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Original Article

Bioremediation and detoxification of a textile azo dye-metanil orange by bacterial strain PIA-5

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ABSTRACT

Environmental pollution has been recognized as one of the major problems of the modern world. The increasing demand for water and the dwindling supply has made the treatment and reuse of industrial effluents as an attractive option. 6 bacterial isolates, designated as PIA-1 to PIA-6 were found to be effective in decolorizing Metanil Orange. The superior strain with the highest decolourization efficiency was identified as *Alkalibacillus Sp* strain PIA-5. Dye decolourization of Metanil Orange was found to be maximized after 32 hrs of incubation. Increase in dye concentration was found to be inversely proportional to the decolourization of Metanil Orange. HPLC and FTIR analysis revealed the degradation of the parental dye molecule.

Key words: Textile effluents, Metanil orange, *Alkalibacillus Sp* strain PIA-5 and Detoxification

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INTRODUCTION

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 [1-2]. Textile industry is the one of the most important industry in all over world and this industry uses large volumes of water in wet processing operations and thereby, generates substantial quantities of wastewater containing large amounts of dissolved dyestuffs and other products. More than 10,000 dyes are used in the textile industry and < 280,000 tones of textile dyes are discharged every year worldwide as untreated effluents in the form of wastewater into public drains that eventually empty into rivers [3-4]. Physico-chemical techniques are of great value specifically when effluents from textile industries contain chemical compounds that are hazardous to the biological resources like bacteria and fungi, which play a vital role in nourishing other biotic sources by providing the basic nutrients after the mineralization of organic contaminants in soil and water [5-6]. Biological dye removal techniques are based on the microbial biotransformation of dyes. Many researchers have demonstrated the partial or complete biodegradation of dyes by pure and mixed cultures of bacteria, actinomycetes, fungi and algae [7-8]. Microorganisms for dye decolonization may be obtained simply by the isolation of existing dye degrading cultures from the textile effluent samples, by the adaptation of promising strains to the conditions present in the textile effluents or by the construction of suitable organisms employing genetic engineering methods [9]. Bioremediation is a pollution control process that uses the biological systems to catalyze the degradation or transformation of various toxic chemicals into less harmful forms. The general approaches to bioremediation include:

Hence, the present investigation was intended to assess the potential of PIA-5 strain to decolorize the synthetic textile azo dye, Metanil orange under aerobic conditions, and to optimize the culture conditions to maximize the biomass and decolorization efficiency of PIA-5 Strain.

MATERIAL AND METHODS

Sampling site and sample collection: The sampling area was the textile industries and dyeing units located in and around Kanchipuram, Tamil Nadu, India. The effluent samples from both textile industries and dyeing units were characterized by its dark color and extreme turbidity.

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.

Isolation, Enrichment and Screening of Bacterial Strains Decolorizing Metanil Orange: The effluent samples 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 [10]. 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 decolorizing bacterial strains were selected and re-streaked several times to obtain pure cultures. The pure cultures of dye decolorizing bacterial strains were maintained on dye-containing nutrient agar slants at 4°C.

Decolorization 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 hrs 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 decolorization 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. Decolorization extent was determined by measuring the absorbance of the culture supernatant at 621 nm using UV-visible spectrophotometer (Hitachi U 2800), according to [11].

$$\text{Decolorization efficiency } (\%) = \frac{\text{Dye(i)} - \text{Dye(r)}}{\text{Dye(i)}} \times 100$$

Where, Dye (i) refers to the initial dye concentration and Dye (r) refers to the residual dye concentration. Decolorization experiments were performed in triplicates.

Bacterial Strain and Culture Conditions: Bacterial strain that showed maximum decolorization 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.

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 35ºC 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 [12]. 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 [13].

Optimization of Various culture Conditions for Bacterial Biomass and Metanil Orange Decolorization by *Alkalibacillus* sp. Strain PIA-5
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 decolorizing ability of *Alkalibacillus* sp. Strain PIA-5was 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 decolorization percentage was measured at optimum growth (24 h).

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 decolorization extent of Metanil Orange by *Alkalibacillus* sp. Strain PIA-5, was investigated after 24 h of incubation at 35°C.

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 ($(NH_4)_2SO_4$, KNO_3, NH_4Cl, NH_4NO_3 and $NaNO_3$) were investigated on the bacterial growth and dye decolorization ability of *Alkalibacillus* sp. Strain PIA-5, after 24 h of incubation at 35°C **.**

Effect of Various Metal Ions: The effect of various metal ions on bacterial growth and dye decolorization 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 $MnCl_2$, $MgCl_2$, $HgCl_2$, $ZnSO_4$, $CoCl_2$ and $FeSO_4$, incubated at 35°C for 32 h.

RESULT

Enrichment, Isolation and Screening of Bacterial Strains Decolorizing Metanil Orange: Totally 6 bacterial isolates, designated as PIA-1 to PIA-6 was found to be effective in decolourizing Metanil Orange (Table 1 and 2). PIA-1 to PIA-6 bacterial strains was isolated from 3 different locations (TE1, TE2, and TE3). Out of the 06 bacterial isolates that showed more than 50% decolourization ability on Metanil Orange, PIA-5 (Plate.1 and 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 1.

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. 1), 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% boot strap 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. 2).
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.3 and 4). Hence the optimum cultural conditions for elevating bacterial biomass and dye decolourization in shake flasks were carried out after 32 hrs of incubation.

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 and 6).

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.7 and 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. 9 and 10).

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.11 and 12) 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.

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 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.

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 4). Among the various inorganic nitrogen sources investigated, Potassium nitrate positively regulated the dye decolourization process whereas, NH_4Cl, NH_4NO_3 and $NaNO_3$ slightly suppressed the decolourization efficiency of *Alkalibacillus* sp. Strain PIA-5 (Table 5).

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, Mg2+ showed increased decolourization ability (Table 6). In the presence of Hg2+ and Fe2+ ions, decolourization efficiency was found to be drastically reduced. Incorporation of Co2+ and Zn2+ 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

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.13). 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. 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 NH2 scissoring 3240 cm-1 represented the presence of free NH group (Fig. 15).

The FTIR spectrum of 24 hrs 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 CH2. Peaks at 3240 and 3280 cm^{-1} represented stretching vibrations at O-H. Stretching of N-H was reported at 3296cm-1, clearly expressing the degradation of Metanil Orange (Fig.16).

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.17).

