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Decolorization of Xenobiotic Azo Dye- Black WNN by Immobilized *Paenibacillus alvei* MTCC 10625

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Abstract The present study, *Paenibacillus alvei* MTCC 10625 elucidates the ability for the decolorization of synthetic dye effluent containing Black WNN dye, isolated from textile wastewater contaminated site. 96.4% decolorization of Black WNN (100 mg/l) was attained at pH 9, temperature 35°C within 48 h under optimized condition with free cells of *P. alvei* MTCC 10625. Moreover, *P. alvei* MTCC 10625 immobilized over polyurethane foam (PUF) and nylon mesh (NM), achieved 95.2 and 97.0% decolorization under microaerophilic conditions within 24 h. Biodegradation analysis by FTIR and HPTLC confirms the degradation of Black WNN dye and formation of different intermediate metabolites. Further, microbial toxicity as well as phytotoxicity assay confirms the nontoxic nature of the effluent released from the outlet of column. The former is in relation to using soil microbial flora; *Bacillus cereus*, *Bacillus subtilis* and *Kocuria rosea* MTCC 5932; whereas the later by using *Triticum aestivum* and *Vigna radiata* plants.

Keywords: *biodegradation, black WNN dye, decolorization, nylon mesh, polyurethane foam*

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1. Introduction

Synthetic dyes are used in textile, paper, and printing industries, as well as in dye houses because of their ease of production, fastness, and variety of colors compared with natural dyes. Azo dyes are the largest group of organic dyes with –N=N- group as a chromophore in the molecular structure and represent more than half of the global dye production, specially because of their wide usage in dyeing industries due to the simple dyeing procedure [1]. These dyes belong mostly to the non-biodegradable and recalcitrant type of water pollutants, which make activated sludge treatment methods inadequate. The structural diversity of dyes derives from the use of different chromophoric groups and the major chemical pollutants present in textile effluent are dyes containing carcinogenic amines, toxic heavy metals, chlorine bleaching and softeners etc. [2]. Most (80%) reactive dyes are azo or metal complex azo compounds but also anthraquinone and phthalocyanine reactive dyes are applied, especially for green and blue.

The discharge of highly colored wastewater is currently an important environmental dilemma because of their color, bio-recalcitrance, and potential toxicity towards animals and humans [3,4]. The strong color of discharged dyes even at very small concentrations has a huge impact on the aquatic environment caused by its turbidity and high pollution strength. Removal of color is desirable in the disposal of textile wastewater due to aesthetic deterioration as well as the obstruction of penetration of dissolved oxygen and sunlight into water bodies which seriously affect the life of aquatic flora and fauna [5,6]. Majority of synthetic dyestuff is hardly removed from textile wastewater by conventional wastewater treatment methods [7]. Dye removal from wastewater with traditional physico-chemical methods, such as coagulation, adsorption and oxidation with ozone being expensive, can generate large quantity of sludge and usually require the addition of environmental hazardous chemical additives [8]. Considering drawbacks in aforementioned treatments, microbial remediation techniques have gained much attention in the last few decades. The development of efficient and environment friendly bioremediation technologies [9], to reduce dye content in wastewater up to an acceptable level at affordable price, is of paramount significance. The general approach of bioremediation is to improve the natural biodegradation capacity of the native organisms by providing suitable environmental condition for degradation. Several microbial sources have been reported for dye decolorization including, fungal and bacterial cultures [10,11]. However, ubiquitous nature of bacteria makes them very useful tool for the textile wastewater treatment. Researcher has investigated a number of genera that are capable of degrading azo dyes including *E. coli* and *Pseudomonas luteola* [12], *Alcaligenes faecalis* PMS-1 [13], *Acinetobacter radioresistens* [14], *Bacillus amyloliquefaciens* [15], *Enterobacter* sp. [16], *Enterobacter* sp. GY-1 [5], *Exiguobacterium profundum* strain N4 [17], *Salmonella subterranean* and *Paenibacillus polymyxa [18]*, *Pseudomonas aeruginosa*, *Pseudomonas putida* [19] and *Staphylococcus epidermidis* [20,21].

Research at a glance was carried out for dye decolorization by using *Paenibacillus* species. Decolorization (70%) of textile wastewater was investigated by microbial flocs consisted *Acromobacter* sp., *Bacillus* sp. and *Paenibacillus* sp. to accumulate heavy metals present in the textile wastewater. Moreover, Palamthodi et al. [22] make use of *Paenibacillus azoreducers* for the decolorization of Green and Blue dye. The objective of the present research paper is focused on the potential of *Paenibacillus alvei* MTCC 10625 for the decolorization of Black WNN dye from the aqueous solution and can be used successfully in the commercial treatment of textile wastewater.

2. Materials and Methods

2.1. Dye, Media and Chemicals

Black WNN, a di-azo dye used in this study was provided by Messers. Abhishek Industries Ltd., Barnala, Punjab (India). An absorbance maxima (λmax) for this dye was obtained by scanning dye solution over visible range of 350-750 nm. All Growth media and chemicals are of analytical grade and procured from Hi-Media Laboratories and SD Fine-Chem Ltd., Mumbai respectively. The composition of the mineral salt (MS) medium used for the decolorization study was as follows: $(NH_4)_2SO_4$: 1.0 g/l; K_2HPO_4: 6.0 g/l; KH_2PO_4: 1.0 g/l; $MgSO_4.7H_2O$: 0.1 g/l; NaCl: 5.0 g/l and Glucose: 3.0 g/l [23]. Stock solutions of dye and glucose were prepared, filter sterilized and added into MS medium at the time of inoculation in requisite concentration.

