4.1 INTRODUCTION:

The effluents discharged by industries such as carpet, textile, leather, pharmaceuticals, food, cosmetics and printing contain azo dye (10–15 %) [Forgacs *et al.* (2004); Vimonses *et al.* (2010); Robinson *et al.* (2001)], which are considered to be the most hazardous among xenobiotics. The Acid Red G is one of the popular azo dyes that have been extensively used for the dyeing wool, nylon, and silk fibers [Ozcan *et al.* (2004); Vimonses *et al.* (2010)]. Azo dyes are being recalcitrant and toxic in nature which creates havoc to flora and fauna. The discharge of these effluents into nearby water bodies leads to the reduction in sunlight penetration and dissolved oxygen content of waterways; hence, increase in biological oxygen demand and chemical oxygen demand adversely affect the water quality [Jonstrup *et al.* (2011); Meng *et al.* (2012)]. It is also well documented that synthetic dyes and their metabolites are toxic, carcinogenic and mutagenic in nature [De AragaoUmbuzeiro *et al.* (2005); Tan *et al.* (2005)]. To overcome these problems, effluent standard has to be achieved prior to disposal to surface bodies. There are various physical and chemical methods for treatment such as adsorption, photoionization, electrolysis, oxidation, and neutralization that have limitations like high cost, low efficiency, and production of secondary toxic intermediates [Asad *et al.* (2007); Zainal *et al.* (2005); Harrelkas *et al.* (2008)]. Due to cost effectiveness and ecological compatibility, bioremediation of such effluents is most promising technology [Baban *et al.* (2010); Spagni *et al.* (2010); Kolekar *et al.* (2012)].

Bhadohi (Uttar Pradesh) carpet cluster of India is one of the oldest clusters in the world, continuously working from the 16th century onward. There are number of dying plants; majority of them are discharging their effluent only after lime treatment. The ground and surface water of the region are badly affected. There is enough literature reported pertaining to dye effluent treatment for textile industries, but very few of them

are adding the need of carpet clusters. Few articles available for this cluster have used only fungi. Literally, there is no literature for the treatment of carpet dye effluent using indigenous bacteria. This study is an attempt to identify indigenous bacteria to treat these effluents at lower cost.

In the present chapter deals with the isolation and identification of bacterial strain BHUSSp X2 and its subsequent application for the bioremediation of carpet dyeing effluent of carpet industries (Bhadohi) Uttar Pradesh, India. Contaminated soil sample from the site was used for isolation of bacterial strain, and their identification was done using 16S rRNA gene sequence technique. Important operating parameters were optimized for degradation in batch mode. Relative toxicity of degraded products was investigated through phytotoxicity analysis of degraded product. GC-MS studies were performed for the detection of biotransformation pathway of RED G dye. The enzyme responsible for azo dye degradation was also identified.

4.2 Result and Discussion:

4.2.1 Isolation, screening and identification of bacterial strain

The microorganisms were isolated from the soil sample collected from effluent discharged site of carpet cluster industries as these were well acclimatized with the system having better potential for degradation of dyes. The bacterial strain BHUSSp X2 showed remarkable decolorizing ability for RED G dye, among various strains isolated. The bacterial identification was based on Bergey's methods employed, and to finalize the species using isolated bacterial strain 16S rRNA gene sequence analysis was used. The *Stenotrophomonas* sp. BHUSSp X2 (*Figure 4.1*) thus identified was used for decolorization in present investigation. The isolated gene sequence was deposited in the Gene Bank of NCBI having accession number (KJ740220), which has till date not been utilized for decolorization of dye.

