3.1 INTRODUCTION:

The synthetic azo dyes are widely used in carpet, textile, paper, cosmetics, pharmaceuticals, food and many other industries [Saratale *et al.* (2011)]. Their annual production exceeds 7×10^7 metric tons [Robinson *et al.* (2001a); Akhtar *et al.* (2005)] and demand is continuously increasing. About 70% of azo dyes produced are applied in textile industry due to their availability in variety of colors, high stability and low cost [Wang *et al.* (2008); Saratale *et al.* (2009)]. A significant proportion of total production (10–15 %) remains unutilized and released in the environment at various stages that contaminate water bodies like river, lake, ground water [Ozdemir *et al.* (2013); Moosvi *et al.* (2007); Prasad *et al.* (2013)]. The recalcitrant azo dye effluents adversely affect living organisms and their ecosystem. They are mutagenic and carcinogenic for human being. The intense color reduces photosynthetic activity of aquatic plants leading to low dissolved oxygen level and anoxic conditions, causing killing of aquatic organism, including fishes. The effluent discharged from several types of industries has proven mutagenic activity [Coelho *et al.* (1992)]. It is therefore necessary to treat such industrial wastewater. There are variety ofphysico-chemical methods such as flocculation/coagulation [Rodrigues *et al.* (2013)] precipitation, sedimentation, reverse osmosis and nanofiltration [Myung *et al.* (2005)]. However, these methods are associated with low dye removal efficiency and high cost due to high energy requirements [Forgacs *et al.* (2004); Zhang *et al.* (2004)]. In contrast, bioremediation of dye effluents by microorganisms is cost effective and eco-friendly [Kuhad *et al.* (2004); Mohana *et al.* (2007)]. Recently, researchers have focused on isolating microorganisms from

contaminated sites to degrade dye effluents under aerobic and anaerobic conditions. Several microorganisms such as *Morganella* sp. HK-1, *Bacillus cereus* strain HJ-1 and *Yarrowia lipolytica* have been identified for efficient degradation of aromatic dyes [Pathak *et al*. (2011)]. During the anaerobic degradation, harmful aromatic amine intermediates are produced, which are carcinogenic and mutagenic. These harmful intermediates are converted to less toxic products by hydroxylation and deamination reactions using aerobic biodegradation steps [Tripathi et al. (2011); Levine *et al*. (1991)].

This chapters deals with the literature regarding the degradation and detoxification of Navy N5RL1 dye, being used for coloring the carpet, present investigation aims to investigate its degradation using bacterial strain (*Staphylococcus saprophyticus* BHUSS X3) isolated from dye-contaminated site. The various factors affecting dye degradation such as agitation speed, pH, initial concentration and temperature were optimized. The decolorization and degradation products were analyzed by UV–Visible Spectrophotometer, FTIR and HPLC. Further, phytotoxicity of degradation products of Navy N5RL1 dye was tested by germinating the *Phaseolus aureus* seeds on these degradation products.

3.2 Result and Discussion:

3.2.1 Bacterial identification by Morphological, Biochemical and 16S rRNA gene sequencing

The fast-growing Gram-negative bacteria *Staphylococcus saprophyticus* strain BHUSS X3 on the dye-amended nutrient agar was initially identified morphologically and biochemically (*Table 3.1*) before their molecular identification using 16S rRNA gene

sequence technique. The bacterial 16S rRNA sequence was most closely related to *Staphylococcus saprophyticus* BHUSS X3. The evolutionary relations of the strain among the genus of *Staphylococcus* are shown in the phylogenetic tree *Figure 3.1*. The sequence of the strain was directly deposited in Gene Bank (Accession No. KJ439577) (http://www.ncbi.nlm.nih.gov/nuccore/KJ439577). The analysis of 1150 base pair of 16S rRNA gene sequence obtained was 99% identical to that of *Staphylococcus saprophyticus* as compared to the other genera.

Table 3.1: Characteristics of isolated bacterial starin *Staphyolococcus saprophytic* BHUSS X3

Characteristic	***Staphyolococcus saprophytic* BHUSS X3**
Gram Staining	-Ve
Shape	Cluster,Chain
SIM Motality	Non-Motile
TSI	+Ve/NoGrowth
H_2S Production	+Ve
Glucose Utilization	+Ve
Sucrose Utilization	+Ve
Lactose Utilization	+Ve
Citrate Utilization	-Ve
Urease	-Ve
Oxidase	-Ve
Indol	+Ve

