Analysis

It is observed that with the progress in time there is a steady rise in the O.D which indicates the degradation of the supplied H_2O_2 thereby confirming the presence of peroxidase enzyme in the isolated organism**.** Refer to Table 3.

3.1.1.2 Analysis of Dye Degrading Enzymes

Azoreductase, Alkane Hydroxylase and Catechol 1, 2- dioxygenase were obtained in both the dye sample as well as the pure culture sample of the organism by performing their respective assays. It was also seen that in the presence of the dye the organism depicts the expression of all the mentioned enzymes i.e., azoreductase, alkane hydroxylase and catechol 1, 2- dioxygenase showing elevated levels up to 17.5 times, 2.4 times and 6.22 times respectively. Refer to table 4.

3.4 SDS-PAGE

SDS-PAGE result confirms the presence of the dye degrading enzymes in the isolated organism by the presence of bands in their respective molecular weight position on the gel. Azoreductase with molecular weight 20-22 kDa, Alkane Hydroxylase with molecular weight 45-52 kDa, Peroxidase with molecular weight 40-44 kDa and Catechol 1, 2-dioxygenase with molecular weight 28-35 kDa can be clearly distinguished on the gel. The result can be seen in Fig 3.

3.5 Gas Chromatography

Gas chromatogram obtained from this technique shows the presence of different products formed by the enzymatic activities of *Aeromonas aquariorum.* The organism helps in degrading the complex hydrocarbons into simpler and non-hazardous forms. From the pre-made standards few of the products were found to be derivatives of C14, C16 and C24. Rest of the peaks were unidentified. Also the quantitative estimation of the products revealed the total hydrocarbon content to be 32 µg/ml, 12 µg/ml and 18 µg/ml respectively. Refer to Figure 4.

3.6 16S rDNA sequencing

Based on maximum identity score, the first fifteen sequences were selected and aligned using multiple alignment software program, Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree (Refer to Fig. 6) was constructed using MEGA 5. The organism was identified to be similar to *Aeromonas aquariorum* strain N2 (GenBank Accession Number: JF775500.1).

IV. Tables And Figures

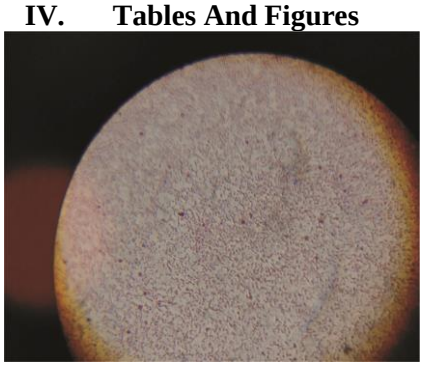

Fig 1. Gram negative short rods under 45X.

Table 1: Percentage of dye degradation at different bacteria: dye ratio. Control: +0.092

Bacteria: Sample Ratio	Incubation Time	Optical Density (Measured at 575 nm)	% Dye Degradation
1:1	48 hours	+0.04	56.5
	96 hours	+0.01	89.13
1:2	48 hours	+0.061	33.69
	96 hours	+0.021	77.17
2:1	48 hours	+0.02	78.26
	96 hours	+0.007	92.39

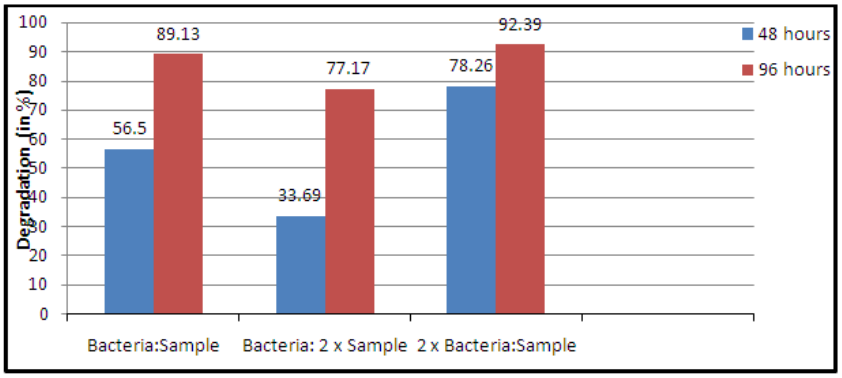

Fig 2: Graph depicting percentage of dye degradation at different bacteria: sample ratios.

Table 2: Effect of $FeCl_3$ and H_2O_2 in the dye degradation. Control: +0.365

Reaction Mixture	**Incubation Time**	**Optical Density (Measured at 590 nm)**	**% Dye Degradation**
Bacteria + Sample	24 hours	+0.12	67.12
	48 hours	+0.169	53.69
	72 hours	+0.098	73.15
H_2O_2+ Sample	24 hours	-	-
	48 hours	-	-
	72 hours	-	-
$FeCl_3$+ Sample	24 hours	-	-
	48 hours	+0.02	94.5
	72 hours	+0.008	97.8
Bacteria + Sample +$FeCl_3$	24 hours	+0.084	76.98
	48 hours	+0.145	60.27
	72 hours	+0.123	66.3
Bacteria + Sample + $FeCl_3$+ H_2O_2	24 hours	-	-
	48 hours	+0.032	91.23
	72 hours	+0.035	90.41

Table 3: Spectrophotometric Analysis for Peroxidase Assay.