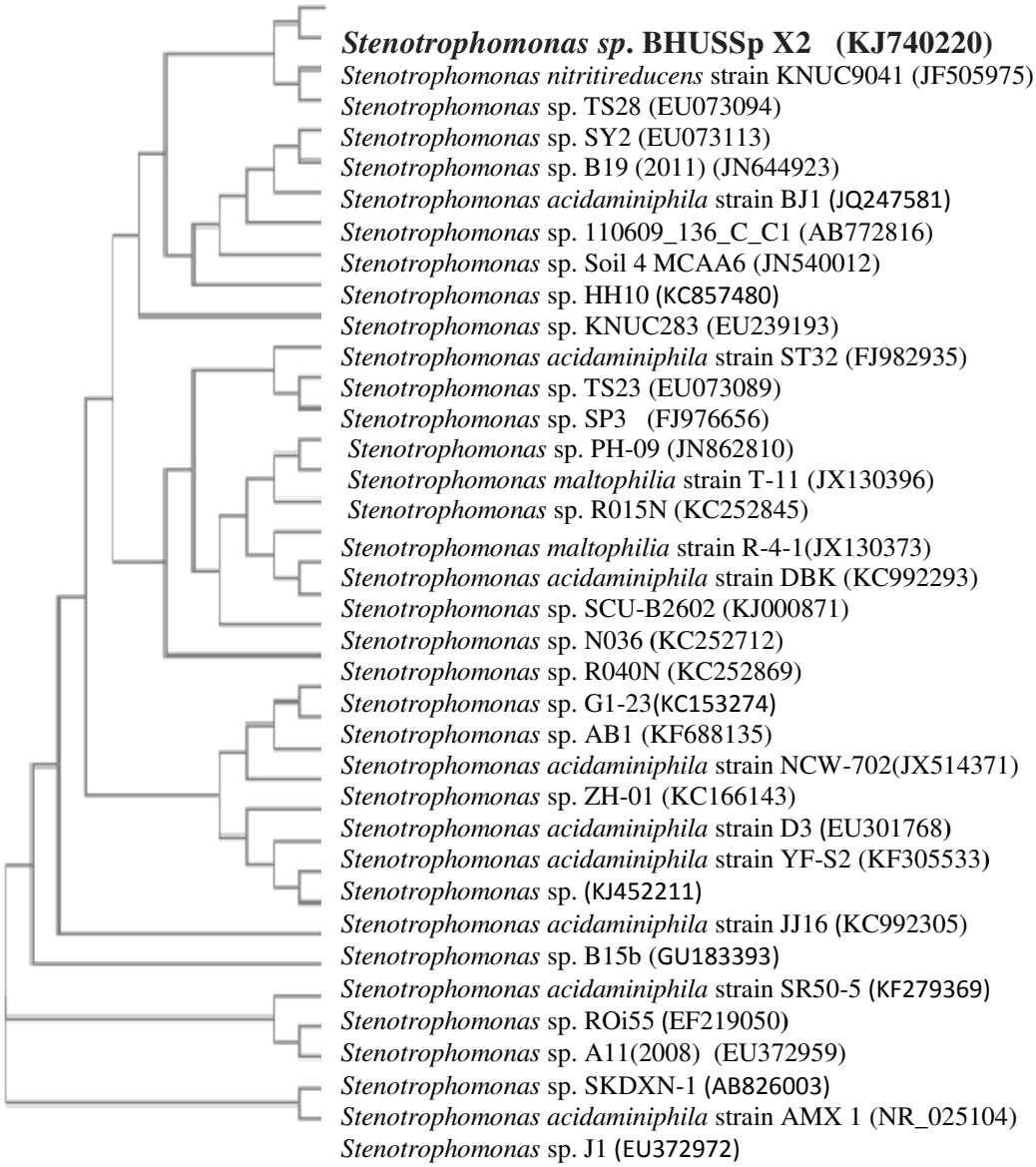


Figure 4.1: Phylogenetic tree: Neighbor joining phylogenetic tree based on 16S rRNA gene sequence showing the relationship between isolated bacterial strain *Stenotrophomonas* sp. BHUSSp X2 and other relatives within genus.

4.2.2 Optimization of important operating parameters

As shown in *Figure 4.2*, 95% of decolorization was achieved at static condition as compared to 45% decolorization under shaking condition at 100 rpm. This trend is reversed to the growth of bacterial strain. This observation is well supported by [Moosui *et al.* (2005); Khehra *et al.* (2012); Oturkar *et al.* (2011)]. This is possibly due to reduced activity of azoreductase with increase dissolved oxygen level under shaking condition whereas at static condition azo dyes itself acts as an electron carrier resulting into increase decolorization efficiency [Pearce *et al.* (2003)]. So, optimization of other important operating parameters for dye degradation static conditions was preferred.

The effluent being discharged from dying plants of carpet industries cluster generally have basic pH (7–10), and this has been validated during the testing of effluent of Bhadohi carpet cluster. *Figure 4.3* shows the effect of pH on decolorization. It was observed from the figure that minimum decolorization achieved at pH 5 was below 23% whereas when the pH was 7 and 8, decolorization efficiency improved drastically, and more than 95% decolorization have been achieved. With further increase in pH 9 and 10, efficiency of decolorization again reduced, and only 30% decolorization was observed. This might be due to the conducing environment of growth of bacterial strain in alkaline environment. However, further increases in pH were again nonfavorable for the system due to the prevailing condition.

Temperature plays a vital role in growth and performance of bacterial strain. So, the performance of BHUSSp X2 was evaluated in the temperature range of 20–45 °C for decolorization as shown in *Figure 4.4*. It is clear from this figure that only 33%

decolorization was obtained at 20°C. However, with increases in temperature, decolorization efficiency of bacterial strain was improved, and 97% decolorization was observed at 35°C. With further increase in temperature, decolorization efficiency started declining resulting only 55% decolorization at 45 °C. The reduced decolorization efficiency at 20 and 45 °C are probably due to deactivation of enzyme at these extreme conditions. But, further increase in temperature showed adverse impact on decolorization as even less than 55% decolorization could be achieved at 45 °C. The decolorization rate depends on temperature because at very low and high temperature, deactivation of enzymes takes place. At lower initial dye concentrations of 100–200 mg/L, more than 95% decolorizations have been achieved within 6 h in *Figure 4.5*. With further increase in dye concentration (300– 500 mg/L), reduced and delayed decolorization was observed, and maximum decolorization of 90% could be achieved after 18 h. Beyond 700 mg/L, the bacterial strain seems to be ineffective as maximum of 30% decolorization only be achieved even after 24h. This decrease in decolorization at higher dye concentration can possibly be attributed to lower growth and higher toxicity due to accumulation of large quantity of metabolites.

The nutrient plays a vital role in dye degradation as they control the growth of bacterial strain. The impact of various carbon and nitrogen sources on dye degradation was investigated and summarized in *Table 4.1*. Efficacy of agricultural residues such as wheat bran, rice husk, and bagassa was also investigated as carbon sources. It can clearly be seen from *Table 4.1* that addition of either one carbon or one nitrogen sources has minimal effect on decolorization with exception of yeast as nitrogen source which

rendered 80% decolorization with 10 h even without any carbon source. The combination of yeast and glucose as nitrogen and carbon sources resulted into 90% decolorization within 8 h closely followed by ammonium chloride and glucose as carbon source resulting into 82% decolorization in 12 h, whereas the nutrient broth comprising multiple nitrogen sources and single carbon have rendered 98% decolorization within 6 h. Agriculture residue such as wheat bran and bagassa proved to be worthy of replacing the combination of carbon and nitrogen sources as 85 and 72% decolorization could be achieved within 8 and 10 h, respectively.