2.2. Microorganism

Bacterial culture isolated from soil sample contaminated with textile effluent and screening performed by microtiter plate technique in mineral salt (MS) medium has been earlier discussed in our research article [24]. Bacterial isolate, NF23 was submitted to Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, for biochemical characterization and Chromous Biotech Ltd, Bangalore, India, for molecular identification. The bacterial isolate NF23 was identified as *Paenibacillus alvei* and utilized for further study.

2.3. Effect of Process Parameters on Dye Decolorization

Mid-log phase culture of the *P. alvei* MTCC 10625 was inoculated into MS medium, supplemented with Black WNN dye, for its decolorization under shaking/static conditions to ensure the requirement of oxygen for decolorization. Further, the effect of different process parameters, such as pH (4-11), temperature (20-50°C), dye concentration (50-600 mg/l) and inoculum concentration (1,3,5,7 and 10%) was examined. In addition to this, the effect of additional co-substrates, like carbon (glucose, maltose, sucrose and starch) and nitrogen sources (ammonium chloride, ammonium nitrate, ammonium sulphate, urea and yeast extract) on dye decolorization were also investigated.

2.4. Immobilization of *P. alvei* MTCC 10625 on Support Matrices

Polyurethane foam (PUF) and nylon mesh (NM) were selected as support matrices due to their high porosity and non-reactive (inert) nature, easy availability and low cost. The polyhedral and filamentous structure of PUF and NM provide a considerable large surface area for the immobilization of *P. alvei*. Matrices were sliced into 1x1x1 cm cubes and thoroughly washed with distilled water, dried and sterilized. Mid-log phase culture (1%) of *P. alvei* MTCC 10625 was inoculated into a 250 ml Erlenmeyer flask containing 50 ml of NB and pre-sterilized pieces of support matrices were kept for incubation at 37°C and 125 rpm in a shaker incubator for 24 h. Fresh NB medium (25 ml) was added into it after every 24 h and this step of process was consecutively repeated for 5 d. This enabled the bacterial culture to get immobilized over the PUF and the NM. Thereafter, immobilized bacterial biomass was used as an inoculum for decolorization studies.

2.5. Decolorization of Black WNN Dye in Batch, Fed Batch and Continuous up Flow Column System Using Immobilized *P. alvei* MTCC 10625

Decolorization of Black WNN dye was performed in an Erlenmeyer flask containing 50 ml MS media containing 100 mg/l concentration of dye and inoculated with immobilized matrices (PUF and/or NM) in a batch system. In case of fed batch system, MS medium was supplemented with WNN dye in the Erlenmeyer flask, followed by 24 h incubation, with batch wise addition of medium and dye for the duration of 8 days. The samples were further analyzed for decolorization.

For decolorization in continuous system, an upflow acrylic column (Height: 30 cm; OD: 3.0 cm and ID: 2.5 cm) having 100 ml working volume was fabricated. The column was packed aseptically with previously acclimatized and immobilized *P. alvei* MTCC 10625 up to bed height of 25 cm. The void volume of the column, packed with matrices, was found to be 70 ml. MS medium containing 100 mg/l Black WNN dye was pumped into the column using peristaltic pump (*Khera Instruments Pvt. Ltd. Delhi, India*) with a specific flow rate in the range of 6-7 ml/h. Decolorized sample was collected from the outlet periodically for 12 d and further assayed for decolorization, biodegradation and toxicity.

2.6. Dye Decolorization Assay

Decolorization measured as decrease in absorbance was expressed in terms of percentage. The decrease in absorbance was measured at λmax (597 nm). Decolorized sample (5 ml) was withdrawn after periodic interval of time, centrifuged at 10000 rpm for 15 min and then absorbance was measured. The uninoculated dye free medium was used as blank. All assays were performed in triplicate and compared with uninoculated control. The color removal efficiency of bacterial isolate was expressed as per following equation [25].

$$\text{Decolorization}(\%) = \left[(I - F)/I\right] \text{x} 100$$

Where, I = initial absorbance; F = final absorbance of decolorized sample

2.7. Biodegradation Assay

The decolorization of Black WNN dye by *P. alvei* MTCC 10625 was monitored using UV-vis spectrophotometer (*Spectronix, ST 2800, Germany*) and analyzed by the change occurred in the absorption spectra (200-800 nm). Metabolites produced after bacterial treatment, were extracted with equal volume of ethyl acetate and then dried over anhydrous sodium sulphate to remove the moisture content. Thereafter, the extracted metabolites were evaporated to dryness in a rotary vacuum evaporator (*Khera Instruments Pvt Ltd., Delhi*) and dissolved in small volume of methanol to concentrate the metabolite and used for FTIR and HPTLC analysis. For FTIR, extracted samples dissolved in methanol were dried and then mixed with spectroscopic pure KBr (1:300) to form a pellet and analysis was carried out by placing pellet in a sample holder of FTIR spectrophotometer (*Shimadzu, 8400S, UK*). Changes in frequencies (4,000-400 cm^{-1}) were observed [10].