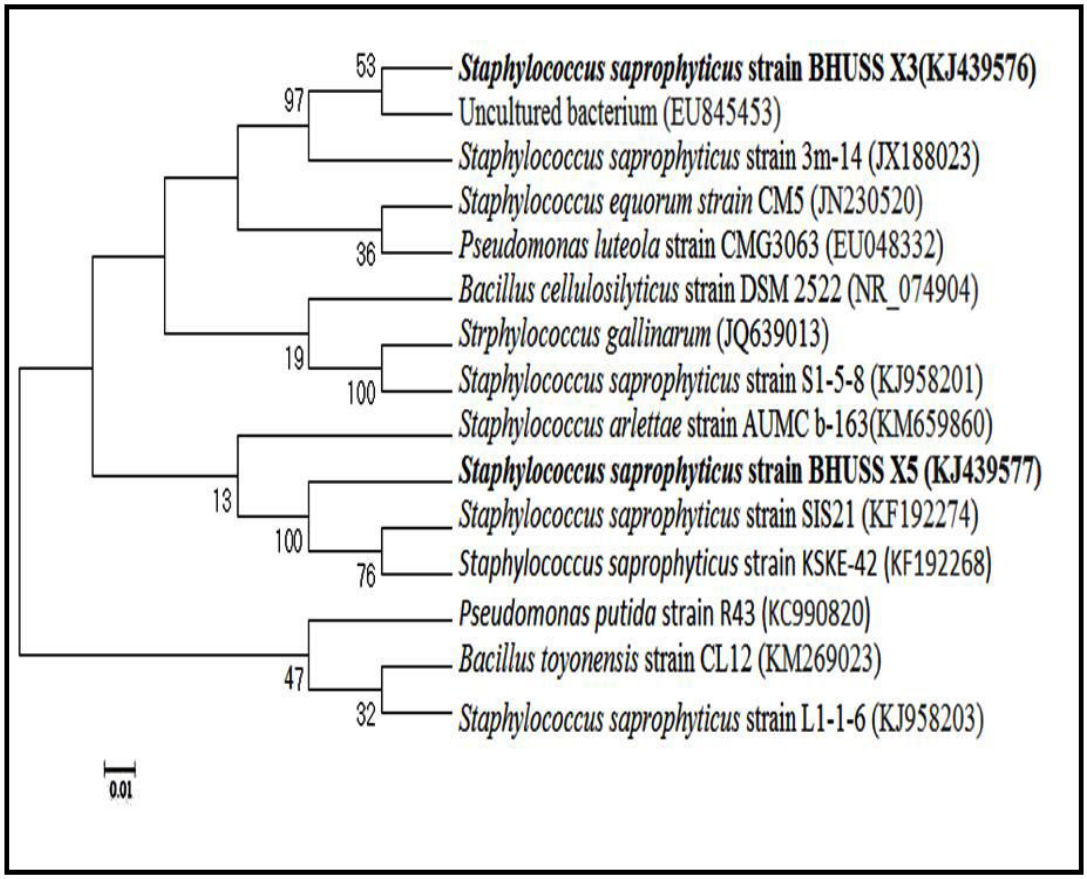

Figure 3.1: The evolutionary history of bacterial strain BHUSS X3 was analysed by using the Neighbour-Joining Method, Bootstrap values (n=1000) are indicated at the nodes and scale bars represent 0.01 substitution/site; the sequences have been retrieved from NCBI database, showing the phylogenetic relationships of *Staphylococcus saprophytic* BHUSS X3 with different strain of *Staphylococcus* and other species of genus *Pseudomonas and Bacillus*.

3.2.2 Parameter Optimization for Degradation Studies

3.2.2.1 Effect of Agitation

The bacteria decolorized Navy N5RL1 dye more efficiently under static anoxic conditions (95.7%) as compared to the agitation at 100 rpm (25%) for initial dye

concentration of 100 mg/l, for 6h incubation as shown in *Figure 3.2(a)*. The bacterial growth was fast under shaking condition but colour removal was slow. In contrast under static condition bacterial growth was slow but dye decolourization was comparatively fast. The result could be explained in terms of facultative anaerobic nature of isolated bacteria *Staphylococcus saprophyticus* strain BHUSS X3. Under static condition partial anaerobic or microaerobic condition is generated and bacteria excrete extracellular and intracellular enzymes for dye decolourization. In the agitated samples of the dye the bacterial respiration consumes most of the NADH necessary for the azoreductase activity in the dye decolourization [Stolz (2001)]. Therefore, static conditions were adopted to investigate dye decolorization in the following experiments.

