Chapter-III
CHAPTER 3

ECOFRIENDLY BIOREMEDIATION OF METANIL ORANGE
BY TEXTILE EFFLUENT ACCLIMATIZED BACTERIAL
STRAIN, ALKALIBACILLUS SP. STRAIN PIA-5

5.1 INTRODUCTION

Textile industry is providing one of the most basic needs of the people and maintains sustained growth for improving quality of life. It has a unique position as a self reliant industry, from the production of raw materials to the delivery of finished products, with substantial value-addition at each stage of processing (Hemapiya et al., 2010). Textile industries utilize enormous amount of water and chemicals for the wet processing of textiles and also use various types of synthetic dye to impart attractive colors of commercial importance (Khan and Srivastava, 2014). Dyes released from the textile processing and dyestuff industries result in the increase of organic load of the natural reservoirs. The effluents from these industries are very complex; contain a wide variety of colorants and other chemicals such as dispersants, acids, bases, salts, detergents, humectants, oxidants etc. Even a very small amount of dye in water (10 to 50 mg l\(^{-1}\)) will affect the aesthetic value, transparency and gas solubility of water bodies affecting the aquatic flora and fauna (Banat and Nigam, 1996; Shah et al., 2013).

The major classes of synthetic dyes that are used for textile dyeing and other industrial applications include acidic, basic, azo, reactive, anthraquinone and tri-aryl methane dyes (Padmavathy et al., 2003; Garcia-Montano et al., 2007). Azo dyes, which are aromatic compounds with one or more -N=N- groups, constitute the largest class of synthetic dyes used in commercial applications such as textile, leather and tanneries (Akhtar et al., 2005; Kumar et al., 2007). According to a survey in 1994, the world’s production of dyes was around one million ton, of which more than 50% were azo dyes (Stolz, 2001). Azo dyes are widely used in a number of industries such as textiles, dyeing, food, cosmetics and paper printing, with the
textile industry as the largest consumer (Frank et al., 2002; Bourie and Din, 2016). There are more than 100,000 commercially available dyes with over $7 \times 10^7$ tons of dye stuff produced annually worldwide (Asad et al., 2007; Lalnunhlimi and Krishnaswamy, 2016). The textile effluents may contain dyes of various intense colours, such as dyes having the functional groups of alkenes, aromatic, C-N and S-O bonds of red color. Some are made by inorganic molecules such as Al-O, Si-O, K-O and N=N bonds which are responsible for color development in the wastewater (Manikandan et al., 2009). Color is the first contaminant in the wastewater. In addition to their visual effect and their adverse impact in terms of COD, many dyes are toxic, mutagenic and carcinogenic. The toxicity of industrial effluents may be attributed to the presence of metals, chlorides, etc. in them and the break down products of dyes (Isik and Sponza, 2003; Acemioglu, 2004).

Contamination of surface water and ground water by the textile effluents significantly affects agricultural practice. Irrigation with untreated industrial effluent drastically reduced the germination and vigour index of crops like rice, Sorghum, wheat, cow-pea and maize and decreased the nitrogen fixation ability in green gram. But the diluted effluent improved the germination rate and increased the chlorophyll, carbohydrate and protein content. However, the biologically treated effluent enhanced the yield and quality of many cereals and pulses (Manikandan et al., 2009). Dyes are identified as the most problematic compounds in textile effluents due to their high water solubility and low degradability. Moreover, the high volumetric rate of industrial effluent discharge in combination with the increasing stringent legislation, make the search for appropriate treatment technologies on important priority (Romero et al., 2006; Khelifi et al., 2009). With the increased use of a variety of dyes and synthesis of new ones to satisfy the ever-growing and changing needs of man, pollution by dye wastewater is currently recognized as a serious environmental issue (Mendez-Paz et al., 2005; Bouraie and Din, 2016).