HPTLC analysis was performed, using HPTLC system (*CAMAG, Switzerland*), by applying 5 µL of control dye and 10 µL extracted metabolites on the pre-coated silica gel plates (*TLC silica gel 60 F 254S, Merck Germany*) by micro syringe, using spray gas nitrogen semiautomatic sample applicator (*Linomat 5, CAMAG, Switzerland*). The dosage parameters for plates were set at 8 mm bands, 15 mm apart from Y axis. First application position was 10 mm from left edge. The composition of developing solvent system used as mobile phase was toluene: ethyl acetate: methanol (4:3:3). The twin trough chamber was pre-equilibrated with developing solvent for a period of 15-20 min prior to plate development. TLC plates were developed by placing in the twin trough chamber containing developing solvent until the desired running distance was attained and then dried with hot air blower for 5 min. After development, densitometric evaluation of spots was carried out at 254 nm and 366 nm using deuterium (D_2) and mercury (Hg) lamp, respectively with slit dimension 4x0.3 mm using CAMAG TLC Scanner-4 [28]. The chromatograms were integrated using HPTLC Wincats evaluation software.

2.8. Scanning Electron Microscopy (SEM)

For scanning electron micrograph, support matrices (PUF and NM) were collected from the continuous upflow column after the operation of 12 d. Matrices were fixed with 2% glutaraldehyde and 4% formaldehyde solution at room temperature for 1 h and subsequently washed with 0.15 M phosphate buffer (7.0) for 15min (3x15). Further, the fixed samples were dehydrated in graded series of ethanol solutions 30, 50, 70 and 90% (3x15 each) and 100% (1x15) for 15 min. The un-inoculated support matrices were also treated as per the described procedure [27]. Micrographs were taken on a scanning electron microscope (*JSM-6150, JEOL, JAPAN*).

2.9. Toxicological Assays

Phytotoxicity test was carried out on *Triticum aestivum* and *Vigna radiata* [28] the two common Indian agricultural crops, to assess the toxicity of Black WNN dye and its metabolites formed after decolorization by *P. alvei* MTCC 10625. Ten healthy seeds of *T. aestivum* and *V. radiata* were sown in the plastic pot containing garden soil. Toxicity study was carried out at room temperature by daily irrigating the pots with 10 ml of synthetic Black WNN dye (at 50 mg/l concentration) and degraded metabolites after bacterial treatment. Control was maintained by treating seeds with distilled water (daily 10 ml). Germination rate, length of plumule and radical, were recorded after ten days [29]. The microbial toxicity of dye control and treated dye metabolites (extracted and dried) were examined in relation to soil microbial flora; *Bacillus cereus*, *Bacillus subtilis* and *Kocuria rosea* MTCC 5932 were studied. Mid-log phase bacterial cultures were serially diluted and subsequently 10^{-3} dilution was inoculated on nutrient agar plate. Thereafter, two wells of 5.0 mm diameter were made on the nutrient agar plates. One well was filled with 0.1 ml of dye (control) and another with dye metabolites. Zone of inhibition (diameter in cm) surrounding the well represented the index of toxicity [3].

2.10. Statistical Analysis

Statistical methods such as standard deviation and standard error were employed using Microsoft Excel (version 2007 for Windows). Confidence level at 95% intervals was calculated for each set of samples to determine the margin of error.

3. Results and Discussion

3.1. Microorganism

On the basis of gram staining and other morphological characteristics, the isolated bacterial strain was recognized as a gram positive, off white in color, slightly raised, smooth, opaque, rod shaped (bacilli), non-motile and spore forming bacterium. Biochemical characterization substantiates that it's a starch hydrolysing bacteria and shows positive result for indole, methyl red, voges-proskauer, catalase and oxidase test. Moreover, this is a nitrate reducing and glucose fermenting bacterium, deposited via accession number *Paenibacillus* sp. MTCC 10625. Further, the bacterial isolate was identified as *Paenibacillus alvei* after molecular identification. *P. alvei* was found to be an efficient bacterial culture, decolorizes effectively a wide variety of textile dyes. Therefore, bacterial culture was selected to investigate the decolorization potential against Black WNN dye and routinely maintained on nutrient agar slants and preserved in glycerol stock at -20°C for further studies.

The complete 16S rRNA gene sequence of bacterial isolate NF23 (1457nt) was obtained. BLAST search results using the 16S rRNA gene sequence of isolate NF23 indicated that the similarities with members of the genus *Paenibacillus alvei* ranged between 99.2 and 99.5%, showed highest sequence similarities with *Paenibacillus alvei* CCM2B (99.4% score; GenBank entry: FN433032) and the next closest homologue was found to be *Paenibacillus* sp. (Genbank entry: GQ927307). Phylogentic tree was developed by Weighbour tree method, a weighted version of neighbor joining method

that gives significantly less weight to the longer distances in the distance matrix (Figure 1).