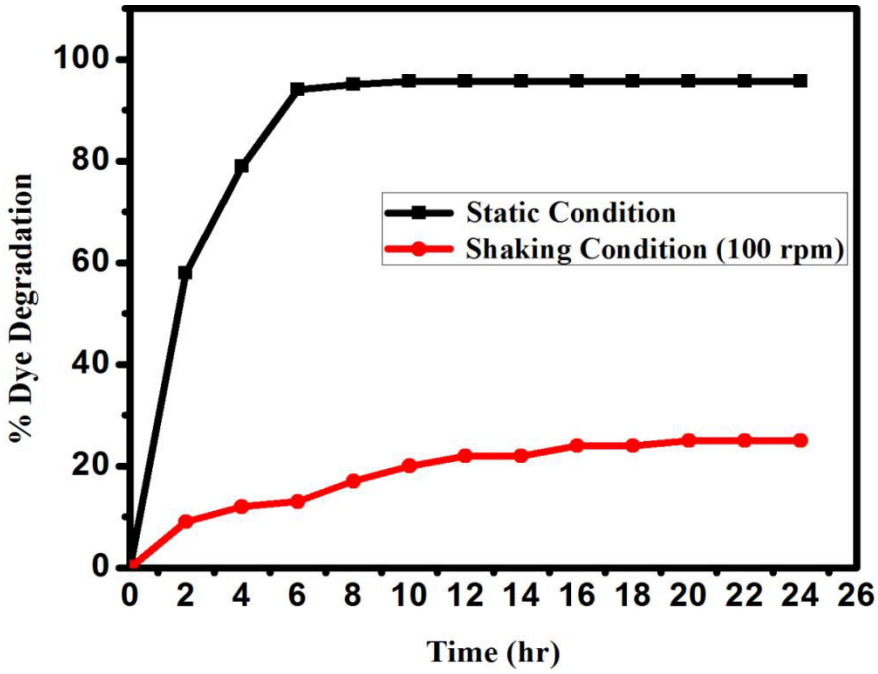

Figure 3.2: (a) Effect of Static/Shaking condition for efficient decolorization of dye Navy N5RL1

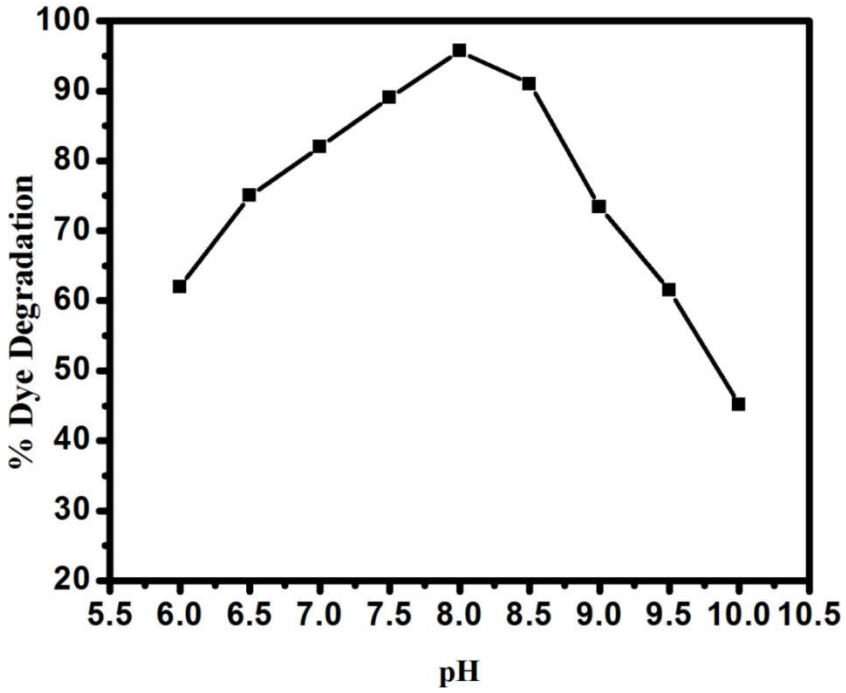

Figure 3.2: (b) Effect of pH on dye Navy N5RL1 decolorization by *Staphylococcus saprophytic* BHUSS X3 optimized static culture condition at temperature 35 °C and 100 mg dye concentration.

3.2.2.2 Effect of pH

The bacteria *Staphylococcus saprophyticus* BHUSS X3 was able to tolerate broad pH range of 6–10, so decolourization was tested for this range. For initial dye concentration, 100 mg/l, degradation increased with increasing pH upto 8 (95.5 %) and further increase in pH reduced the decolorization efficiency (*Figure 3.2(b)*). The decolorization was affected by the extreme pH. The dye degradation under alkaline conditions has been reported to be favored by various bacteria [Chen *et al*. (2003); Guo *et al*. (2007); Kilic *et al*. (2007); Bhatt *et al*. (2005)] probably due to preferred growth in basic pH.