Time Interval	**Optical Density (Measured at 430 nm)**
0 sec	-
60 sec	+0.001
90 sec	+0.002
120 sec	+0.003
150 sec	+0.003
180 sec	+0.004

Table 4: Detection of Dye Degrading Enzymes.

Dye Degrading Enzyme	**Concentration (in U/mg/ml).**	
	In Dye Sample (With respect to control).	**In pure culture.**
Alkane Hydroxylase	0.12	0.05
Azo Reductase	0.35	0.02
Catechol 1,2-dioxygenase	0.56	0.09

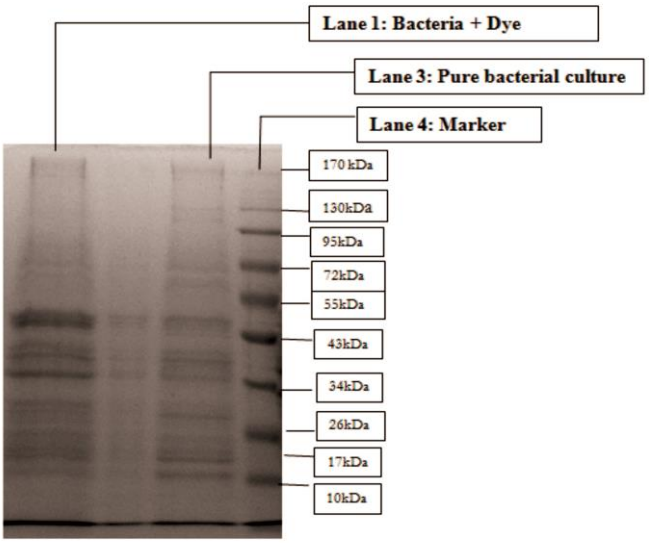

Fig 3. SDS-PAGE gel run.

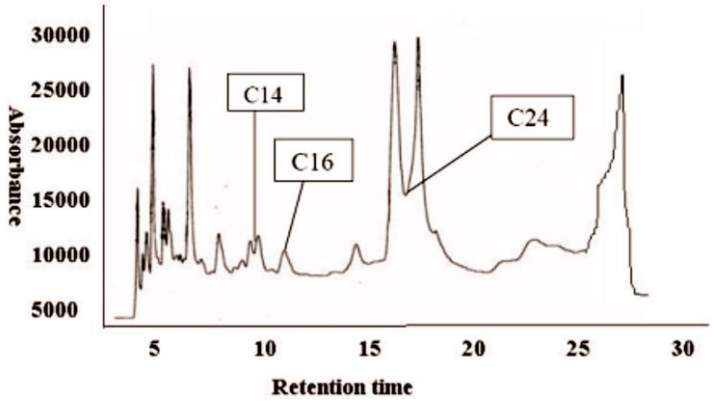

Fig 4. Gas chromatogram.

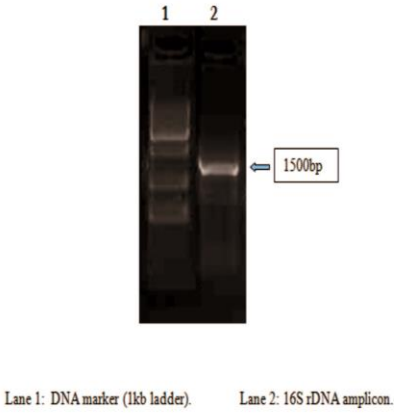

Fig 5: 1.2% Agarose gel showing single 1.5 kb and 16S rDNA amplicon.

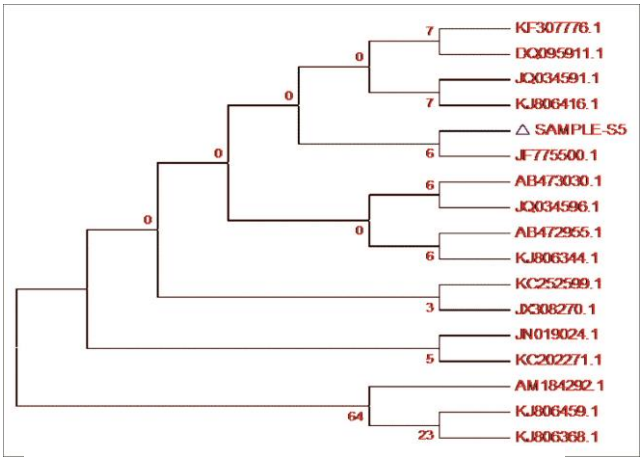

Fig 6: Phylogenetic tree showing the evolutionary relationships of eleven taxa.