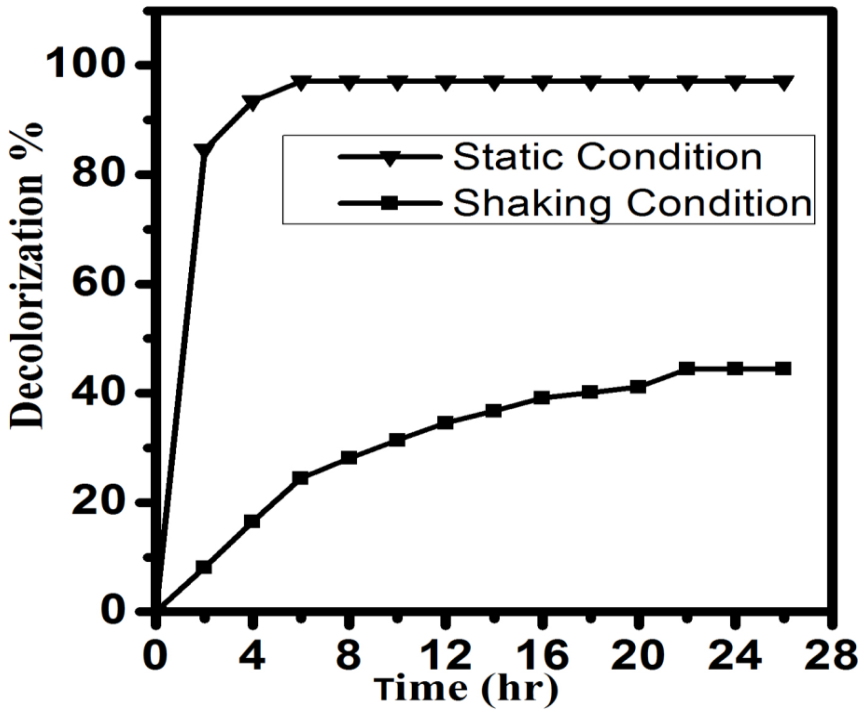


Figure 4.2: Effect of shaking and static condition on decolorization of C.I. Acid Red 1 dye by isolated bacterial strain in minimal media.

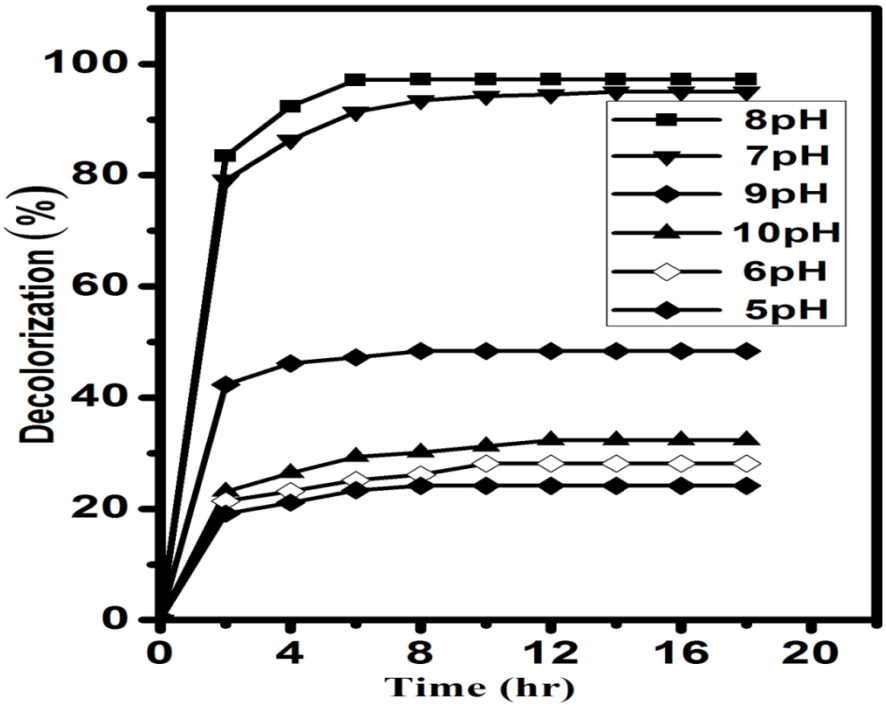


Figure 4.3: Effect of pH on decolorization of C.I. Acid Red 1 by strain BHUSSp X2 in minimal media. The reaction was performed in static condition with a concentration of dye 200 mg/L at temperature 35 °C. Error bars present $p < 0.05$.

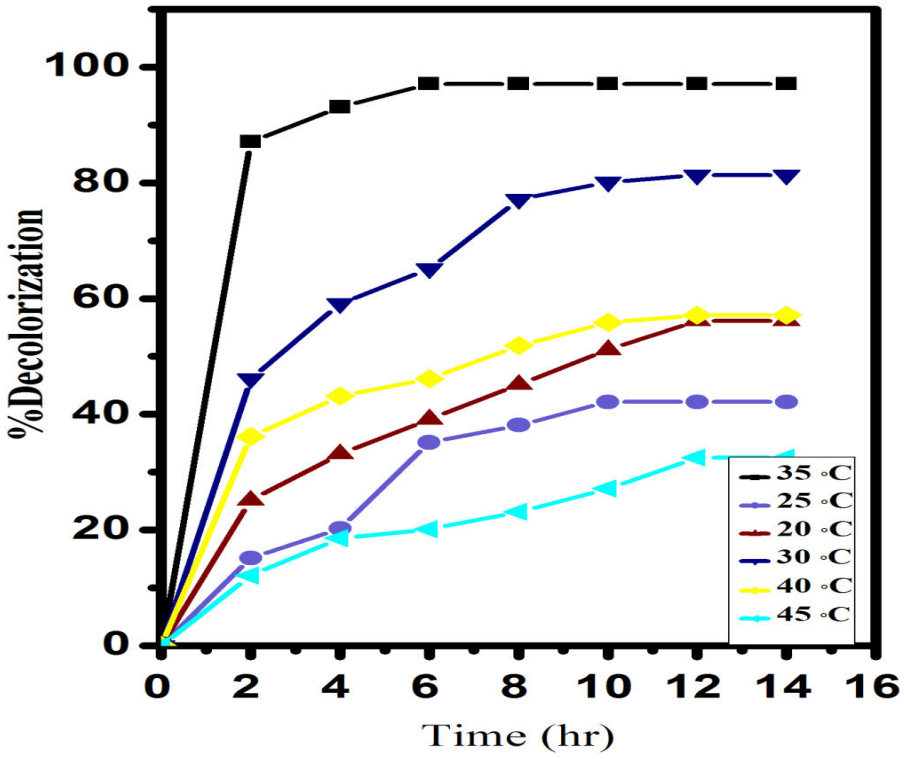


Figure 4.4: Effect of various temperature on the decolorization of C.I. Acid Red 1 by isolated bacterial strain *Stenotrophomonas* sp. BHUSSp X2 in minimal media. Conditions: pH 8; under static condition. Error bars present $p < 0.05$.