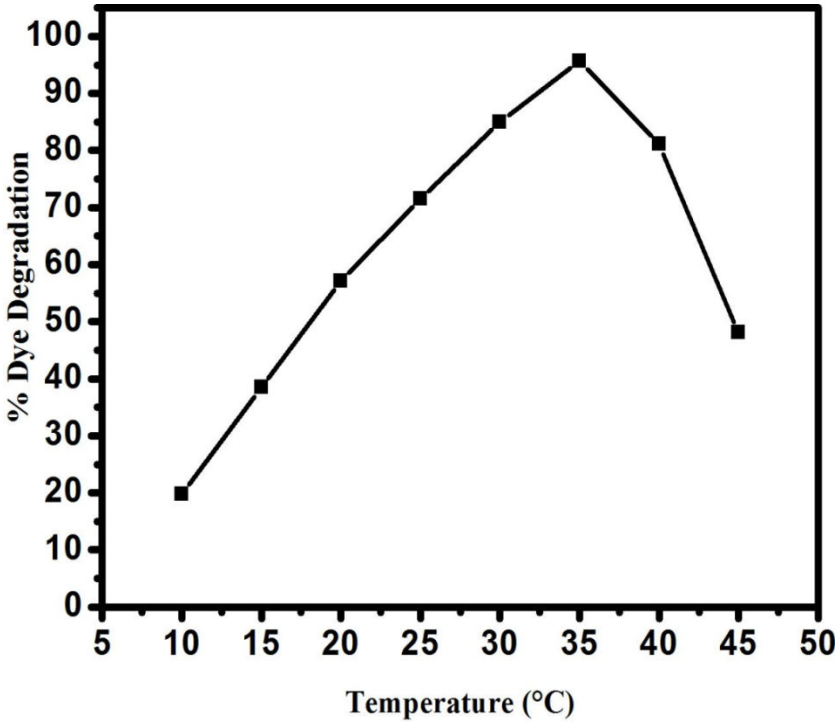

Figure 3.2: (c) Effect of temperature for decolorization of dye Navy N5RL1 by *Staphylococcus saprophytic* BHUSS X3.

3.2.2.3 Effect of Temperature

The incubation temperature is an important parameter for bacterial growth. The selected mesophilic bacteria were utilized for dye degradation in temperature range 20–45 °C as shown in (*Figure 3.2(c)).* It is clear from the figure that percentage decolorization increased with increase in temperature up to 35 °C and further increase in temperature beyond 35 °C resulted in decreased dye degradation. The result could be explained in terms of the inactivation of enzyme and loss of cell viability [Saratale *et al.* (2009)].

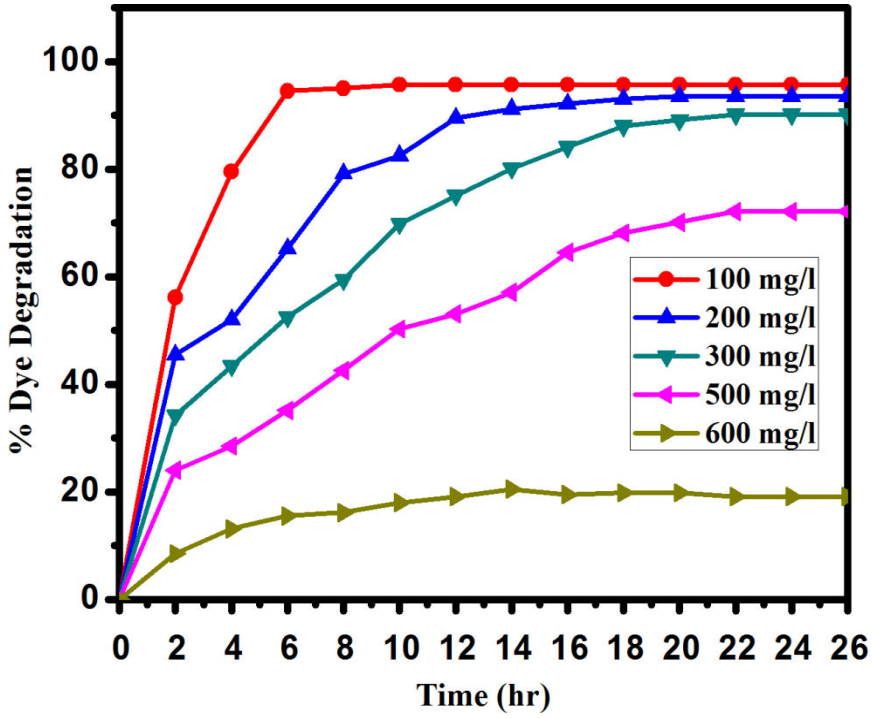

Figure 3.2: (d) Effect of initial dye Navy N5RL1 concentrations (100-1000 mgl^{-1}) under optimized static culture condition at pH 8.0 and temperature 35 ℃.