Environmental biotechnology is constantly expanding its efforts in the biological treatment of dye-contaminated wastewaters (Junghanns et al., 2008). Synthetic dyes are recalcitrant to microbial degradation because they contain substitutions such as azo, nitro or sulpho groups (Pagga and Brown, 1986).
Microbial decolourization is environment-friendly and cost competitive alternate to physico-chemical treatment decomposition processes (Umbuzeiro et al., 2005; Dafale et al., 2008). It is known that several microorganisms, including bacteria, yeasts, fungi and algae, can decolorize and even completely mineralize many azo dyes under certain environmental conditions. Many researchers have reported on the microbial decolourization of azo dyes (Ponraj et al., 2011; Dellamatrice et al., 2017). The decolourization process is achieved by the reductive cleavage of azo bond in anaerobic conditions, but the end-products of this reaction are often more dangerous than the parent compounds (Hemapriya et al., 2010). Moreover, their complete mineralization only occurs in the presence of molecular oxygen, so that a further step is required (Lalnunhlimi and Krishnaswmy, 2016).

This study deals with the biodegradation of Metanil Orange by a newly isolated bacterial strain *Alkalibacillus sp.* Strain PIA-5 under aerobic conditions. The optimal culture conditions for maximizing the bacterial biomass and decolourization ability of *Alkalibacillus sp.* Strain PIA-5 were investigated. The biodegradation by the isolate was monitored by HPLC, FT-IR and GC-MS. Finally, phytotoxicity assay was performed to assess the toxicity of treated and untreated dye sample on selected plants.

### 5.2 MATERIALS AND METHODS

#### 5.2.1 Textile Azo Dye used

Metanil Orange, the commonly used textile azo dye used in this study was procured from a local textile dyeing unit. Stock solution was prepared by dissolving 1 g of Metanil Orange in 100 ml distilled water. Since azo dyes may be unstable to moist-heat sterilization, the stock solution of Metanil Orange was sterilized by membrane filtration (Millipore Millex ® - GS, 0.22 Mm filter unit). All the chemicals used in this study were of the highest purity available and of an analytical grade.
5.2.2 Isolation, Enrichment and Screening of Bacterial Strains

Decolourizing Metanil Orange

The effluent samples collected from the textile industries (Kanchipuram) were serially diluted and spread over basal nutrient agar medium containing 50 ppm of Metanil Orange. pH of the culture media was adjusted to 7.0 before autoclaving and incubated at 37°C for 5 days (Hemapriya et al., 2010). Bacterial colonies surrounded by halo (decolorized) zones were picked and streaked on nutrient agar plates containing Metanil Orange. The plates were re-incubated at 37°C for 3 days to confirm their abilities to decolorize Metanil Orange. Colonies of different dye decolourizing bacterial strains were selected and re-streaked several times to obtain pure cultures. The pure cultures of dye decolourizing bacterial strains were maintained on dye-containing nutrient agar slants at 4°C.

5.2.3 Decolourization Assay

A loopful of overnight bacterial culture was inoculated in Erlenmeyer flask containing 100 ml of nutrient broth and incubated at 150 rpm at 37°C for 24 h. Then, 1 ml of 24 h old culture of PIA-5 strain was inoculated in 100 ml of nutrient broth containing 50 ppm of Metanil Orange and re-incubated at 37°C till complete decolourization occurs. Suitable control without any bacterial culture was also run along with experimental flasks. 1.0 ml of sample was withdrawn every 12 h and centrifuged at 10,000 rpm for 15 min. Decolourization extent was determined by measuring the absorbance of the culture supernatant at 621 nm using UV-visible spectrophotometer (Hitachi U 2800), according to Chen et al. (2003).

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\text{Dye (i) } - \text{ Dye (r)}
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\text{Decolourization efficiency (\%)} = \frac{\text{Dye (i)} - \text{Dye (r)}}{\text{Dye (i)}} \times 100
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Where, Dye (i) refers to the initial dye concentration and Dye (r) refers to the residual dye concentration. Decolourization experiments were performed in triplicates.
5.2.4 Bacterial Strain and Culture Conditions

Bacterial strain that showed maximum decolourization percentage on Metanil Orange was aerobically cultured in nutrient broth containing 50 ppm of the above mentioned dye. The pH was adjusted to 7.0. For frequent use, the culture was maintained by transfer to a fresh medium at 24 hrs intervals. When required for prolonged periods, it was maintained by sub-culturing once every 7 days on slants, prepared by solidifying the above mentioned medium with 2.0 (w/v) agar.