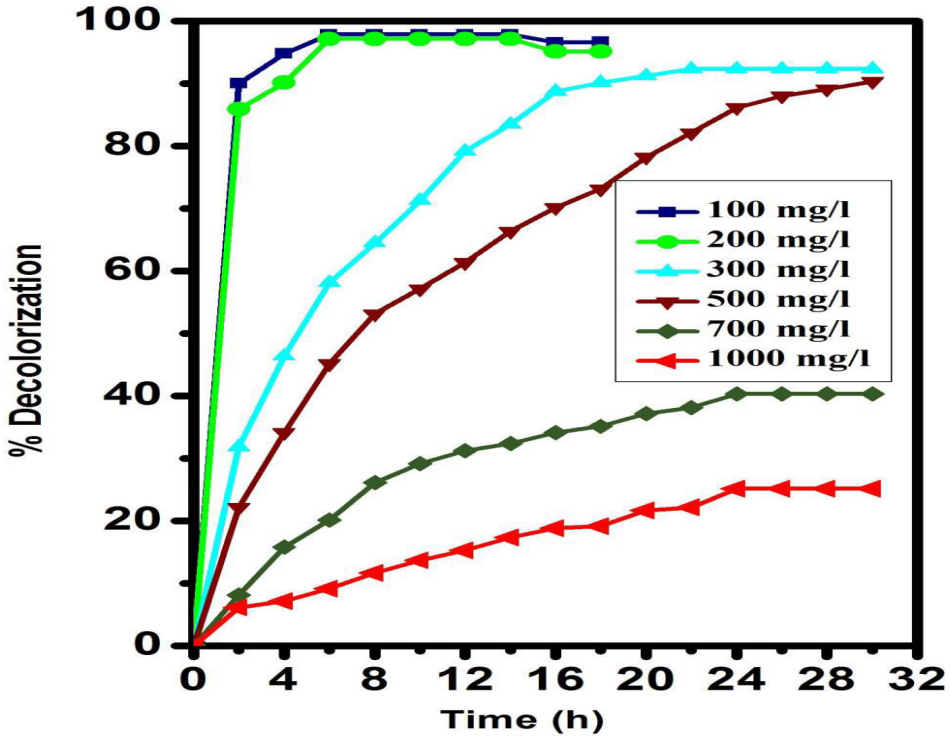


Figure 4.5: Effect of initial dye concentration on the decolorization of C.I. Acid Red 1 by isolated bacterial strain *Stenotrophomonas* sp. BHUSSp X2 in minimal media. Conditions: pH 8; temperature 35°C; under static condition. Error bars present $p < 0.05$.

Table 4.1: Effect of various carbon and nitrogen sources supplemented with MSM media on decolorization by isolated bacteria *Stenotrophomonas* sp. BHUSS X2.

Media	Decolorization (%)	Time (h)
MSM	12	28
MSM + Glucose	52	16
MSM + Dextrose	48	18
MSM + Fructose	40	22
MSM + Lactose	20	13
MSM + Sucrose	35	26
MSM +Peptone	16	20
MSM + Yeast	80	10
MSM+ Starch	16	18
MSM + Ammonium Chloride	22	15
MSM+ Urea	13	24
MSM+ Breef	30	12
MSM + Yeast + Glucose	91	8
MSM+ Ammonium Chloride+ Glucose	82	12
MSM + Urea+ Glucose	25	16
MSM+ Rice Husk	34	14
MSM+ Wheat bran	85	8
MSM + Bagassa	72	10
MSM + Woods chips	62	14
Nutrient Broth	98	6

4.2.3 Repeated applicability test of isolated bacteria

The decolorization efficiency of isolated bacterial strain for Acid RED G (200 mg/L) under static condition was measured for ten cycles. Fresh addition of dye was made for every cycle. The result thus obtained shown in *Figure 4.6*. It is obvious from figure that decolorization efficiency decreases with increase in cycle as only 25% decolorization was observed in 48 h for tenth cycle whereas for first two cycles, more than 95% decolorization was achieved only in 6 h. The decrease decolorization efficiency on repeated use of isolated bacterial strain could be attributed to reduced ability of nutrient leading to stationary phase and subsequently to death phase (Kolekar *et al*. 2013).

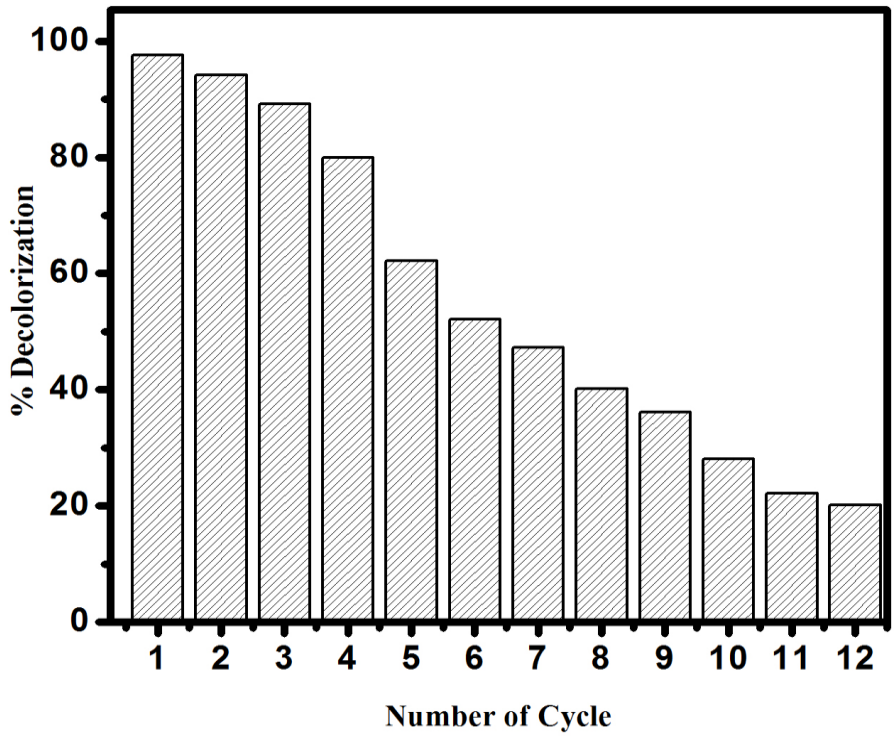


Figure 4.6: Repeated Use of isolated bacterial strain BHUSS X2. Error bars present $p < 0.05$.