3.2.2.4 Effect of initial dye concentration

The dye degradation experiment was conducted for the initial concentration range of 100–1,000 mg/dye. The dye decolourized most effectively in initial concentration of 100 mg/l dye (95.7%) within 6h (*Figure 3.2(d)*). The decolorization decreased as the initial concentration of dye increased. In case of 500 mg/l initial dye concentration degradation declined to 65% in 18 h. Such reduction in the decolourization rate at higher concentrations has been attributed to toxic effect of dye [Jadhav *et al.* (2011); Kalme *et al.* (2007); Khera *et al.* (2005)].

3.2.3 Analytical investigation

3.2.3.1 UV–Vis spectral analysis

In the UV–Vis spectral analysis of pure dye solution, there were two minor peaks at 210, 260 nm and a major peak at 560 nm as shown in *Figure 3.3*. The peak in the visible range disappeared after decolorization of the azo dye by bacteria and two peaks in the UV range were replaced by single peak at 250 nm (*Figure. 3.3*) probably due to the formation of some aromatic intermediate products.

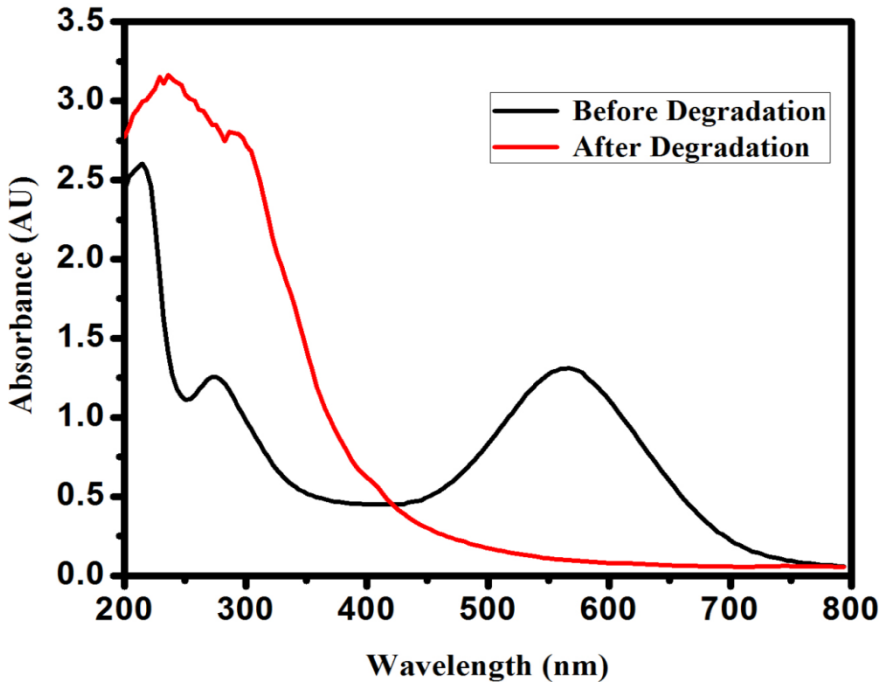

Figure 3.3: UV-Vis spectrophotometer of Navy N5RLI (100 mg/l) biodegraded by *Staphylococcus saprophytic* BHUSSX3 before and after optimized condition at T = 35 °C, pH = 8.0, bacterium = 2.6×10^6 cells/ml.

3.2.3.2 HPLC analysis

The HPLC analysis of dye before degradation has two peaks at the retention time 5.99 and 6.00 min, whereas degradation products showed in *Figue.3.4 (a)-(b)* peaks at lower retention time namely 3.99 and 4.10 min, probably due to degradation of dye into small intermediate products.