5.2.5 Molecular Phylogeny / 16S rDNA Analysis of PIA-5

A loopful of PIA-5 cells were inoculated into 100 ml of basal nutrient broth medium and incubated at 35°C for 24 hrs. The culture was centrifuged at 8,000 rpm for 4 min to separate cell pellet. Then, the total genomic DNA was extracted using bacterial genomic DNA isolation kit. The 16S rDNA sequence of the isolate PIA-5 was amplified via the polymerase chain reaction (PCR), using two universal primers: the 16S forward primer 5’-AGAGTRTGATCMTYGCTWAC-3’ and the 16S reverse primer 5’-CGYTAMCTTWTTACGRCT-3’, which yielded a product of approximately 1431 bp (~1.4 kb). The purified PCR product was directly sequenced using Big Dye Terminator version 3.1 cycle sequencing kit according to procedure of Pidiyar et al. (2004). The sequencing reactions were run on AB-PRISM automated sequencer (ABI-3730 genetic analyzer).

The nucleotide sequence analysis was done at BLAST-n site at NCBI server www.ncbi.nlm.nih.gov/BLAST. The alignment of the sequences was done using CLUSTAL W program VI.82 at European Bioinformatics site (www.ebi.ac.uk/clustalw). The analysis of 16S rDNA gene sequence was done at Ribosomal Data Base Project (RDP) II (http://rdp.cme.msu.edu). The sequence was refined manually after crosschecking with the raw data to remove ambiguities. The phylogenetic tree was constructed using the aligned sequences by the neighbour joining method using kimura-2 parameter distances in MEGA 2.1 software (Kumar et al., 2001).
Optimization of Various culture Conditions for Bacterial Biomass and Metanil Orange Decolourization by *Alkalibacillus* sp. Strain PIA-5

5.2.6 Effect of Temperature, pH, Agitation Rates and Dye Concentrations

The effect of temperature, pH, agitation rates and dye concentration on both bacterial biomass and dye decolourizing ability of *Alkalibacillus* sp. Strain PIA-5 was studied. This was carried out by incubating the bacterial strain at different temperatures (20-60°C), different pH values of the medium (pH 4.0-10.0), different agitation speeds (0-200 rpm) and various dye concentrations (50-1000 ppm). Bacterial biomass and decolourization percentage was measured at optimum growth (24 h).

5.2.7 Effect of Carbon Sources

The effect of various soluble carbon sources (1% w/v) such as glucose, sucrose, lactose, maltose and starch on bacterial growth and dye decolourization extent of Metanil Orange by *Alkalibacillus* sp. Strain PIA-5, was investigated after 24 h of incubation at 35°C.

5.2.8 Effect of Various Nitrogen Sources

The effect of two different categories of nitrogen sources (1% w/v), *viz.*, organic nitrogen sources (tryptone, beef extract, peptone, yeast extract and meat extract) and inorganic nitrogen sources ((NH₄)₂SO₄, KNO₃, NH₄Cl, NH₄NO₃ and NaNO₃) were investigated on the bacterial growth and dye decolourization ability of *Alkalibacillus* sp. Strain PIA-5, after 24 hrs of incubation at 35°C.

5.2.9 Effect of Various Metal Ions

The effect of various metal ions on bacterial growth and dye decolourization percentage by *Alkalibacillus* sp. Strain PIA-5 was investigated by cultivating the bacteria in basal nutrient broth media containing 50 ppm Metanil Orange, in the presence of various metal ions (5 mM) such as MnCl₂, MgCl₂, HgCl₂, ZnSO₄, CoCl₂ and FeSO₄, incubated at 35°C for 32 hrs.
Analysis of Biodegraded samples by HPLC, FT-IR and GC-MS

Biodegradation of Metanil Orange was monitored by High Performance Liquid Chromatography (HPLC), Fourier Transform Infra Red (FT-IR) spectroscopy and Gas Chromatography-Mass Spectroscopy (GC-MS).