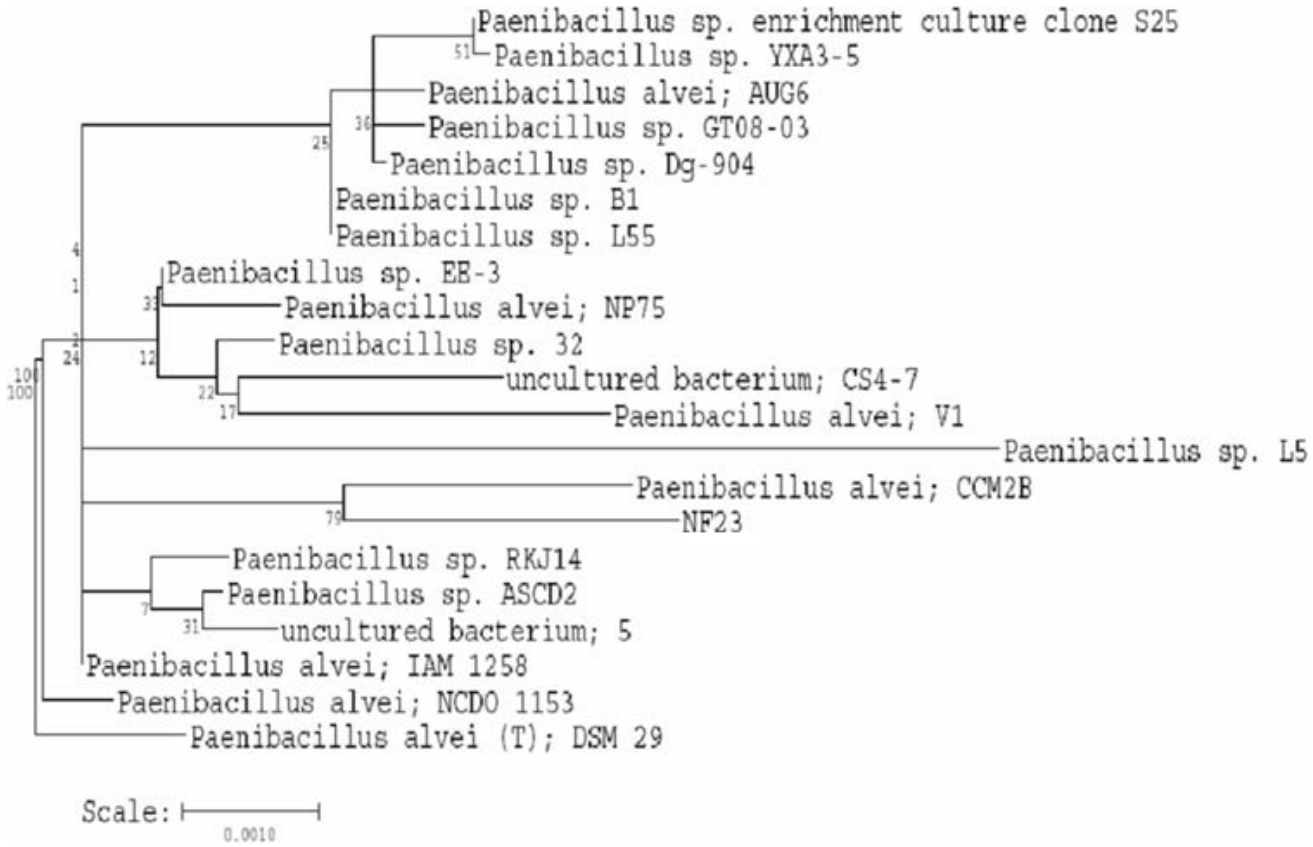

Figure 1. Phylogenetic tree of bacterial isolate NF-23 identified as *Paenibacillus alvei* MTCC 10625

3.2 Decolorization Experiments and Effect of Physicochemical Parameters

95.1% decolorization was achieved within 72 h under anoxic or microaerophilic condition, while it was 19.4% under aerobic condition at pH 7.3 and temperature 37°C (Figure 2a). *Klebsiella* sp. strain VN-31 [30] and an endophytic bacterium *Exiguobacterium profundum* strain 4 isolated from roots of *Amaranthus spinosus [17]* was able to decolorize 94.0% and 83.3% of Reactive Black 5 under static (anoxic) condition and no significant color changes were detected in the shaking condition. Similarly, *Staphylococcus hominis* RMLRT03 strain brought 85.5% decolorization of Acid Orange in static condition which was reduced to 32.4% in shaking condition [31]. Efficient dye removal in static (anoxic) condition has a great significance in terms of less energy consumption and stipulation of simpler reactor designing for scale up studies. Different researchers also suggested that a few bacteria under aerobic condition are unable to decolorize textile azo dyes, as it hampers the breakdown of azo dyes, by using oxygen as a terminal electron acceptor preferably in place of azo groups [11].