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 7 and 8).

DISCUSSION

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 [14]. The presence of dyes imparts an intense color to effluents, which leads to environmental as well as aesthetic problems. The treatment of azo-dyecontaining wastewaters still presents an arduous task and a technical challenge [15]. 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 [16-17]. 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 [18], Navy Blue RX by *Streptomyces krainskii* SUK-5 [19] and Orange 16 by *Aeromonas* sp. Etl-1949 was achieved within 24 h of incubation [4].

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 [20], 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 [21] and Congo red by *Bacillus* sp. [22] was achieved at 37°C. Decolourization of Congo Red by *Bacillus* sp. VT-II was maximized at 40°C [23].

Optimum decolourization of Reactive Red-22 by *E. coli* strain NO3 and *Pseudomonas luteola* was achieved at 42°C [24-25]. 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 [26-28]. 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 [29-30]. Bacterial community is highly sensitive to the variation in the oxygen requirement. Agitation was another important parameter that affected the biomass and dye decolourizing ability of *Alkalibacillus* sp. Strain PIA-5. Bacterial growth was found to be maximized under shaking conditions at 200 rpm. According to [31], 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 [19], [32]. The bacterial growth and decolourization efficiency are independent as enzymatic decolourization via azoreductases and laccases may be repressed under aerobic conditions. According to [33], 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* [34].

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 [35]. Among the various organic N2 sources tested, yeast extract was found to be the most superior in maximizing the bacterial growth and decolourizingability of *Alkalibacillus* sp. Strain PIA-5. Similarly, yeast extract maximized the decolourization of many azo dyes [34], [36], [37]. 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, Mg2+ ions positively regulated the growth and decolourizing percentage of the isolate. According to [30] 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 Co2+, Zn2+ and Fe2+ may be due to their oligo-dynamic effect [38].

Azo dye reduction may involve different mechanisms like enzymatic and meditated, locations like intracellular and extracellular process in which reducing equivalents from either biological or chemical sources are transferred to the dye [39]. Oxidative biodegradation takes place upon the action of enzymes such as Azoreductases, peroxides and laccases [40-41]. 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 [42]. 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.

CONCLUSION

Economical and eco-friendly approaches are needed to degrade dye-contaminated wastewater from various industries. Alkalibacillus sp. (PIA-5) isolated from textile effluents completely bio transformed Metanil Orange. The dye degradation was found to be dependent on various optimization parameters such as temperature, pH, dye concentration, agitation speed and additional nutritional sources. The results obtained in this

study for the mechanisms involved in dye removal can be considered as a fundamental step for the representation of the experimental behavior and for development of process design. HPLC and FT-IR analysis substantiated the complete degradation of Evan's Blue, Malachite Green and Metanil Orange by the isolates, involving the complete breakdown of functional bonds to form non-toxic intermediates.

GC-MS chromatogram of the degraded samples revealed complete absence of carcinogenic amines, which are prohibited in accordance with the Consumer Goods Ordinance, Textiles Committee, Ministry of Textiles, Govt. of India. Phytotoxicity studies with *Sorghum vulgare* and *Phaseolus mungo* revealed the complete detoxification of the textile azo dyes. Thus, with the results obtained from the GC-MS analysis and phytotoxicity assay, the bacterial strains were proved to be highly capable of detoxifying the potent harmful and carcinogenic azo dyes and their breakdown products.

In addition, degradation and detoxification ability of the *Escherichia coli, Chromohalobacter Salexigens and Alkalibacillus sp.* could be advantageous to integrate degradation process prior to conventional processes. Further, pilot or large scale simulations are required to investigate the ability to establish a continuous process for effluent treatment using our microbial culture. Thus this work may provide a reasonable basis for development of an effective biotechnological process for the environmentally safe remediation of dye pollutants present in textile effluents.

Based on the behavioural, morphological and histopathological assay of gill showed its possible waste minimization through compilation effect treatment and through advanced dyeing techniques such as lowering of liquors ratio during dyeing, optimizing dyeing processes and recipes, process of innovation and by substituting offending dyestuffs with more eco-friendly substitutes etc. for pollution control. This can be effectively applied by source reduction instead of "end of pipe" solution. In the present study a comparative study was made to know the impact of untreated textile dye effluent with treated textile dye effluent with bacterial strains. Textile effluent was found to be more toxic when compared to the treated textile effluent with control. With respect to toxicity behavioural and morphological histopathological changes of fish in untreated textile effluent was found to be more toxic highly damages followed by treated textile effluent with bacterial strains because gills are direct contact with environment.

From the present study it is understood textile industry effluent shows the degree of damage caused by the pollutants on the different target organs of the fishes. Thus it could be suggested that precautionary measures should be taken against the discharge or the treatment of this effluent before releasing it in the fresh water bodies. Control of this type of pollution can be best achieved by reduction or prevention and dilution at the source. Legal administrative and technical measures are also necessary to reduce or eliminate the undesirable effects of industrial effluents in receiving waters. Based on the behavioural, morphological and histopathological assay of gill showed its possible waste minimization through compellation effect treatment and through advanced dyeing techniques such as lowering of liquors ratio during dyeing, optimizing dyeing processes and recipes, process of innovation and by substituting offending dyestuffs with more ecofriendly substitutes *etc* for pollution control. This can be effectively applied by source reduction instead of "end of pipe" solution. This can be practiced by standards imposed by the authorities.

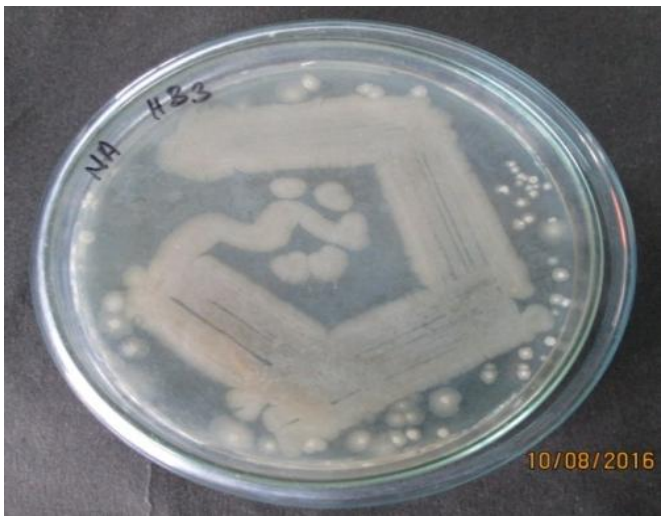

Plate 1. ***Alkalibacillus*** **sp. Strain PIA-5 grown in Nutrient Agar plate**

Plate 2. Decolorization of Metanil Orange by *Alkalibacillus* sp. Strain PIA-5

ALIGNED DATA (1431 bp)