5.2.10 HPLC Analysis of Decolorized Sample

10 ml of decolorized sample was taken after 24 hrs of incubation, centrifuged at 12,000 g for 30 min, and filtered through 0.45 µm membrane filter (Millipore). The filtrate was then extracted with diethyl ether and flash evaporated in rotary vacuum evaporator in temperature controlled water bath (50°C) and residue was dissolved in 2 ml of HPLC grade methanol and used for analysis. This extracted sample was analyzed by HPLC having a mobile phase of 50:49.6:0.4% (methanol: water: disodium hydrogen phosphate). HPLC conditions: HPLC analysis was carried out using -18 columns with a flow rate of ml min⁻¹, chart speed of 1cm min⁻¹ and UV detector at 280 nm.

5.2.11 FT-IR Analysis of Decolorized Sample

The biodegraded Metanil Orange was characterized by FT-IR spectroscopy (Perkin-Elmer, Spectrum one). The analysis results were compared with the control dye. The FT-IR analysis was done in the mid IR region (400-4000 cm⁻¹) with 16 scan speed. The samples were mixed with spectroscopically pure KBr in the ratio (5:95). The pellets were fixed in sample holder and then analyzed (Hemapriya and Vijayanand, 2014).

5.2.12 GC-MS Analysis of Biodegraded Sample

Decolourized sample was centrifuged and filtered through 0.45 µm membrane filter. The filtrate was then extracted with diethyl ether and flash evaporated in rotary vacuum evaporator in temperature controlled water bath (50°C) and residue was dissolved in HPLC grade methanol for GC-MS analysis. GC-MS conditions: The GC-MS analysis of metabolite(s) was carried out using a QP5050
gas chromatography coupled with mass spectrophotometer (Shimadzu double beam spectrophotometer, UV1601). The analysis was performed in the temperature programming mode at an ionization voltage of 70eV. The temperature of the restex column (0.25 mm, 60 m; XT1-5) was kept at 60°C for initial 2 min, then raised upto 220°C with rate of 8°C / min, and then raised upto 220°C with rate of 2°C / min and held at 260°C for 15 min. The temperature of the injection port and the GC-MS interface was maintained at 270°C and 280°C respectively. The flow rate of helium as a carrier gas was 0.1 ml / min. The compounds were identified using WILEY and NIST library on the basis of mass spectra and retention time.

5.2.13 Phytotoxicity Studies

Ethyl acetate extracted products of Metanil Orange degradation were dried and dissolved in 5 ml sterile distilled water to make a final concentration of 100 ppm for phytotoxicity studies. The phytotoxicity tests were carried out on two kinds of seeds, one from grains, Sorghum vulgare Pers. (monocot) and other from pulses Phaseolus mungo L. (dicot) commonly practiced in Indian agriculture (Parshetti et al., 2006). The study was carried out at normal room temperature. 10 healthy plant seeds of each variety were treated separately with 5 ml of control dye, Metanil Orange and its degraded products (100 ppm) per day. Control set was carried out using distilled water at the same time. Germination percentage as well as the length of plumule and radical was recorded after 7 days (Saratale et al., 2009).

5.3 RESULTS

5.3.1 Dye Stuff Used

The dye stuff used in this study was Metanil Orange with color index number 22312 (www.sigmaaldrich.com) and molecular formula of C_{16}H_{10}N_{2}S_{2}O_{6}Na_{2}. The absorption maximum of this dye was 510 nm. They are widely used in textile, leather and pharmaceutical industries. The structure of Metanil Orange is shown below:
5.3.2 Enrichment, Isolation and Screening of Bacterial Strains Decolourizing Malachite Green

Totally 06 bacterial isolates, designated as PIA-1 to PIA-6 was found to be effective in decolourizing Metanil Orange (Table 5.1 and 5.2). PIA-1 to PIA-6 bacterial strains was isolated from 3 different locations (TE$_1$, TE$_2$, and TE$_3$). Out of the 06 bacterial isolates that showed more than 50% decolourization ability on Metanil Orange, PIA-5 (Plate 5.1 and 5.2) was found to be the superior strain with the highest decolourization efficiency of about 91% and was selected for the further studies. Morphological, cultural and biochemical characteristics of PIA-5 strain is tabulated in Table 5.1.