The optimum pH and temperature for Black WNN dye decolorization was 9.0 (Figure 2b) and 35°C (Figure 2c), respectively. A change in pH was observed during decolorization i.e. from 9.0 to 7.5, but the decolorization took place due to bacterial activity and not by change in pH. *Escherichia coli* and *Pseudomonas luteola* were reported for the complete decolorization and degradation of Reactive Black B, Remazol Blue and Reactive Red RB at pH 7.0 with consistent decolorization up to pH value 9.5 [13]. *Sphingomonas paucimobilis* also decolorized Methyl Red at pH 9.0 [32]. Similarly, *Staphylococcus epidermidis* MTCC 10623 completely decolorized Black WNN [33] and Basic Red 46 [23] at pH 9.0. Decolorization of Navy Blue-3G by *Brevibacillus laterosporous* MTCC 2298 in broad pH (7.0-11.0) range corresponds to our results [25]. Bacterial isolates with permissiveness to a wide range of pH will be more adaptable and effective in dye decolorization of effluent with unpredictable alkaline pH range [34]. Further, temperature is another factor of great importance for the decolorization potential of bacterial isolates which affect their growth and physiological functioning. Bacterial cultures, such as *Aeromonas hydrophilla* [35], *Staphylococcus hominis* [31] and *Staphylococcus epidermidis* MTCC 10623 [21,33] revealed effective decolorization and degradation of Methyl Red (80%), Acid Orange (92.3%), Black WNN (97%) and Basic Red 46 (99%) at 35°C favors our results. Complete decolorization of Direct Blue 1 by *Marinobacter* sp. strain HBRA was observed at 37°C [36]. Joe et al. [37] reported decolorization of Remazol Black B by *Pseudomonas aeruginosa* CR-25 in the temperature range from 23-37°C, whereas at 40°C decolorization was inhibited.

Decolorization of Black WNN dye at different concentration (50-600 mg/l) by *P. alvei* MTCC 10625 investigated 98.0% dye decolorization at 50 mg/l after 96 h and showing reduction in decolorization to 96.7, 95.9, 94.8, 93.8, 88.2 and 78.0% at 100, 200, 300, 400, 500 and 600 mg/l (Figure 2d), whereas the decolorization of Black WNN by *Staphylococcus epidermidis* MTCC 10623 was limited to 68% at 250 mg/l dye concentration [33]. Decolorization of Reactive Black 5 by *Exiguobacterium*

profundum strain N4 was slightly affected (85.9% to 84.7%) owing to increase in dye concentration from 150 to 901 mg/l [17]. Bacterial strain *Rhizobium radiobacter* MTCC 8161 showed the decolorizing efficiency of 60-90%, with increasing concentration of Reactive Red 141 dyes from 100-500 mg/l [38]. In contradiction to our results, a sharp reduction was observed in decolorization efficiency of bacterial isolates *Alcaligenes faecalis* [13] and *Marinobacter* sp. strain HBRA [36] at higher concentrations of Reactive Orange 13 and Direct Blue 1; while complete decolorization (100%) was achieved at 50 and 100 mg/l concentration, respectively. In contrast to our results, Watanapokasin et al. [18] showed only 24% removal of Reactive Blue 4 (300 mg/l) with bacterial consortium of *Salmonella subterranea* and *Paenibacillus polymyxa* at 35°C, under anaerobic condition after 24 h incubation.