TGCAGTCGAGCGCGGGAAGCGAGCCGAAGCCCTTCGGGGTGGACGCTCGTGGAACGAGCG
GCGGACGGGTGAGTAACACGTGGGCAACCTACCTGTGAGACGGGGATAACTCCGGGAAACC
GGGGCTAATACCGGATAACGCATCGAACCGCATGGTTCGATGATCAAAGATGGCTTCTAGCT
ATCACTCACAGATGGGCCCGCGGCGCATTAGTTAGTTGGTGAGGTAACGGCTCACCAAGGC
GACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGA
CTCCTACGGGAGGCAGCAGTAGGGAATCATCCGCAATGGACGCAAGTCTGACGGTGCAACG
CCGCGTGAACGATGAAGGTCTTCGGATCGTAAAGTTCTGTTGTGAGGGAAGAACAAGTGCC
GTTCGAATAGGGCGGCACCTTGACGGTACCTCACCAGAAAGCCCCGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCG
CAGGCGGTTCCTTAAGTCTGATGTGAAAGGCCACAGCTCAACTGTGGAGGGCCATTGGAAAC
TGGGGAACTTGAGGACAGAAGAGGAGAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGA
TATGTGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCGCG
AAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGAGTGCT
AGGTGTTAGGGGGTCCAACCCTTAGTGCTGCAGTTAACGCAATAAGCACTCCGCCTGGGGA
GTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCTGCACAAGCGGTGGAGCAT
GTGGTTTAATTCGACGCAACGCGAAGAACCTTACCAGGTCTTGACATCTTCGGACAACCCAA
GAGATTGGGTCTTCCCTTCGGGGACCGAATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCCTGATCTTAGTTGCCAGCATTC
AGTTGGGCACTCTAGGATGACTGCCGGTGACAAACCGGAGGAAGGCGGGGATGACGTCAAA
TCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGAAGCC
AAACCGCGAGGTCGAGCTAATCCCATAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAACTC
GCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGAATGCCGCGGTGAATACGTTCCC
AGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTGGCAACACCCGAAGTCGGTGGGGT
AACCTTCACGGAGCCAGCCG

Fig. 1. 16S rDNA sequence of *Alkalibacillus* sp. Strain PIA-5

ALIGNMENT VIEW and DISTANCE MATRIX TABLE
(With Sample KV001 -sequence taken as reference sequence)

S_ab score	Organism Name	NCBI Accession No
0.998	Alkalibacillus salilacus (T); BH163	AY671976
0.997	Alkalibacillus halophilus (T); YIM 012	DQ359731
0.998	Alkalibacillus sp. HNPhu	EF517965
0.998	Alkalibacillus sp. HS20D	EF517966
0.997	Alkalibacillus salilacus; HBCC-3	EU377478
0.997	Alkalibacillus sp. M18-1	EU868875
0.996	Alkalibacillus sp. M18-3	EU868876
0.997	Alkalibacillus sp. M18-4	EU868877
0.998	Alkalibacillus sp. R327	HM179168
0.998	Alkalibacillus salilacus; R559	HM179185

Fig. 2: Neighbourhood Analysis and closest homology of PIA-5 Strain

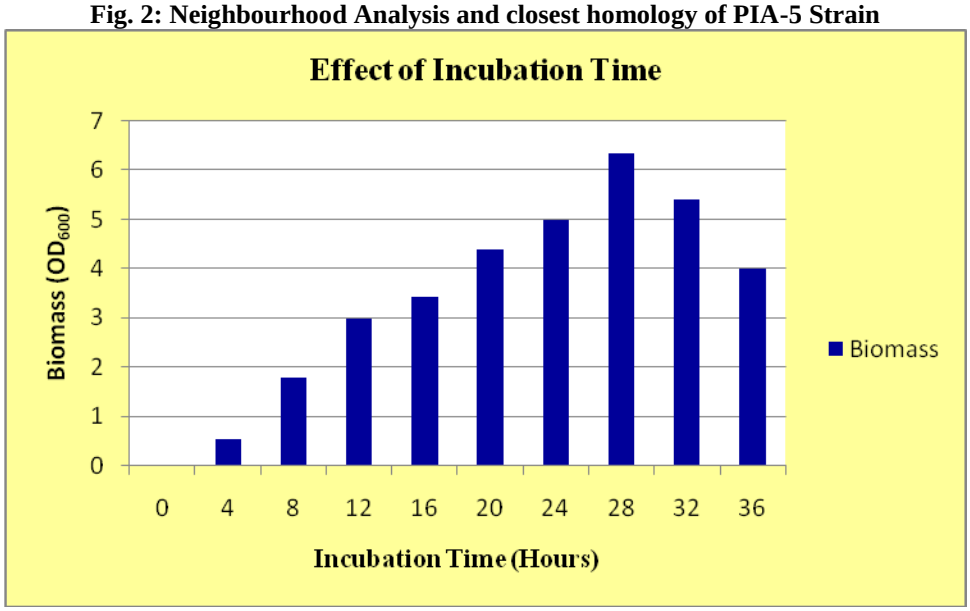

Fig. 3. Effect of incubation Time on bacterial biomass of *Alkalibacillus* sp. Strain PIA-5