4.2.4 Enzyme Analysis

Enzyme and their activities prior and after decolorization are summarized in *Table 4.2*, and values presented are mean of three replicates. Increased amount of enzymes such as laccase, lignin peroxidase, tyrosinase, azoreductase, and NADH reductase in the degraded product suggest their prominent role in the decolorization process. It also suggests that extracellular and intracellular enzyme activities are induced in the presence of dye. Azoreductase seems to be most prominent enzyme in decolorization using *Stenotrophomonas* sp. BHUSSp X2 followed by laccase and NADH as significant increase in their amount has been observed as compared to control. The relative contribution of these enzymes may vary with the change in bacterial strain. The decolorization observed in the enzymes in present study such as azoredustase, laccase, NADH, tyrosinase, and ligin peroxidase was, respectively, 500, 103, 97, 60, and 17% reflecting their relative importance. These findings are well supported by [Vijaykumar *et al.* (2007); Jadhav *et al.* (2010)].

Table 4.2: Effect of various enzyme activities before and after decolorization of RED G dye by isolated bacteria *Stenotrophomonas sp*. BHUSSp X2.

Enzyme	**Before Degradation**	**After Degradation**
Laccase[1]	0.34 ± 0.018	0.689 ± 0.108
Tyrosinase[1]	0.50± 0.012	1.25±0.005
Ligin Peroxidae[1]	0.12 ± 0.023	0.14 ± 0.027
NADH-DCIP Reductase[2]	8.23 ± 0.012	16.23 ± 0.005
Azoreductase[3]	0.29 ± 0.012	1.8 ± 0.002

Values are mean of three replicates.

[1]Enzyme activity: µM/min/mg protein

[2]Enzyme activity: µg of DCIP reduced/min/mg protein

[3]Enzyme activity: µMg of RED G reduced/min/mg protein

4.2.5 Analytical investigations

UV-Visible spectrometric analysis was made in the range (200–800 nm) to investigate decolorization for the dye samples prior to and after degradation as shown in (*Figure 4.7*). Complete disappearance of peak in the visible region after 6 h was observed for bacterially degraded sample, confirming the efficacy of isolated strain BHUSSp X2. The results of analytical investigation by HPLC (Water HPLC, Model no. 2690) are presented in *Figure 4.8 (a)* (Prior to degradation) and *Figure 4.8 (b)* (after degradation). The retention time for one peak observed in case of dye sample before degradation as shown in *Figure* 4.8 (a) was 6 min whereas two peaks were observed at retention times 3.99 and 5.1 min, respectively, for bacterially degraded sample (*Figure 4.8 (b)*). These suggest degradation or fragmentation of the compound present in the dye sample. FTIR spectra of control and bacterial degraded samples are shown in *Figure 4.9 (a)* and *Figure 4.9 (b)*. The complete absence of certain peaks and shifting of others in the FTIR spectra of dye degraded sample indicate complete elimination of certain component and degradation of others. The presence of characteristic of peaks 3451.23 and 1597.26 cm^{-1} indicates the presence of N-H stretching and azo group, respectively, in dye sample prior to degradation (*Figure 4.9 (a)*). The absence of peak at 1597.26 cm^{-1} and shifting of peak 3451.29 to 3414 cm^{-1} represent complete degradation of azo group and change of N-H stretching to O-H stretching, respectively. The other prominent peaks present in the spectra as shown in *Figure 4.9 b* for dye sample before degradation or 1496.81 and 1620.26 cm^{-1} confirming the presence of aromatic nitro compound, whereas the peaks at 2964.26, 1668, 1411.09, 1319.21, 1151.54, and 839 cm^{-1} confirm the presence of C-H

stretching of CH group, aromatic amines, O-H deformation, –SO stretching, and sulfonated groups, respectively. The absence of peaks at 1319.21 21 and 1151.54 cm^{-1} for FTIR spectra of degraded sample represent the removal of azo bond and sulfonated group; this finding is aligned to Waghmode *et al.* (2012) and Senan and Abraham (2004), confirming microbial degradation.

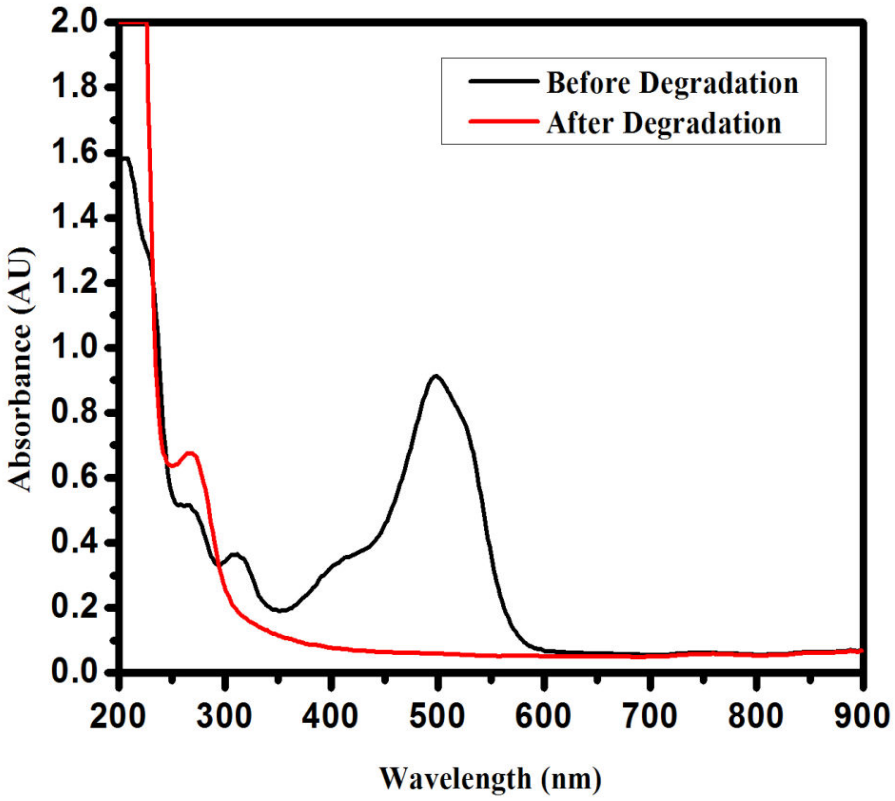


Figure 4.7: UV-visible spectra of C.I. Acid Red 1 and after decolorization by isolated bacterial strain BHUSSp X2 at optimized condition pH 8, Temperature 35 °C, Static condition and 200 mg/L dye concentration.