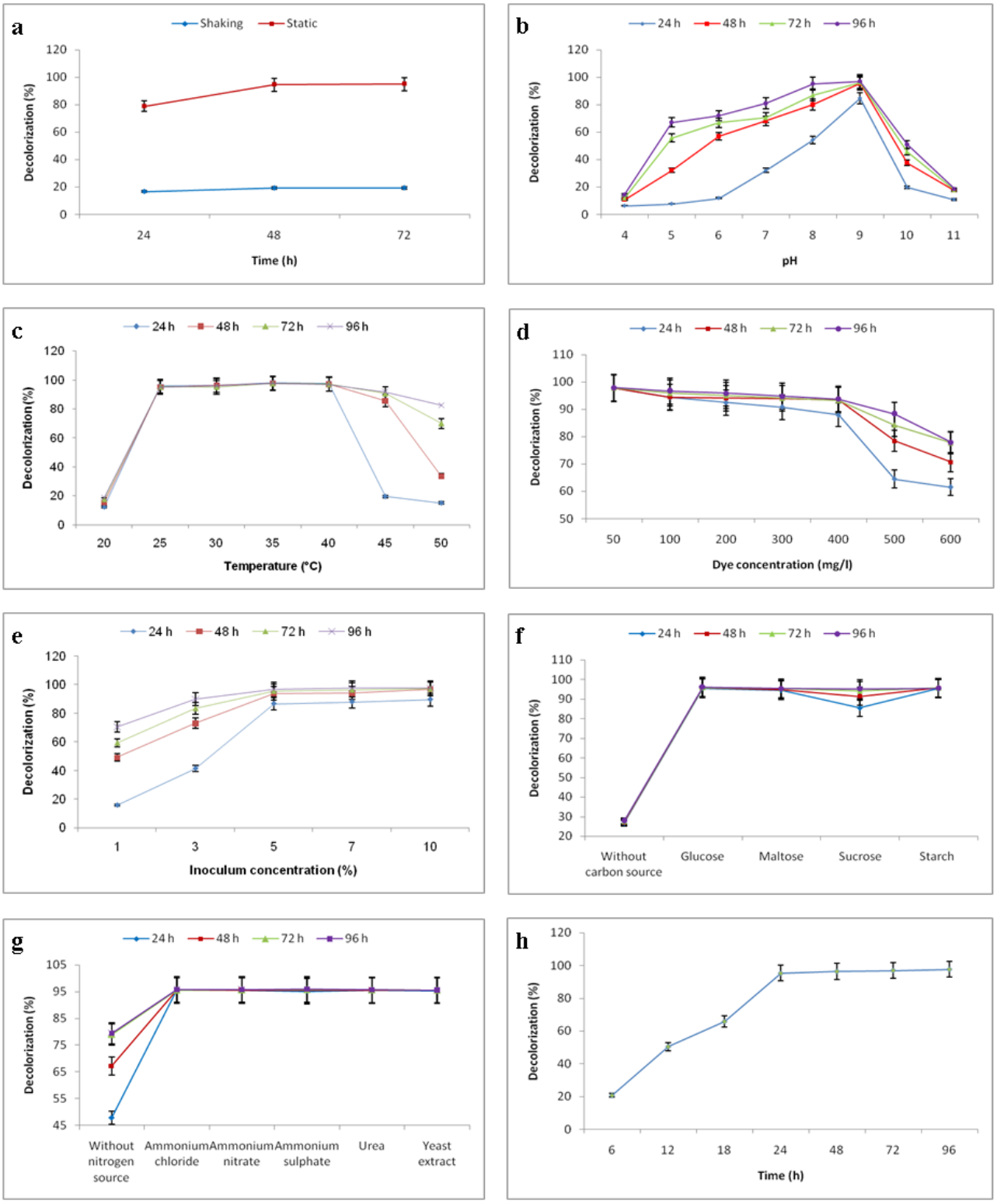

Figure 2. Effect of [a] growth conditions; [b] pH; [c] temperature; [d] dye concentration; [e] inoculum concentration; [f] carbon source and [g] nitrogen source on decolorization of Black WNN dye by *Paenibacillus alvei* MTCC 10625. Figure [h] illustrates the decolorization of Black WNN dye by *P. alvei* MTCC 10625 under optimized conditions (Temp.: 35°C, pH: 9, Dye conc.: 100 mg/l; co-substrates: glucose and ammonium sulphate and Growth condition: static)

A proportionate increase was found in decolorization of Black WNN dye with increase in inoculum concentration. Decolorization was gradually increased to 70.5, 89.8 and 96.9% with increased inoculum concentration of 1, 3, and 5%, whereas 97.6% decolorization was achieved with 7 and 10% inoculum showing no further increase in decolorization (Figure 2e). Similar findings were reported by other researchers for Black WNN (96.0%) [21] and Basic Red 46 (99.5%) [33] dye decolorization with 5% (v/v) inoculums of *Staphylococcus epidermidis* MTCC 10623. Furthermore, no significant difference in dye removal was found at 7 and 10% (v/v) doses of inoculum. Therefore 5% inoculum was optimal for further decolorization studies, because beyond this, decolorization was independent of increase in inoculum concentration. Similarly, 75.1% decolorization of Reactive Black 5 with 2 ml aliquot of *Enterobacter* sp. GY1 progressively increased to 79.0, 81.0, with 4 and 6 ml of culture aliquot within 24 h, but no significant change was observed with 8 and 16 ml culture [5]. Moreover decolorization of Orange II (86.9%) and textile effluent (72%) was evident with 4% inoculum dose of *Pseudomonas* SKG-1 [39], *Bacillus subtilis* and *Streptococcus faecalis* [40] inoculum in 48 and 36 h respectively. Furthermore, these authors did not find significant difference in decolorization 6, 8, and 10% (v/v) doses of inoculum.

The concurrent increase in bacterial growth along with dye decolorization by the supplementation of carbon and nitrogen sources attributed the fact that dyes are lacking free carbon content, and initialization of biodegradation of textile azo dyes necessitates the addition of any extra carbon source in the medium [41]. Decolorization of Black WNN dye was enhanced by the addition of different co-substrate. Only 26.6% decolorization was attained in MS medium without any carbon source that enhanced to 95.6, 95.3, 94.5 and 85.4% in MS medium substituted with glucose, starch, maltose and sucrose, respectively after 24 h and further increased to 96.3% with glucose and more than 95.0% with all other carbon sources after 96 h (Figure 2f). This shows that this microorganism can also utilize starch as a replacement for glucose as a cheaper co-substrate. Glucose is an easily metabolizable carbon source, which not only acts as a reducing agent for dyes, thereby increasing dye decolorization, but also promotes bacterial growth by affecting biochemical processes and metabolism. Similar to our observation, Wang et al. [16] reported 96.2% decolorization of Reactive Red 180 anaerobically by *Citrobater* sp., when added with glucose at 4 g/l concentration. Garg et al. [39] and Pokharia and Ahluwalia, [21] also supplemented glucose as carbon source for decolorization of mono azo dye Orange II and Basic Red 46 by *Pseudomonas putida* SKG-1 and *Staphylococcus epidermidis* MTCC 10623 respectively. Decolorization of Black WNN dye was >95.0% with all nitrogen sources (ammonium chloride, ammonium nitrate, ammonium sulphate, urea and yeast extract) at 0.1% (w/v) including organic and inorganic nitrogen source; while 47.8% decolorization was found without nitrogen source after 24 h incubation (Figure 2g). This is inferred from the results that the decolorization process is not affected by the chemical nature of nitrogenous compound, whereas Ramya et al. [42] had revealed that decolorization was strongly inhibited by incorporation of inorganic sources (sodium nitrite and ammonium chloride) in the medium. Similarly, Garg et al. [39] reported the maximum decolorization of Orange II containing effluent supplemented with ammonium sulphate (92.8%), while other nitrogen sources may be arranged in the following decreasing order of dye decolorization: ammonium nitrate (70.6%) > ammonium chloride (39.4%) > peptone (30.1%). Further, under optimized conditions 95.2% decolorization was examined after 24 h that increased to 97.6% after incubation of 96 h (Figure 2h).