5.3.3 Molecular Phylogeny / 16S rDNA analysis of PIA-5 Isolate

Genomic DNA of the bacterial strain PIA-5 was isolated from the overnight broth culture. A total of 1431 bases sequence of PCR amplified 16S r DNA gene was determined from the isolate PIA-5 (Fig. 5.2), which corresponds to more than 99% of the gene sequence. In the analysis at NCBI and RDP sites it showed homology to 16S r DNA sequences from *Alkalibacillus* species. In the phylogenetic analysis, the sequence formed a cluster within *Alkalibacillus* species with 100% bootstrap value, confirming the identity of the isolate as strain of this species. The highest similarity value exists with *Alkalibacillus* sp. JH16 (gene bank entry: DQ232744). The bacterial strain was identified as *Alkalibacillus* sp. Strain PIA-5 (Fig.5. 3).
Optimization of Culture Conditions for Maximizing Bacterial Biomass and Dye Decolourizing Ability of *Alkalibacillus* sp. Strain PIA-5

5.3.4 Effect of Incubation Time

The bacterial growth and decolourization of Metanil Orange by *Alkalibacillus* sp. Strain PIA-5 was found to be greatly influenced by the Incubation time. Dye decolourization by the isolate was found to be noticed after 16 h of incubation and the activity reached the maximum level after 32 hrs of incubation as shown in (Fig. 5.4 and 5.5). Hence the optimum cultural conditions for elevating bacterial biomass and dye decolourization in shake flasks were carried out after 32 hrs of incubation.

5.3.6 Effect of Temperature

Bacterial growth and decolourization of Metanil Orange by *Alkalibacillus* sp. Strain PIA-5 was greatly influenced by the incubation temperature. Both bacterial growth and decolourization ability of the bacterial isolate maximized in between 30-45°C, with optimum being 35°C after 32 h of incubation. However, bacterial biomass and decolourization efficiency was found to be greatly reduced at incubation temperatures below 30°C and above 40°C respectively (Fig. 5.6 and 5.7).

5.3.7 Effect of pH

Bacterial growth and decolourization of Metanil Orange by *Alkalibacillus* sp. Strain PIA-5 was investigated under the influence of neutral, acidic and alkaline pH (5.0 – 9.0). Both biomass and decolourization percentage of the strain was found to be maximized at slightly alkaline medium (pH 8.0). Decolourization efficiency of the isolate in a neutral pH 8.0 was comparatively higher than the slightly acidic medium (pH 5.0). However, at an extreme acidity of pH 4.0, both bacterial biomass and decolourization percentage was highly reduced (Fig. 5.8 and 5.9).

5.3.8 Effect of Dye Concentration

Influence of increasing dye concentrations of Metanil Orange were investigated on bacterial biomass and decolourization efficiency of *Alkalibacillus* sp.
Strain PIA-5. The rate of dye decolourization of Metanil Orange increased linearly with increase in the initial dye concentration upto 100 ppm. As the dye concentration increased in the culture medium, a gradual decline in color removal was attained. At high concentration (1000 ppm), Metanil Orange greatly suppressed both bacterial biomass and decolourization ability (Fig. 5.10 and 5.11).

5.3.9 Effect of Aeration/Agitation

Influence of increasing agitation speeds (0-200 rpm) on bacterial growth and decolourization ability of *Alkalibacillus* sp. Strain PIA-5 on Metanil Orange was investigated. The results shown in Fig.5.12 and 5.13 have revealed that the bacterial growth steadily increased with the increasing agitation speeds, showing optimum growth at 200 rpm. In contrast, decolourization ability of the isolate drastically decreased with increase in agitation speed. Static conditions proved to be effective than shaking conditions in optimizing decolourization percentage of PIA-5.

5.3.10 Effect of Carbon Sources

Among the various carbon sources investigated, sucrose (1%) instigated optimum bacterial biomass (6.24) and decolourizing ability (92%) of *Alkalibacillus* sp. Strain PIA-5 (Table 5.3). In contrast, incorporation of glucose, maltose and starch as carbon source, negatively regulated both bacterial growth and dye decolourization efficiency of *Alkalibacillus* sp. Strain PIA-5.