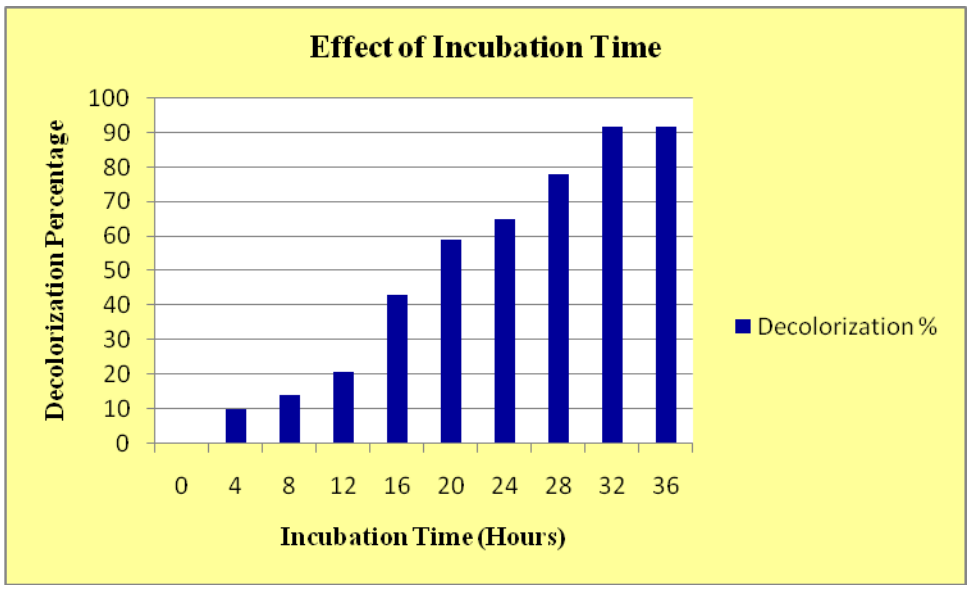

Fig. 4. Effect of Time on decolourization of Metanil Orange by *Alkalibacillus* sp. PIA-5

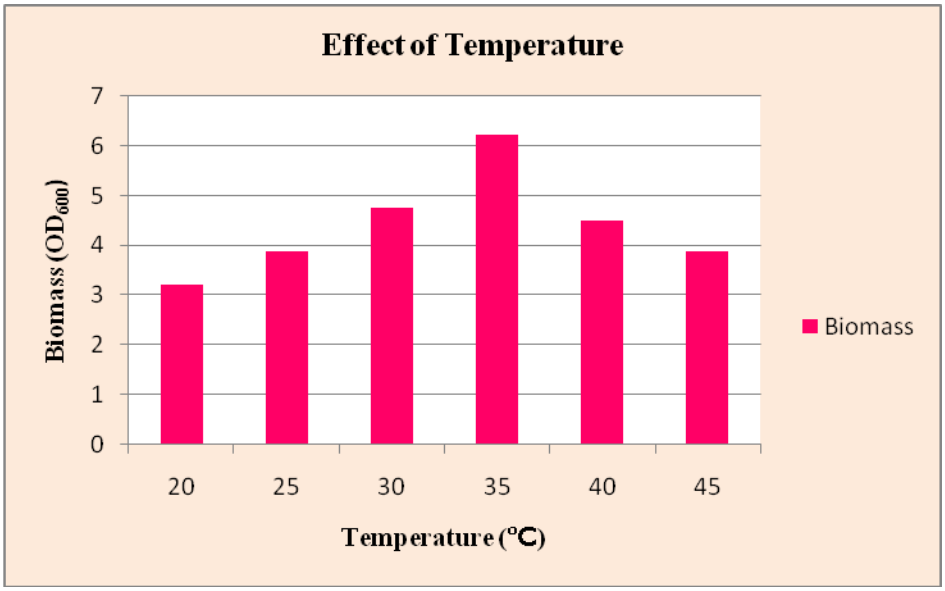

Fig. 5. Effect of Temperature on bacterial biomass of *Alkalibacillus* sp. Strain PIA-5