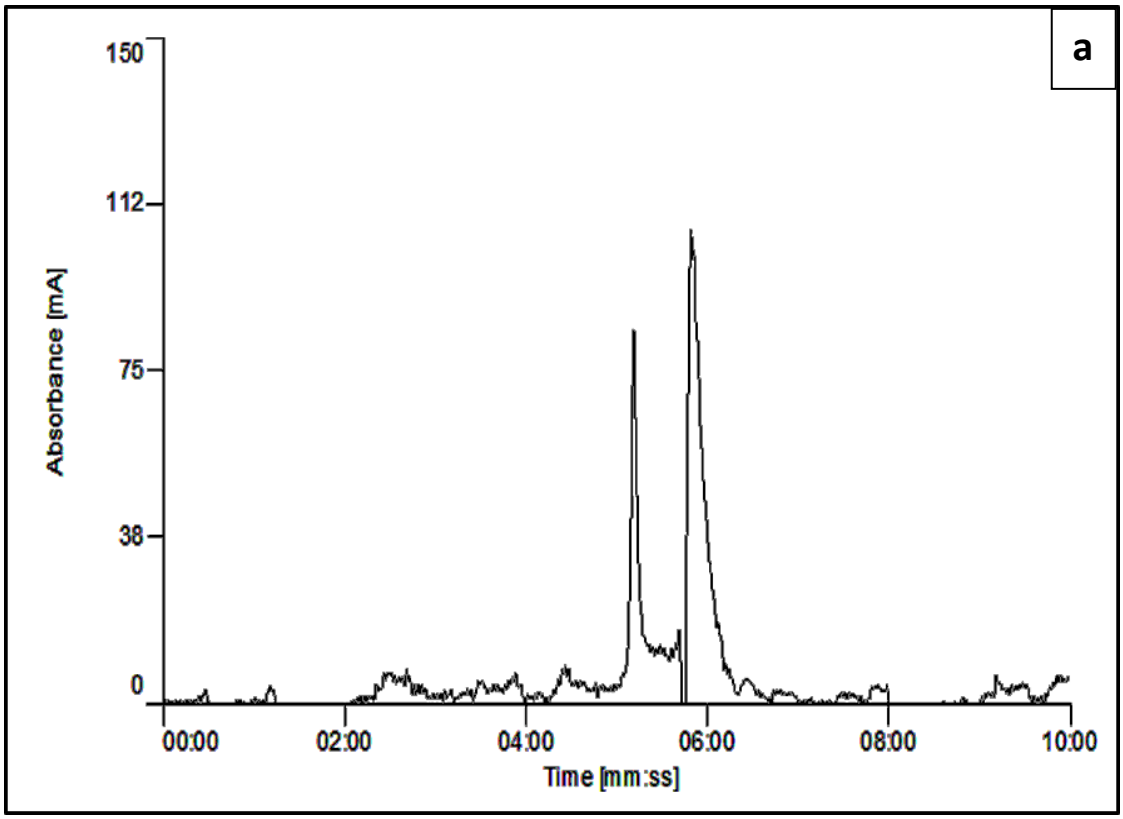

Figure 3.4: (a) HPLC analysis for dye degradation of dye control Sample

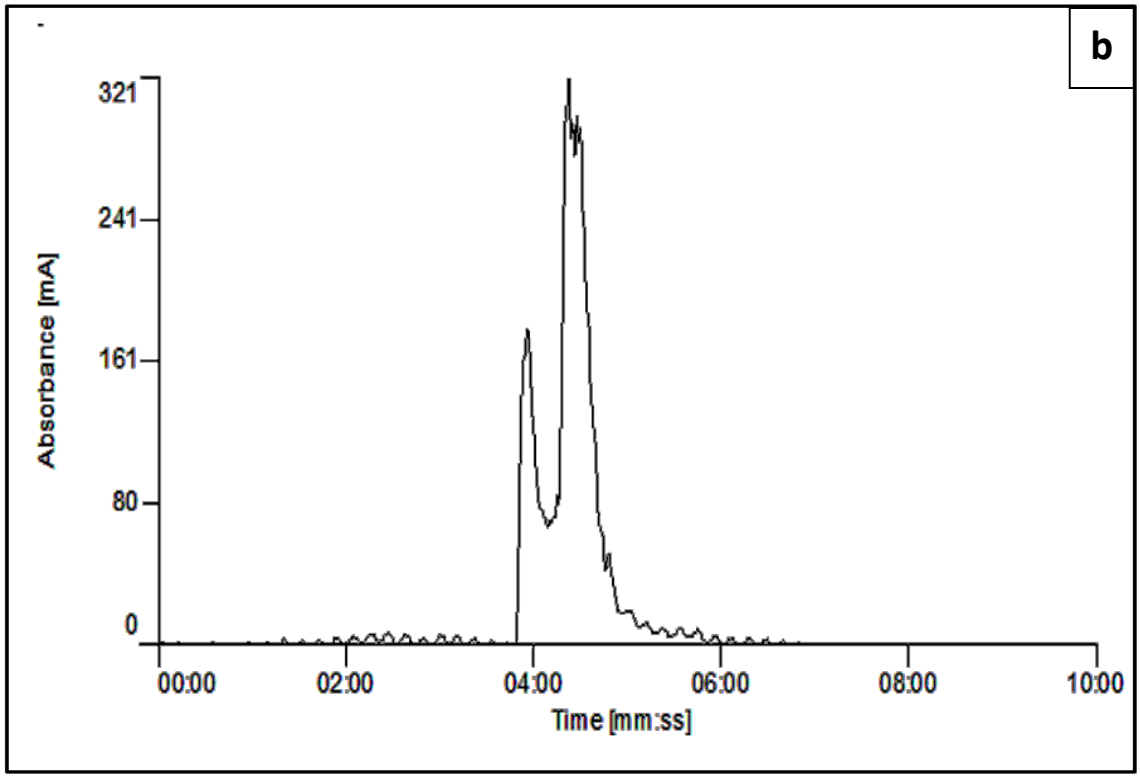

Figure 3.4: (b) HPLC analysis for dye degradation of degraded sample.