5.3.11 Effect of Nitrogen Sources

Among the various organic nitrogen sources tested, Yeast extract had significant effect on bacterial biomass (6.24) and dye decolourization ability (92% respectively). In contrast, meat extract showed negative impact on bacterial growth and subsequent decolourization efficiency (Table 5.4). Among the various inorganic nitrogen sources investigated, Potassium nitrate positively regulated the dye decolourization process whereas, NH₄Cl, NH₄NO₃ and NaNO₃ slightly suppressed the decolourization efficiency of *Alkalibacillus* sp. Strain PIA-5 (Table 5.5).
5.3.12 Effect of Metal Ions

Supplementation of the culture media with suitable metal ions significantly improved the dye decolourization and bacterial biomass of *Alkalibacillus* sp. Strain PIA-5. Among the various metal ions studied, Mg\(^{2+}\) showed increased decolourization ability (Table 5.6). In the presence of Hg\(^{2+}\) and Fe\(^{2+}\) ions, decolourization efficiency was found to be drastically reduced. Incorporation of Co\(^{2+}\) and Zn\(^{2+}\) ions greatly suppressed bacterial growth and decolourization efficiency of *Alkalibacillus* sp. Strain PIA-5.

Analysis of Biodegraded samples by HPLC, FT-IR and GC-MS

5.3.13 HPLC Analysis of Decolourized Sample

HPLC results of the control and the decolorized medium (24 hrs) by of *Alkalibacillus* sp. Strain PIA-5 were obtained. The HPLC analysis of Metanil Orange dye sample collected at 0 hrs incubation showed 1 major peak (Fig. 5.14). As the decolourization progressed, the biodegradation of parent compound was observed with 4 detectable peaks at 24 hrs extracted metabolites, however major peak was not observed, clearly indicating the biodegradation of Metanil Orange dye by *Alkalibacillus* sp. Strain PIA-5 (Fig. 5.15).

5.3.14 FT-IR Analysis of Decolourized Sample

Comparison of FT-IR spectrum of the control dye with extracted metabolites after complete decolourization clearly indicated the biodegradation of Metanil Orange by *Alkalibacillus* sp. Strain PIA-5. Peaks in the control dye spectrum represented the stretching between C-Cl at 536 cm\(^{-1}\), C-C-C bending at 1182 cm\(^{-1}\), stretching vibrations at 1631 cm\(^{-1}\) for NH\(_2\) scissoring 3240 cm\(^{-1}\) represented the presence of free NH group (Fig. 5.16). The FTIR spectrum of 24hrs extracted metabolites showed significant change in positions of peaks when compared to control dye spectrum. Peaks at 507, 526 and 561 cm\(^{-1}\) represented CH deformation. Stretching vibrations at 1483 cm\(^{-1}\) showed C-OH stretching. A new peak at 1631 cm\(^{-1}\) represents C-H deformation of acyclic CH\(_2\). Peaks at 3240 and 3280 cm\(^{-1}\) represented stretching vibrations at O-H. Stretching of N-H was reported at 3296 cm\(^{-1}\), clearly expressing the degradation of Metanil Orange (Fig. 5.17).
5.3.15 GC-MS Analysis of Biodegraded Sample

GC-MS analysis of biodegraded sample was carried out to study the degraded products during the decolourization of Metanil Orange by *Alkalibacillus* sp. Strain PIA-5. The GC-MS chromatogram showed the presence of 15 different peaks. Moreover, it was concluded that the decolourization sample completely lacked the presence of banned carcinogenic amines, which are prohibited in accordance with the Consumer Goods Ordinance (Textiles Committee, Ministry of Textiles, Govt. of India) (Fig.5.18).

5.3.16 Detoxification / Phytotoxicity Assay

*Sorghum vulgare* seeds treated with tap water showed 100% germination, the mean plumule length of 22 ± 0.4 cm and the mean radical length of 8.0 ± 0.6 cm. In contrast, the seeds treated with untreated dye sample showed only 70% germination, the mean plumule length of 13 ± 0.5 cm and the radical length of 3.8 ± 0.2 cm. Whereas, the seeds treated with treated dye sample (degraded) showed 95% germination, the mean plumule length of 21 ± 0.5 cm and the radical length of 6.5 ± 0.2 cm. *Phaseolus mungo* seeds treated with tap water showed 100% germination, the mean plumule length of 20 ± 0.2 cm and the mean radical length of 4.6 ± 0.4 cm. In contrast, seeds treated with untreated dye sample showed only 50% germination, the mean plumule length of 11.5 ± 0.4 cm, the mean radical length 3.2 ± 0.4 cm, whereas, the seeds treated with treated dye sample (degraded) showed 100 % germination, the mean plumule length of 17.4 ± 0.4 cm, the radical length of 3.6 ± 0.2 cm.