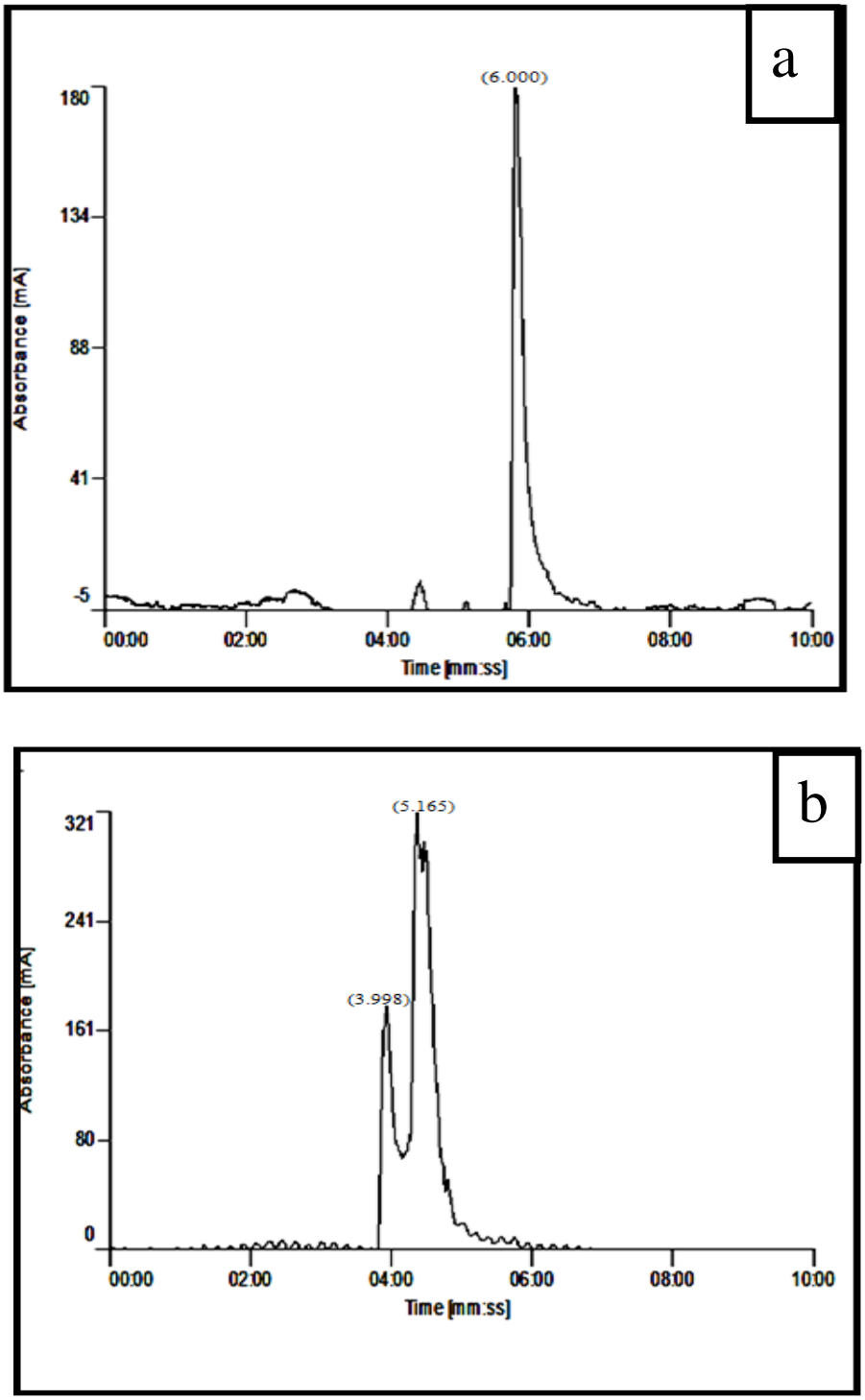


Figure 4.8: HPLC analysis of (**a**) Dye control sample (**b**) metabolites produced after 6h.