3.2.3.3 FTIR Analysis

The FTIR spectra of dye of (*Figure 3.5a*) and dye degradation products differed with number of peaks and their positions (*Figure 3.5b*). The appearance of peak at 1,598.93 cm^{-1} confirmed the presence of aromatic nitro compound and azo group in dye whereas peaks at 1,494.98 cm^{-1} and 1,565.62 cm^{-1} were related to aromatic nitro compounds. The C–C stretching is indicated by peaks at 2,923.65 and 2,853.57 cm^{-1}. The peaks at 1,347.41 and 1,138.75 cm^{-1} are due to the presence of the sulphonated dye compound. The peak at 1,038.81 cm^{-1} indicates primary alcoholic group and peak at 837 cm^{-1} indicates C–H deformation of benzene ring. The sharp peak at 1,598.93 cm^{-1} in azo

compounds absent in the FTIR spectral analysis of degraded products confirms the cleavage of azo bonds. The peak at 3,313.10 cm^{-1} due to O–H stretching indicates hydroxylation of the product. A significant change in FTIR spectrum in degraded dye sample which displayed peaks at 3,230 and 1,667 cm^{-1} for–OH stretching and a peak at for C=N respectively. Fermi resonance band at 2,930 cm supported by a peak at 1,455 -1 for –CH.

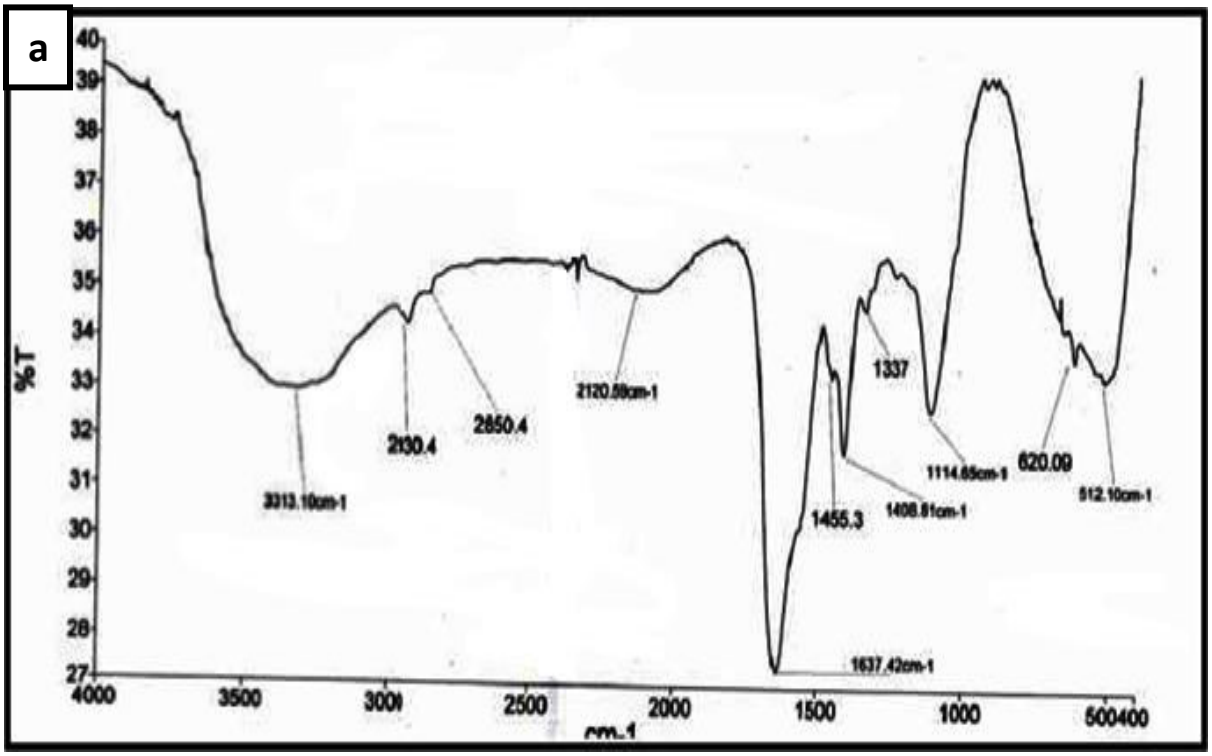

Figure 3.5: (a) FTIR analysis of Navy N5RL Before degradation.