V. Conclusion

Industrial effluents containing recalcitrant compounds have been a major source of pollution. Various experiments have been carried out to remove these pollutants using chemical treatments like electrochemical treatment by chloride ions of Trypan Blue synthetic waste water [5]. However, mostly it has been the biological treatment of these hazardous compounds that has gained an upper hand in the successful removal of toxicity. Toxicity of azodyes has been found to be removed by fungal treatment as reported by Apohan and Yesilada (2005) [6] [7] [8]. Exploiting the enzymatic activity of different microorganisms like *Pseudomonas aeruginosa* [9], *Bacillus* spp. [10] [11] [12], *Klebsiella pneumoniae* [14] have been reported for different dye samples showing the versatility of these organisms in the removal of toxic dye compounds. Also, *Enterococcus faecalis* was found to decolourize Reactive Orange dye upto 77.73 % [15].

The major aim of this paper has been to try and exploit the isolated organism to the fullest in order to remove all compounds having detrimental effects. The dye sample used here, which was later found to be Trypan Blue, was acquired from Jayram Textiles, Barrackpore. The organism, *Aeromonas aquariorum*, was isolated from this sample which could successfully degrade the dye due to the presence of various enzymes like azoreductase, alkane hydroxylase, catechol 1, 2-dioxygenase and peroxidase expressed by the organism itself. Researchers like Umme Kalsoom et al. (2013) had administered enzymes like horseradish peroxidase externally for the same purpose to get similar results [16].

The exo-enzymes released by *Aeromonas aquariorum* can be used in order to degrade the hazardous Trypan Blue dye components to simpler non-hazardous hydrocarbons. Since these recalcitrant dyes are present in industrial effluents which are released into the environment, untreated, they tend to pollute it and are potential source of environmental hazard. So the isolated organism can be deployed into these polluted bodies for the manifestation of its enzymatic activity leading to the biological treatment of the waste water. However, since the organism is an opportunistic pathogen it has to be removed by water purification techniques.

The major usage of this organism can be in bioremediation. *Aeromonas aquariorum* can be used to degrade pollutants to simpler eco-friendly forms.

In the near future, genes encoding for the dye degrading enzymes can be sequenced and amplified by PCR techniques. As *Aeromonas aquariorum* is an opportunistic pathogen, this desired gene sequence can be incorporated into other bacteria like *Escherichia coli* (a commensal) through cloning vectors like plasmids thereby making these organisms capable of dye degradation and eliminating any chance of hazard. With the genes responsible for the secretion of the dyes reported and sequenced, such cDNA libraries can be maintained for further use and need.

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References

[1]. Harrington John M., Widener Justin, Stephens Natalie, Johnson Thomas, Francia Maria, Capewell Paul, Macleod Annette and Hajduk Stephen L. The Journal of Biological Chemistry. ISSN 0021-9258.
[2]. Chung KT, Stevens SEJ and Cerniglia CE 1992 Crit. Rev. Microbiol. 18 175**.**
[3]. Naiem H. Nadaf and Jai S. Ghosh; ResearchJournal of Environmental and Earth Sciences 3(5): 608-613, 2011
[4]. Bishop, D.F. et.al. "Hydrogen Peroxide Catalytic Oxidation of Refractory Organics in Municipal Waste Waters", in Ind. Eng. Chem., Process Design & Development, vol.7, pp. 1110-117 (1968).
[5]. Anantha N. Subbarao and Venkatesha T. Venkatarangaiah; J. Electrochem. Sci. Eng. 3(4)(2013) 167-184
[6]. E. Apohan. O. Yesilada, J. Basic Microbiol., 2005, 45:99-105.
[7]. L. Papinutti, F. Forchiassin, FEMS microbial.lett., 2005**,** 11381:1-5.
[8]. W. M. Abd el-Rahim, H. Moawad; J. Basic Microbiol.,2003**.** 43:367-375
[9]. Annika A. Durve, Arvind R. Gupta and Sayali R. Naphade;Advances in Applied Science Research, 2012, 3 (5):2660-2671
[10]. Sriram N., Reetha D. and Saranraj P. Biological Degradation of Reactive Dyes by Using Bacteria Isolated from Dye Effluent Contaminated Soil. Middle- East Journal of Scientific Research 17(12): 1695-1700, 2013 ISSN 1990- 9233.
[11]. H.G. Kulla, F. Klausener, U. Meyer. Arch. Microbiol**.,** 1983**.** 135, 1-7.
[12]. J. Maier, A. Kandelbauer, A. Erlacher, A. Cavaco-P, M. GubitzAppl. Environ. Microbiol.2004, 70, 837-844.
[13]. Sridevi .Neelam and Rao J. Chandra Sekhara. Biodegradation Studies on Selected Textile Dye by Using Bacterial Isolates. Asian J. Exp. Biol. Sci. Vol 4(2) 2013: 288-292.
[14]. P.K. Wong, P.Y. Yuen Water Res., 199630, 1736-1744.
[15]. M. M. Sahasrabudhe and G. R. Pathade, European Journal of Experimental Biology, 2011, 1 (1), 163-173.
[16]. KalsoomUmme, Ashraf Syed Salman, Meetani Mohammad A, Rauf Muhammad A and Bhatti Haq Nawaz. Mechanistic study of a diazo dye degradation by Soybean Peroxidase. Kalsoom et al. Chemistry Central Journal 2013, 7:93.