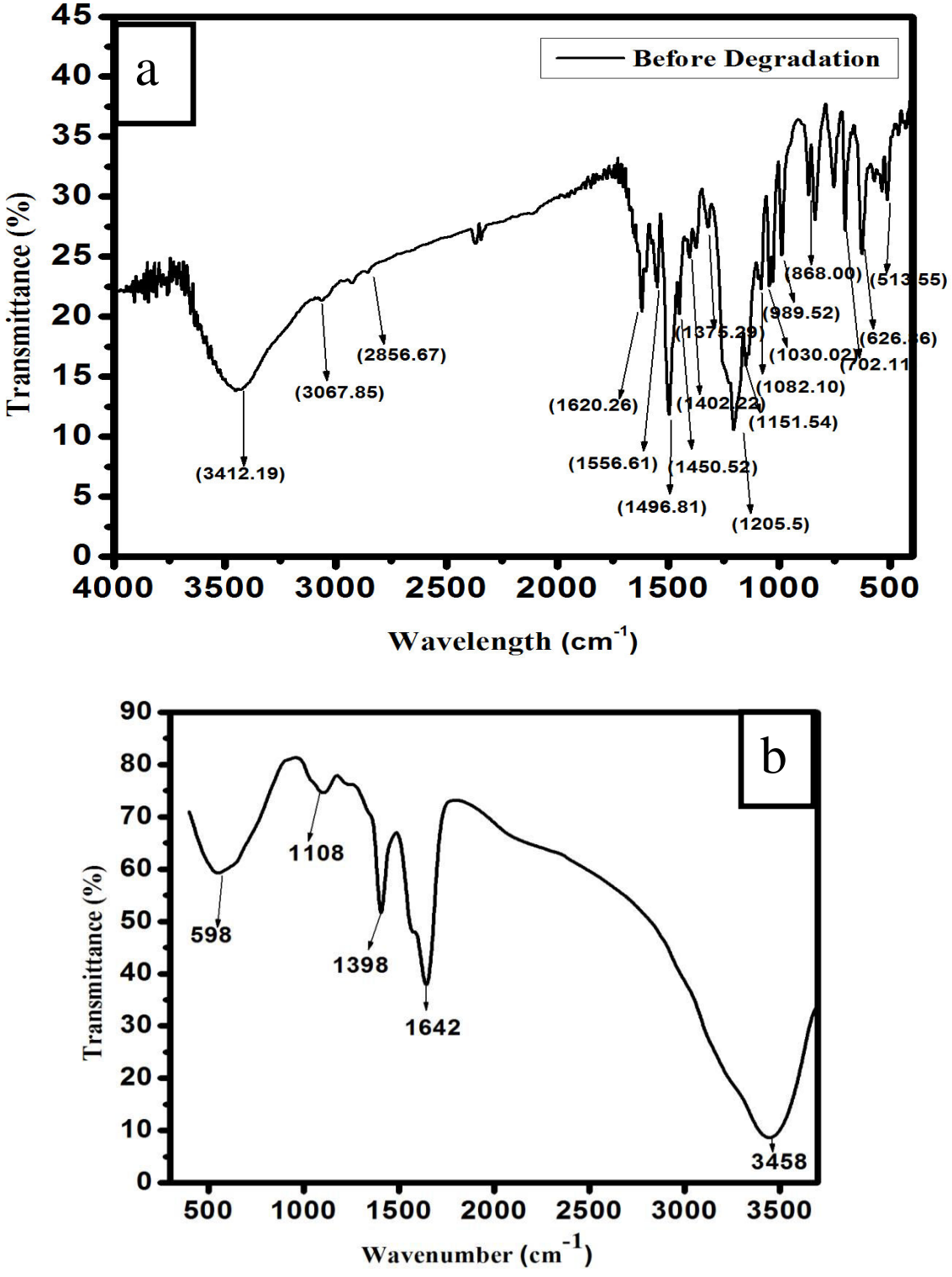


Figure 4.9: FTIR spectrum of (**a**) Dye control sample (**b**) metabolites produced after 6h.

4.2.6 Degradation pathway

To understand the degradation pathway, GC-MS analysis of dye samples prior to and after degradation was carried out and presented in *Figure 4.10*. The presence of various groups in the GC-MS spectra of dye sample before degradation confirms the characteristics of ACID RED 1 dye, whereas GC-MS spectra (*Figure 4.11*) of degraded samples confirms the presence of aniline (M.W. 93 peak at 93 m/z), benzene with M.W. 78 m/z 74, sodium7-amino-6-hydroxynapthalene-2-sulfonate (M.W. 261, m/z 263), 3-hydroxyphthalic acid (M.W. 182, m/z 184) and pyrocatechol (M.W.110, m/z 110). The pathway presented in the present paper suggests that possibly bacterial strain has fragmented the dye initially into aniline and unknown intermediates due to presence of azoreductase enzyme. This is possibly due to the breakage of azo bond. Aniline further breaks into benzene and the unknown intermediate into sodium7-amino-6hydroxynapthalene-2 possibly through desulphonation and deamination which finally converts to pyrocatechol. Dehydroxylation and decarboxylation in addition to desulphonation and deamination were potential degradation mechanism. The fractions of benzene and pyrocatechol possibly have been mineralizing due to ring fission and then TCA cycle.