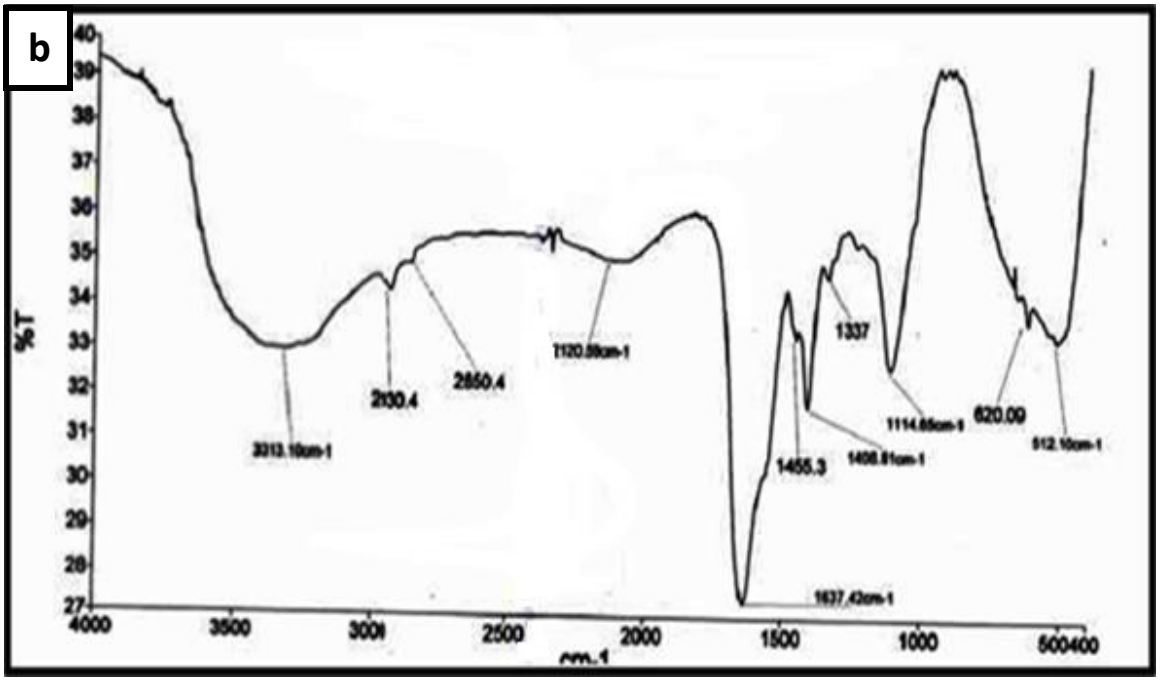

Figure 3.5: (**b**) FTIR analysis of Navy N5RL After degradation.

3.2.4 Phytotoxicity study

The dye wastewater discharged without treatment into nearby water bodies causes serious environmental problems and health hazards [Mansour *et al.* (2011)]. It is clear from *Table 3.2* that up to 100% germination is being observed in cases of treated wastewater and control (pure water), whereas, only 50 and 10% germinations were observed for wastewater having dye concentrations 100 and 1000 mg/l respectively. Similar observations have been made for plumule and radical for control and dye contaminated wastewater, whereas treated water shows better growth in plumule as compared to the control. In the case of radical, both control and treated wastewater show nearly similar result. Enhanced growth in plumule in the case of treated water might be due to leftover nutrients after biodegradation.

Table 3.2: Phytoxicity Study of Navy N5RL1 dye and its degradation metabolites.

	Parameters Studies			
	Water	**Dye (100mg/l)**	**Dye (1000mg/l)**	**Extracted products**
Germination	100	55	10	100
Plumule	2.56 ± 0 .99	1.2 ± 0 .84	0.2 ± 0.17	4.48± 0.99
Radical	0 .766± 0.83	0.23± 0.94	0.15 ± 0.08	0.61 ± 0.62

3.3 CONCLUSION:

The isolated bacterial strain *Staphylococcus saprophytic* BHUSS X3 was very efficient in degrading the dye Navy N5RL1 commonly used in carpet industry. The strain is supposed to be capable in degrading other class of the dyes also in the static micro-aerobic condition. An effluent storage pond near carpet industry without agitation but only amendment of selected locally available bacterial strain could efficiently decrease the dye level.