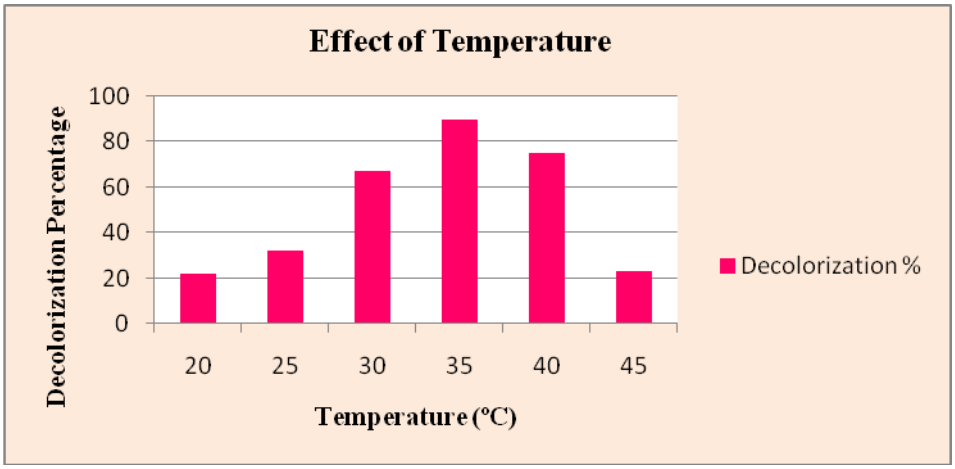

Fig. 6. Effect of temperature on decolorization of Metanil Orange by PIA-5

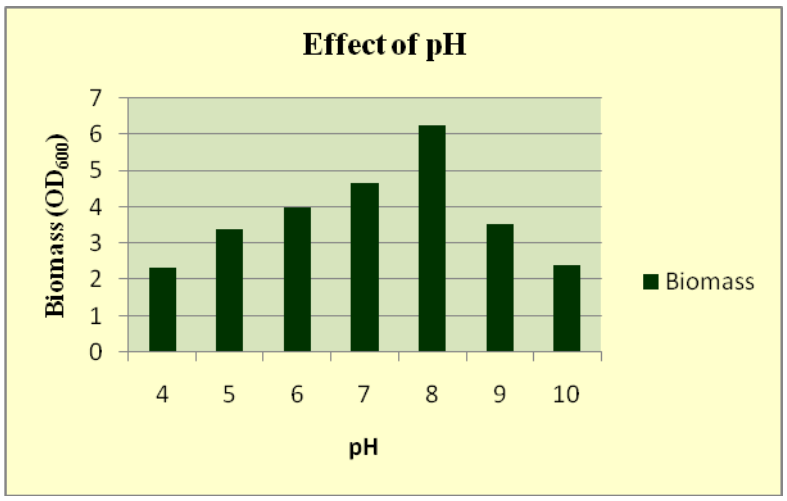

Fig. 7. Effect of pH on bacterial biomass of *Alkalibacillus* sp. Strain PIA-5

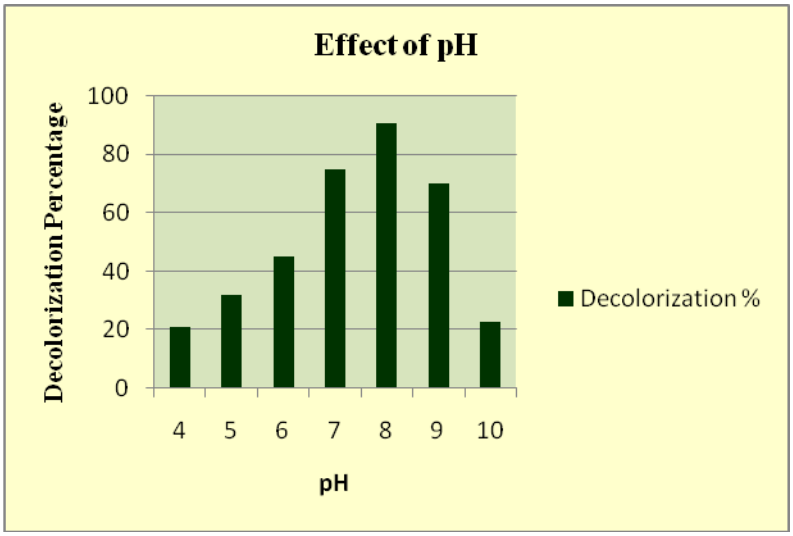

Fig. 8. Effect of pH on decolorization of Metanil Orange by *Alkalibacillus* sp. PIA-5

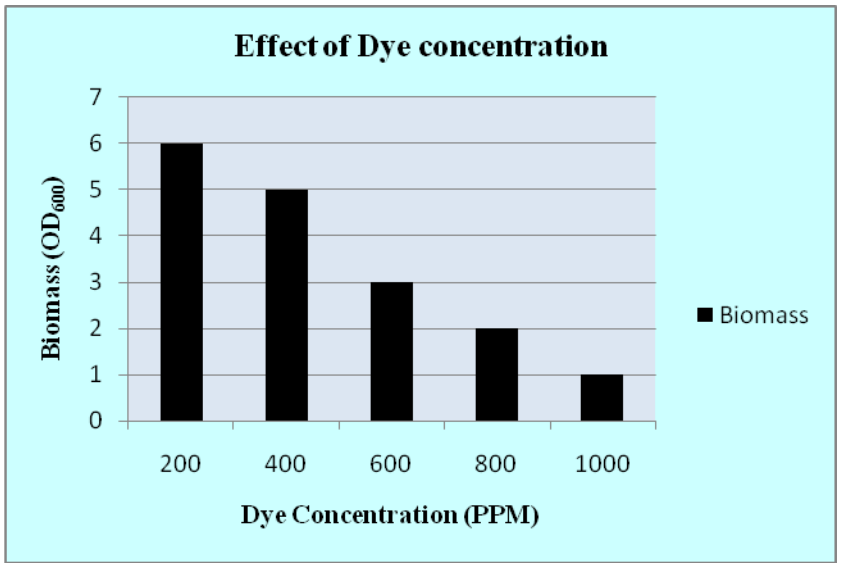

Fig. 9. Effect of Dye concentration on bacterial biomass of *Alkalibacillus* sp. Strain PIA-5

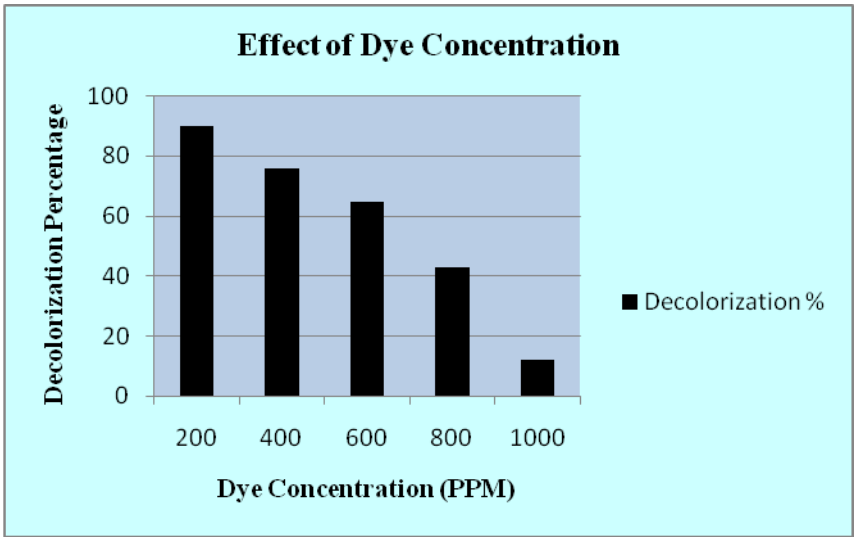

Fig. 10. Effect of Dye concentration on decolorization of Metanil Orange by PIA-5

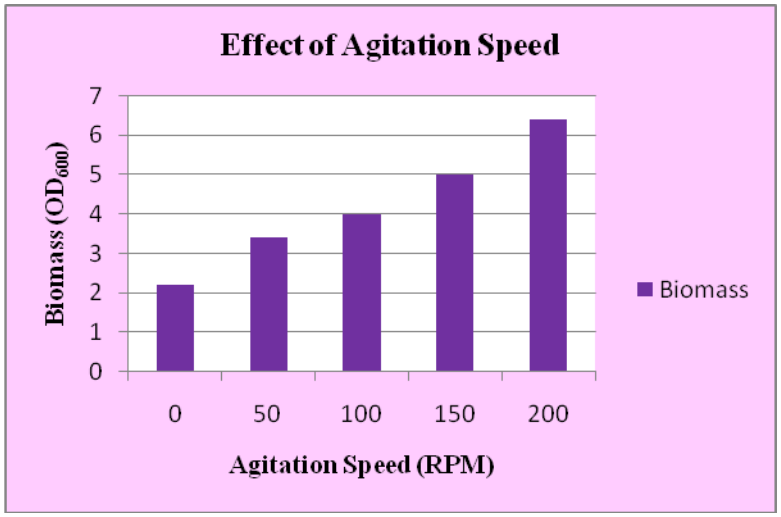

Fig.11. Effect of Agitation Speed on bacterial biomass of *Alkalibacillus* sp. Strain PIA-5