The result indicated that the extracted metabolites (degraded dye) contains non-toxic metabolites, resulting in good germination rate as well as significant root and shoot length of *S. vulgare* and *P. mungo* when compared to dye sample (untreated), where inhibition in all these parameters was observed (Table 5.7 and 5.8).
Wastewater from textile industries pose a serious threat to the environment as large amount of chemically different dyes are used for various industrial applications such as textile dyeing and a significant proportion of these dyes enter the environment via industrial wastewater (Dayaram and Dasgupta, 2008; Bourie and Din, 2016). The presence of dyes imparts an intense color to effluents, which leads to environmental as well as aesthetic problems. The treatment of azo-dye-containing wastewaters still presents an arduous task and a technical challenge (Pandey et al., 2007). As regulations are becoming even more stringent, there is an urgent need for technically feasible and cost-effective methods. Economical removal of color from effluents remains an important problem although a number of successful systems have evolved employing various physico-chemical and biological processes. Regulatory agencies are increasingly interested in decolourization technologies (Anjaneyulu et al., 2005; Khadijah et al., 2009). The overwhelming majority of the current research works in the fields of textile effluent decolourization has been dealing with the various aspects of the applications of microbiological techniques, with the search for new microorganisms providing higher decomposition rates and with the elucidation of the principal biochemical and biophysical processes underlying the decolourization of dyes.

Bacterial growth and dye decolourization ability of *Alkalibacillus* sp. Strain PIA-5 significantly depends upon Incubation time. The bacterial growth and decolourizing capability of the isolate indicated that there was a distinct growth associated nature of the decolourizing efficiency. Maximum decolourization activity was observed at the late exponential phase and stationary phase, suggesting that the factors involved in the decolourization process were produced as the primary metabolites. Both bacterial biomass and percentage decolourization of *Bacillus* sp. strain DRS-1 maximized after 32 hrs of incubation. In contrast, the decolourization of Direct Blue-6 by *Pseudomonas desmolyticum* NCIM 2112 (Kalme et al., 2007), Navy Blue RX by *Streptomyces krainskii* SUK-5 (Mane et al., 2008) and Orange 16 by *Aeromonas* sp. Etl-1949 was achieved within 24 h of incubation (Shah et al., 2013).
Decolourization of Metanil Orange dye by *Alkalibacillus* sp. Strain PIA-5 was found to be maximized at 35°C after 32 hrs of incubation. Similarly Bouraie and Din (2016), reported the maximized decolourization of Reactive black 5 by *Aeromonas hydrophila* at 35°C. In contrast, the decolourization of Acid Orange-10 by *Bacillus fusiformis* KMK5 (Kolekar *et al.*, 2008) and Congo red by *Bacillus* sp. (Gopinath *et al.*, 2009) was achieved at 37°C. Decolourization of Congo Red by *Bacillus* sp. VT-II was maximized at 40°C (Sawhney and Kumar, 2011). Optimum decolourization of Reactive Red-22 by *E. coli* strain NO3 and *Pseudomonas luteola* was achieved at 42°C (Chang and Kuo, 2000; Chang *et al.*, 2001). The initial pH required for obtaining maximum decolourization of Metanil Orange dye by *Alkalibacillus* sp. Strain PIA-5 depends not only upon the bacterial strain, but also upon the ingredients of the culture medium. The best decolourization was achieved at a broad range of pH (6.0-10.0), with optimum being pH 8.0. In contrast, neutral pH was found to be effective in maximizing both bacterial growth and dye decolourization efficiency of many bacterial strains (Kilic *et al.*, 2007; Wang *et al.*, 2009; Khan, 2011). The pH tolerance of *Alkalibacillus* sp. Strain PIA-5 is quite important because the reactive azo dyes bind to cotton fibers by the addition or substitution mechanisms under alkaline conditions (Aksu *et al.*, 2007; Hemapriya *et al.*, 2013).