Thus, the strain under study, *P. alvei* demonstrated significant potential to decolorize the dye as compared to the strains reported by various research groups.

3.3. Decolorization of Black WNN Dye by Immobilized *P. alvei* MTCC 10625 in Batch, Fed Batch and Continuous up Flow Column System

Percent decolorization of Black WNN dye was investigated with *P. alvei* MTCC 10625 immobilized over PUF and NM support matrices and achieved 95.2% and 97.0% decolorization, respectively under static condition within 24 h. It was increased to 97.6% and 97.8% with extended incubation of 96 h in batch system (Figure 3a). Several researchers had also used polyurethane foam (PUF) for the immobilization of microbial consortium [43], *Pseudomonas oleov*orans [27] and fungi in submerged cultures [44]. This study further demonstrated that *P. alvei* MTCC 10625 showed effective decolorization with repetitive addition (Fed-batch system) of Black WNN dye up to 8 d. Decolorization was nearly 96.0% for the five consecutive additions of 100 mg/l of Black WNN dye under optimized conditions, without adding nutrient, during the process by *P. alvei* MTCC 10625 immobilized over PUF and NM, respectively. However, decolorization gradually decreased to 83.8% and 86% after eight cycles; it might be due to decrease in viable cell count and exhaustion of nutrients (Figure 3b). Decolorization of Reactive Red BLI (49.0-90.0%) and Reactive Red 141 (74.0-90.0%) in the medium up to twelve cycles [45] and six consecutive cycles of dye [38] were investigated. Khan et al. [46] reported decolorization of Reactive Red 195 (100 mg/l) was consistent to three consecutive cycles of repetitive addition, which gradually decreased by fourth and fifth cycle and decreased to 50.0% after sixth cycle. *Lysinibacillus* sp. RGS immobilized on loofa showed complete decolorization of Blue HERD dye up to fifth cycle in upflow column bioreactor, consequently reduced to 62.0% after fifth cycle [47]. The decrease in decolorization performance was occurred after few cycles by immobilized bacterial cultures because repeated addition of textile dyes might have imparted toxic effects to the immobilized cells and may have entered in to stationary phase.

A continuous up flow column packed with *P. alvei* immobilized over PUF and NM was utilized to evaluate the decolorization potential of *P. alvei* for twelve days. Black WNN dye at concentration of 100 mg/l in synthetic dye effluent substituted with glucose was fed into the column packed with immobilized PUF and NM matrices at the rate of 6.5 and 6.35 ml/h, respectively and achieved 86.5% (PUF) and 85.4% (NM) decolorization after 12 h (Figure 4). Khehra et al. [48] described an up flow fixed-film column (anoxic-aerobic sequential) bioreactor using a

bacterial consortium based on four acclimatized bacterial strains as *Bacillus cereus, Pseudomonas putida, Pseudomonas fluorescence* and *Stenotrophomonas acidaminiphila* isolated from waste disposal sites of textile processing industries, immobilized on PUF capable of completely synthetic effluent containing 100 mg/l of Acid Red 88 dye with a flow rate of 7 ml/h with HRT of 12 h. The effluent released from up flow column reactor (UFCR) was transferred to continuously stirred aerobic reactor (CSAR) and the sequential anoxic-aerobic treatment of synthetic dye wastewater resulted in the 98.0% color removal. Chen et al. [49] achieved decolorization efficiency of 86.3±4.2% at 50 mg/l concentration of MG with immobilized (in calcium alginate bead) *Pandoraea pulmonicola* YC32 loaded with $5x10^6$ cell/g-bead packed in a continuous column system, when the flow rate was lower than 2 ml/min. Silveira et al. [27] also investigated complete degradation of an azo dye B-15 (25 mg/l) containing synthetic effluent in an anoxic up flow column reactor packed with *Pseudomonas oleovorans* (approximately $9x10^9$ cells/100 ml) immobilized on PUF in only 24 h in comparison to fed batch decolorization of 50 mg/l of dye after a long incubation period of 192 h. It was observed that in addition to using a lower concentration than the ones used in batch and fed-batch reactors, 25 and 50 mg/l, respectively, the reactor was able to decolorize a higher dye concentration with a reduced process time. The color removal in several sequential reactors is basically related with the anaerobic stage, where the further decolorization in the aerobic stage is normally limited to a few percent. According to Khehra et al. [48] the use of an anoxic-aerobic sequential bioreactor in the process, making it much needed to use a second reactor to reach complete breakdown of textile dyes and toxic by- products. Whereas our results showed that a second aerobic stage is not necessary, because *Paenibacillus alvei* was enough potential to decolorize the Black WNN dye in synthetic effluent at higher concentration in only on aerobic stage process supported by Silveira et al. [27].