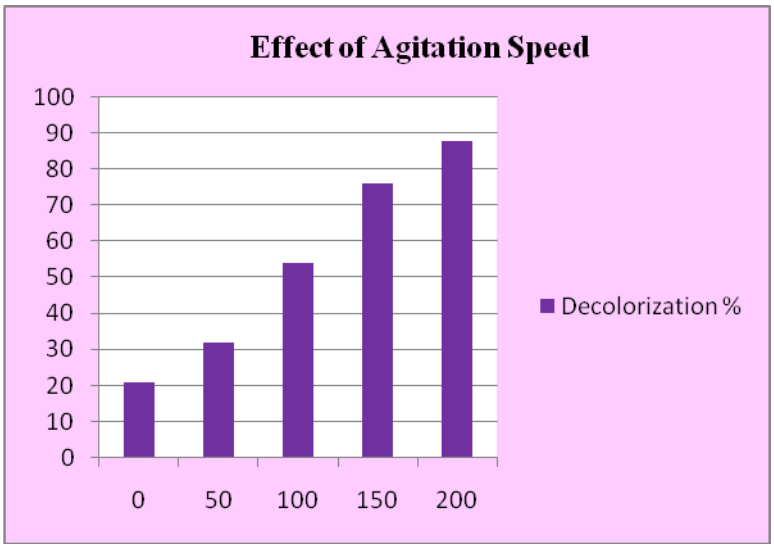

Fig. 12. Effect of Agitation on decolorization of Metanil Orange by *Alkalibacillus* sp.

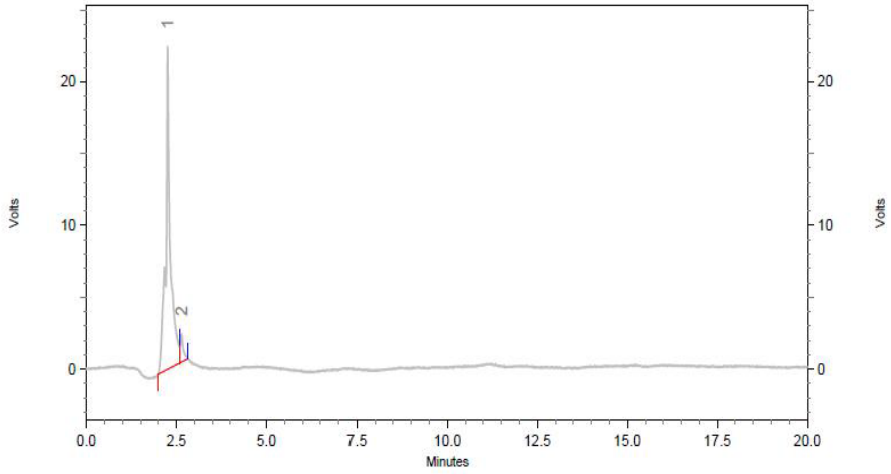

Fig.13. HPLC Chromatogram of Metanil Orange (Control)

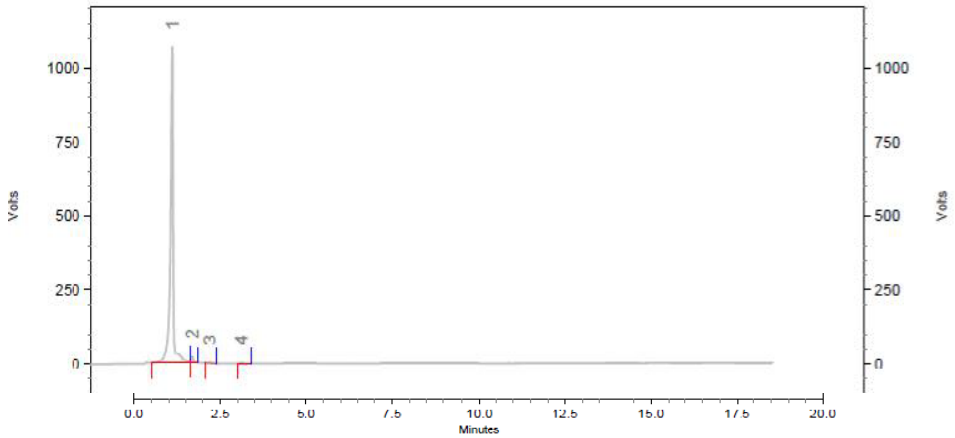

Fig.14 HPLC Chromatogram of Decolorized Metanil Orange (Test Sample)

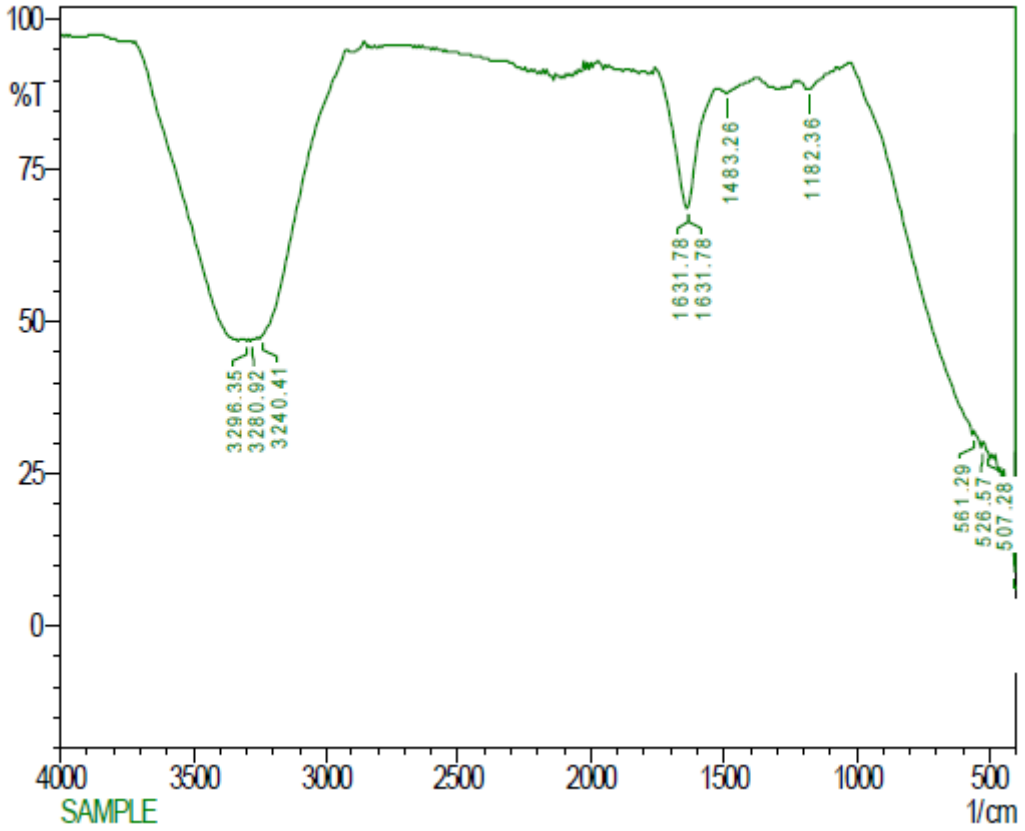

Fig. 15 FT-IR Spectra of Metanil Orange (Control)