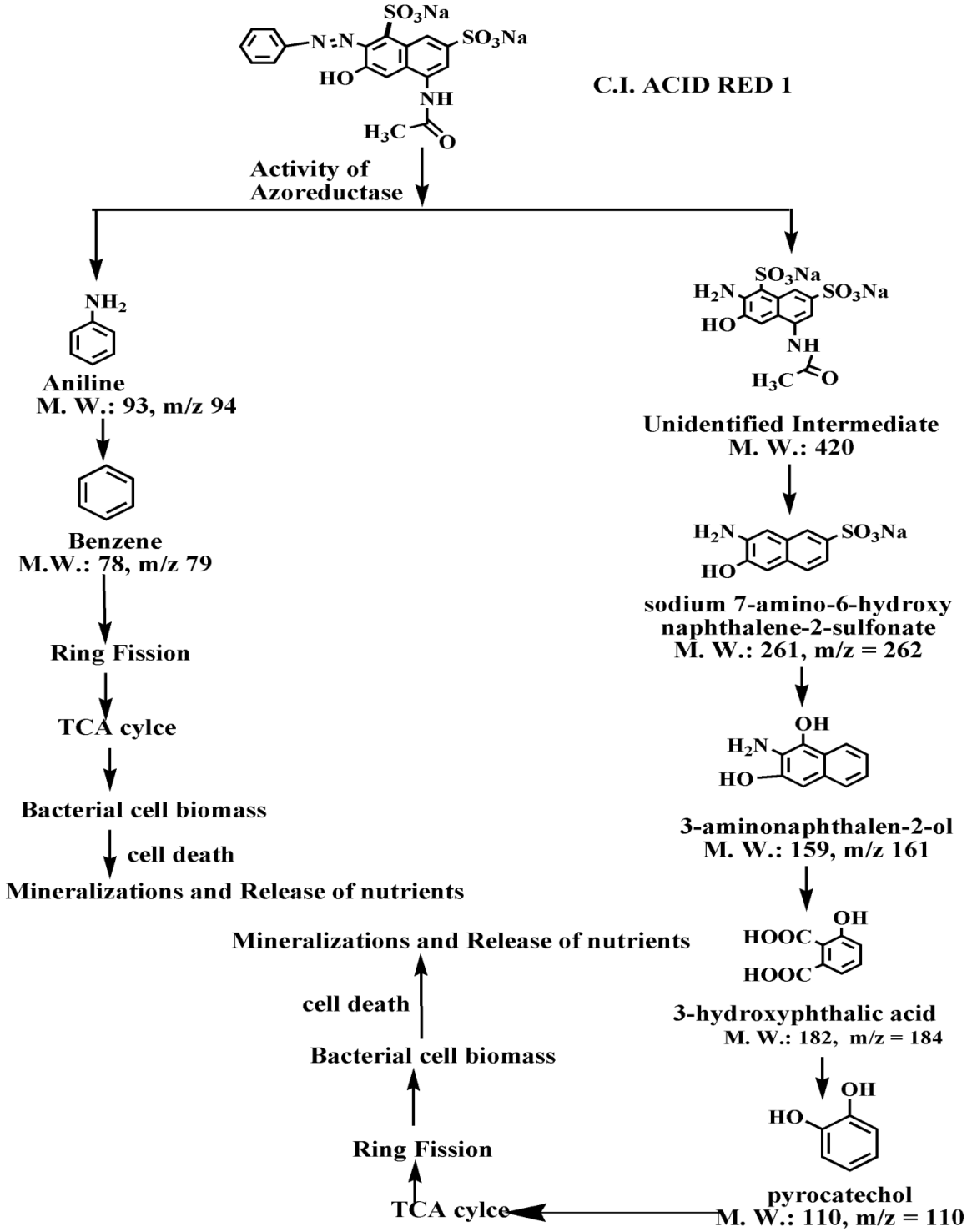
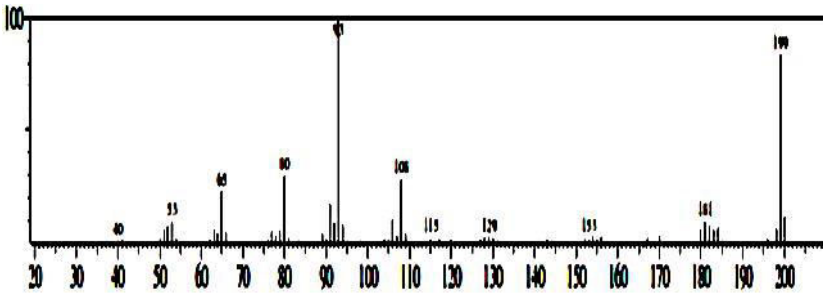
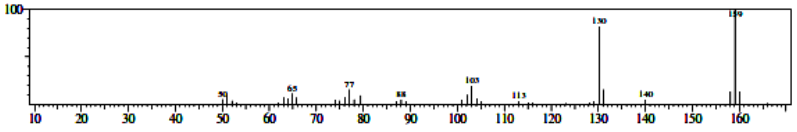
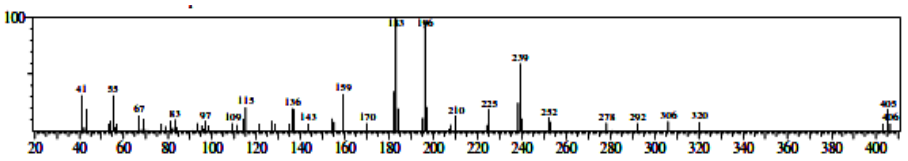
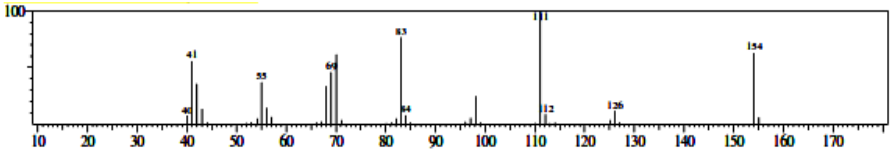


Figure 4.10: Pathway of degradation of Acid Red 1 dye by isolated bacterial strain BHUSSp. X2 by GC-MS.

Table 4.3: GC-MS spectra of degraded metabolites of Acid Red G.

Name of Metabolites	M/W	Gas Chrmatograms
Aniline	93	
3-aminonaphthalen-2-ol	159	
3Hydroxyphthalic acid	184	
Pyrocatechol	111	

4.2.7 Phytotoxicty study

The investigations were made to estimate the phytotoxicity of dye effluents prior to and after degradation. The germination characteristics of gram seeds in pure water, effluents, and extracted metabolites were observed and summarized in *Table 4.4*. Nearly similar germination characteristics were observed in case of water and extracted metabolites whereas only 30 % germination where observed in case of effluent confirming reduced/nontoxicity of treated sample. The degraded sample has not only possessed the noninhibitory effect but also resultant into the improved stem length reflecting availability of additional nutrients as compared to pure water. Reduction in root length as compared to pure water sample in case of degraded metabolites also confirms the easy availability of nutrient in case of degraded metabolites.

Table 4.4: Phytotxicity Study of Acid Red G and its metabolites formed after biodegradation

Parameters Studied *Vinga mungo*	**Water**	**Dye**	**Extracted Metabolites**
Germination	100	30	90
Shoot Length	3.86±0.05	1.26±0.04	3.96±0.06
Root Length	2.56±0.05	0.43.±0.06	2.11±0.07

4.3 CONCLUSION:

BHUSSp X2 microbial strain was isolated from contaminated sites of carpet cluster Bhadhoi. The bacteria were used for the decolorization of effluent from dying plants of the cluster in Uttar Pradesh, India. Upto 97 % decolorization could be achieved at pH 8, temperature 35 °C under static condition, and initial concentration of 200 mg/L. The UV-Visible spectrophotometer, HPLC, FTIR, and GC-MS investigation confirms the microbial degradation as potential mechanism of color removal. GC-MS analysis also rivals that final degradation products were benzene and pyrocatechol due to azo bond cleavage due to microbial action. Phytotoxicity investigation also confirms the noninhibitory effects of degraded metabolites. So, it can be concluded that BHUSSp X2 is having good potential for the treatment of azo dye being discharged from carpet/textile industries. It was also suggested that degraded metabolites were nontoxic in nature and not harmful to the environment. The attempt will be made to transfer the technology after continuous experiments in suspended as well attached culture mode for decolorization of dye.