Bacterial community is highly sensitive to the variation in the oxygen requirement. Agitation was another important parameter that affected the biomass and dye decolourizingability of *Alkalibacillus* sp. Strain PIA-5. Bacterial growth was found to be maximized under shaking conditions at 200 rpm. According to Nascimento and Martins (2004), oxygen acts as a terminal electron acceptor for oxidative reactions to provide energy for all the cellular activities. The variation in the agitation speed has been found to influence the extent of mixing in the shaking flasks and also affect the nutrient availability. In contrast, decolourizingability of some microorganisms was found to be elevated at shaking conditions (Mane *et al.*, 2008; Dellamatrice *et al.*, 2017). The bacterial growth and decolourization efficiency are independent as enzymatic decolourization via azoreductases and laccases may be repressed under aerobic conditions. According to Padmavathy *et al.* (2003), textile azo dyes are deficient in carbon content and as a consequence
biodegradation of dyes is found to be very difficult, without the addition of carbon sources. Among the various carbon sources, sucrose was found to be an ideal candidate in optimizing both bacterial biomass and decolourization efficiency of *Alkalibacillus* sp. Strain PIA-5. Similarly, sucrose elevated the decolourization efficiency (97%) of a heterocyclic monoazo disperse dye by *Bacillus firmus* (Arora et al., 2007).

In microorganisms, organic and inorganic N$_2$ is metabolized into proteins, nucleic acids, amino acids and cell wall components. Decolourization percentage and bacterial biomass of *Alkalibacillus* sp. Strain PIA-5 heavily depends upon the availability of a suitable nitrogen sources in the fermentation medium, which has regulatory effects on bacterial growth (Patel et al., 2005). Among the various organic N$_2$ sources tested, yeast extract was found to be the most superior in maximizing the bacterial growth and decolourizing ability of *Alkalibacillus* sp. Strain PIA-5. Similarly, yeast extract maximized the decolourization of many azo dyes (Arora et al., 2007; Khelifi et al., 2009; Vijayanand et al., 2017). Among the various inorganic nitrogen sources, KNO$_3$ was found to be effective in maximizing bacterial biomass and color removal efficiency of *Alkalibacillus* sp. Strain PIA-5. Supplementation of the culture medium with suitable metal cations improved substantially the growth and decolourization ability of *Bacillus* sp. strain DRS-1. Among the various metal ions tested, Mg$^{2+}$ ions positively regulated the growth and decolourizing percentage of the isolate. According to Hemapriya et al. (2013) the stimulating effect of the metal ions can be attributed either to the stabilization of the outer membrane or to the interaction of metal ions directly with the enzymes. On the other hand, the inhibitory effect of certain metal ions like Co$^{2+}$, Zn$^{2+}$ and Fe$^{2+}$ may be due to their oligo-dynamic effect (Shyamala et al., 2014).

Azo dye reduction may involve different mechanisms like enzymatic and mediated, locations like intracellular and extracellular process in which reducing equivalents from either biological or chemical sources are transferred to the dye (Sanghi et al., 2006). Oxidative biodegradation takes place upon the action of enzymes such as Azoreductases, peroxides and laccases (Kandelbauer et al., 2004; Hassan et al., 2013). To disclose the possible mechanism of the dye decolourization,
the products of biotransformation of Metanil Orange were analyzed by HPLC, FTIR and GC-MS. Many sulfonated aromatic amines accumulate in the environment as evidenced by their occurrence in the surface water, where they are considered to be the substantial polluting factor. Other dye metabolites such as unsulfonated aromatic amines are relatively stable in aquatic conditions and are poorly degraded under anaerobic or aerobic wastewater treatment conditions (Pinheiro et al., 2004). Thus, both sulfonated and unsulfonated aromatic amines are important groups of environmental pollutants formed during the reduction of azo dyes that can potentially pass of big concern to assess the toxicity of the dye before and after biodegradation. The plant seeds germination percentage and the length of the plumule and radical of both S. vulgare and P. mungo seeds was less with control dye, Metanil Orange treatments as compared to its extracted degradation products and water.