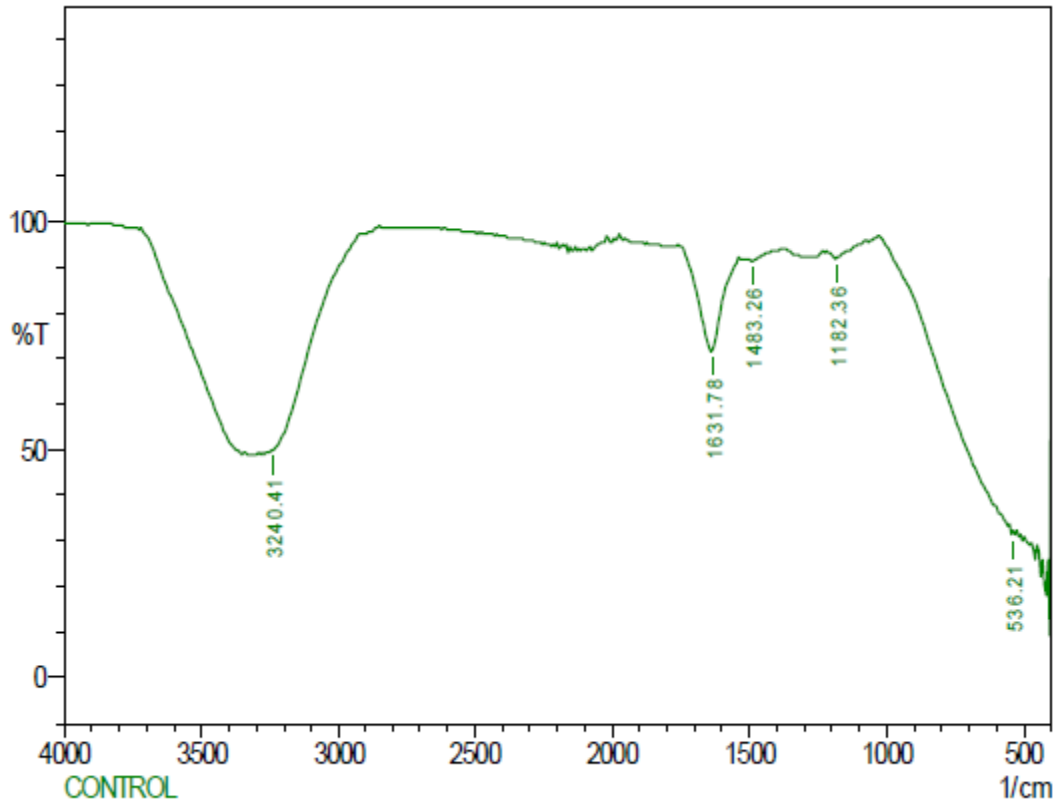

Fig.16 FT-IR Spectra of Decolorized Metanil Orange (Test sample)

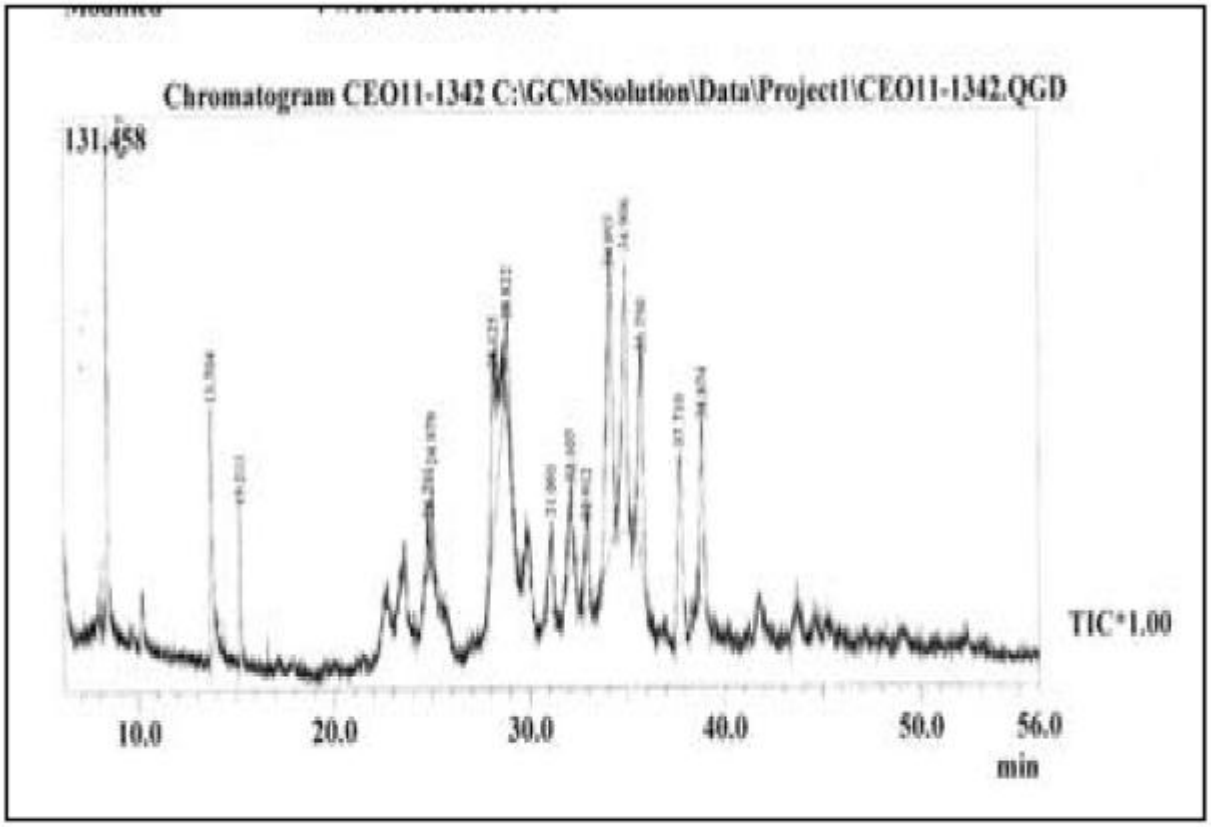

Fig. 17. GC-MS Chromatogram of decolourized Metanil Orange

Table. 1: Bacterial Strains Decolorizing Metanil Orange isolated from textile effluents

Sl. No	Isolates	Sample Collection Site	Time taken for Maximum Decolorization	Decolorization Efficiency
1	PIA-1	TE2	60 h	64.66 %
2	PIA -2	TE3	24 h	70.22 %
3	PIA -3	TE2	40 h	62.00 %
4	PIA -4	TE1	24 h	62.13 %
5	PIA -5	TE2	32 h	91.00 %
6	PIA -6	TE2	24 h	62.52 %

Note: The isolates were considered for the table that showed above 50% decolorization ability.

Table 2: Morphological, Physiological and Biochemical Characteristics of strain PIA-5

Sl. No	Test	Observations
1	Morphology Grams staining Cell shape Motility Cell arrangement	 Positive Pleomorphic rod Motile Single and paired cells
2	Colony Characters on Nutrient agar Colony morphology Colony size Colony elevation Colony density Colony edge Pigmentation	 Circular 1.5 - 2 mm Flat Transparent and glistening Entire Non-chromogenic (Cream color)
3	Sugar Fermentation Lactose Maltose Glucose Sucrose	 Positive -Acid/Gas Positive -Acid/Gas Positive -Acid/Gas Positive -Acid/Gas
4	IMVIC Indole Methyl Red VogesProskauer Citrate	 Positive Negative Negative Negative
5	Enzyme Reaction Urease Production Catalase Activity Oxidase Coagulase	 Negative Positive Positive Negative
6	H_2S Production	Negative
7	Growth on Mac Conkey agar	Colorless

Table 3. Effect of Carbon Sources on Decolorization Efficiency of *Alkalibacillus* sp. strain PIA-5 and its Biomass

Sl. No.	Carbon Sources (gl^{-1})	Decolorization Efficiency	Biomass A_{600}
1	Control	90.22% ± 0.03	5.93 ± 0.25
2	Glucose	42.42% ± 0.20	3.42 ± 0.14
3	Sucrose	92.00% ± 0.08	6.24 ± 0.24
4	Lactose	50.11% ± 0.03	4.62 ± 0.29
5	Maltose	75.32% ± 0.22	5.01 ± 0.17
6	Starch	50.08% ± 0.40	3.83 ± 0.08

Each value is an average of three parallel replicates. ± indicates standard deviation among the replicates

Table 4: Effect of Organic Nitrogen Sources on Decolorization Efficiency of *Alkalibacillus* sp. strain PIA-5 and its Biomass

Sl. No.	Organic Nitrogen Sources (gl^{-1})	Decolorization Efficiency	Biomass A_{600}
1	Control	90.22% ± 0.03	5.93 ± 0.05
2	Tryptone	65.42% ± 0.02	4.24 ± 0.10
3	Beef Extract	72.26% ± 0.02	4.30 ± 0.14
4	Peptone	66.10% ± 0.01	5.42 ± 0.01
5	Yeast Extract	92.00% ± 0.01	6.24 ± 0.10
6	Meat Extract	48.57% ± 0.20	5.12 ± 0.05

Each value is an average of three parallel replicates. ± indicates standard deviation among the replicates

Table 5: Effect of Inorganic Nitrogen Sources on Decolorization Efficiency of *Alkalibacillus* sp. strain PIA-5 and its Biomass

Sl. No.	Inorganic Nitrogen Sources (gl^{-1})	Decolorization Efficiency	Biomass A_{600}
1	Control	92211% ± 0.03	5.93 ± 0.05
2	$(NH_4)_2SO_4$	58.26% ± 0.62	4.35 ± 0.06
3	KNO_3	92.20% ± 0.12	6.23 ± 0.01
4	NH_4Cl	80.05% ± 0.04	6.10 ± 0.03
5	NH_4NO_3	64.32% ± 0.02	5.14 ± 0.05
6	$NaNO_3$	50.07% ± 0.08	5.08 ± 0.06

Each value is an average of three parallel replicates. ± indicates standard deviation among the replicates

Table 6: Effect of Various Metal Ions on Decolorization Efficiency of *Alkalibacillus* sp. strain PIA-5 and its Biomass

Sl. No.	Metal Ions (5mM)	Decolorization Efficiency	Biomass A_{600}
1	Control	90.22% ± 0.03	5.93 ± 0.15
2	$MnCl_2$	78.26% ± 0.12	4.12 ± 0.20
3	$MgCl_2$	91.01% ± 0.22	6.20 ± 0.20
4	$HgCl_2$	48.84% ± 0.12	4.20 ± 0.12
5	$ZnSO_4$	48.66% ± 0.22	4.02 ± 0.22
6	$CoCl_2$	34.27% ± 0.12	4.05 ± 0.02
7	$FeSO_4$	42.92% ± 0.12	3.14 ± 0.20

Each value is an average of three parallel replicates. ± indicates standard deviation among the replicates

Table 7: Phytotoxicity study of Metanil orange and its Degradation Products on *Sorghum vulgare* Pers.

S.NO	Parameters Studied	Tap water	Metanil orange (100 ppm)	Treated sample (100 ppm)
01	Germination (%)	100	60	95
02	Plumule (cm)	22 ± 0.4	13 ± 0.5	21 ± 0.6
03	Radical (cm)	8 ± 0.6	3.8 ± 0.2	6.5 ± 0.2

Table 8: Phytotoxicity study of Metanil orange and its Degradation Products on *Phaseolus mungo* L.

S.NO	Parameters Studied	Tap water	Metanil orange (100 ppm)	Treated sample (100 ppm)
01	Germination (%)	100	50	90
02	Plumule (cm)	20 ± 0.2	11.5 ± 0.4	17.4 ± 0.4
03	Radical (cm)	4.6 ± 0.4	3.2 ± 0.2	3.6 ± 0.2

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