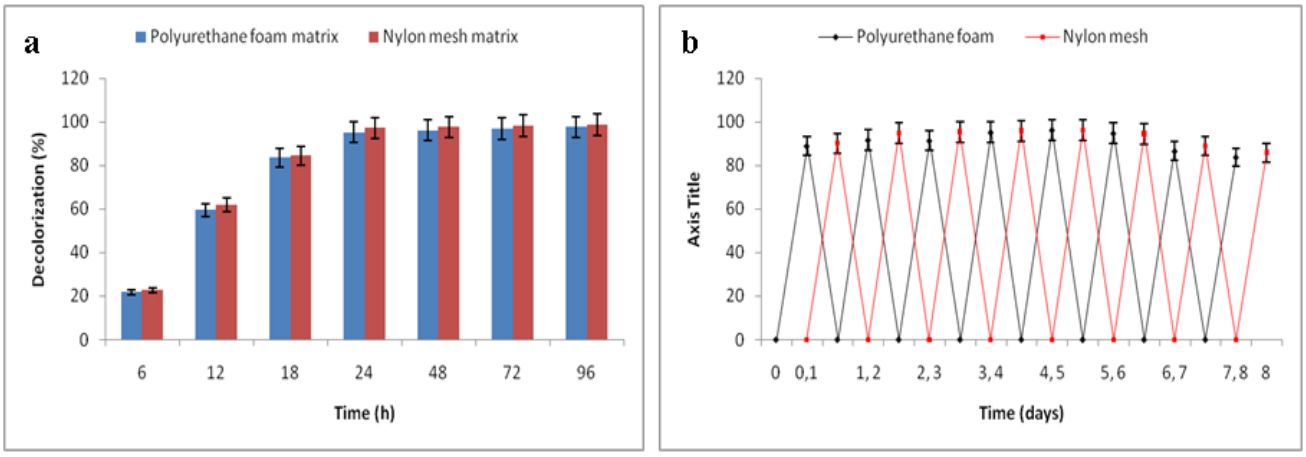

Figure 3. Decolorization of Black WNN dye by *Paenibacillus alvei* MTCC 10625 immobilized on Polyurethane foam and nylon mesh matrix in [a] batch and [b] fed-batch system

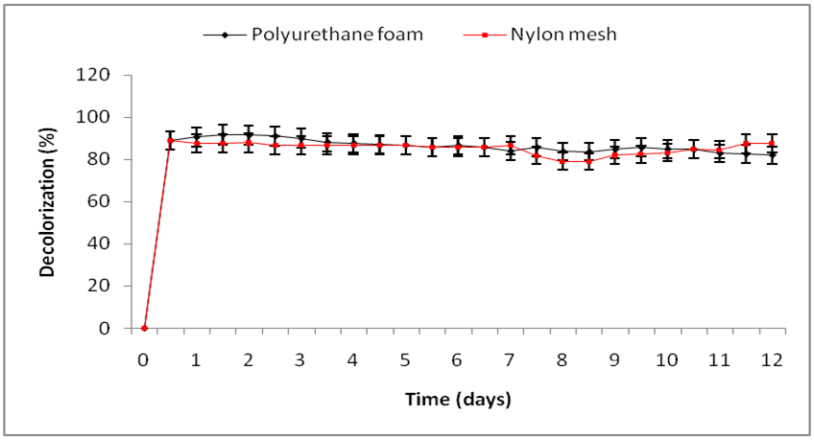

Figure 4. Decolorization of Black WNN dye by *Paenibacillus alvei* MTCC 10625 immobilized on Polyurethane foam and nylon mesh matrix in a continuous up flow column (100 ml) system

3.4 Biodegradation Analysis

Spectral analysis of Black WNN dye (λmax 597 nm) was carried out in the UV-visible range (200-800 nm) to examine the degradation of dye. Black WNN dye showed three absorbance peaks at 203, 259 and 597 nm (Figure 5a), whereas in decolorized sample the absorbance peak in the visible region (597nm) as well as in UV region (203 and 259 nm) completely disappeared after 24 h (Figure 5b) The disappearance of peak after 24 h clearly indicate the degradation of the native compound in the aerophilic conditions as compared to the observation in abiotic control. Decolorization of Black WNN dye is entirely by biodegradation, therefore no pigmentation or color adsorption occurs over bacterial cells; while in adsorption

process bacterial cells adsorb dye providing color to the cells [17]. In the adsorption process, absorption peaks decreased proportionately to each other, whereas in biodegradation, either the major peak in visible region completely disappears or gives rise to a new peak. Similar results were also observed with Reactive Red BLI dye [45], Reactive Black 5, Reactive Yellow 107, Reactive Red 198, Direct Blue 71 [30], Green HE4BD dye [3], Methyl Red [20] and Black WNN [33].

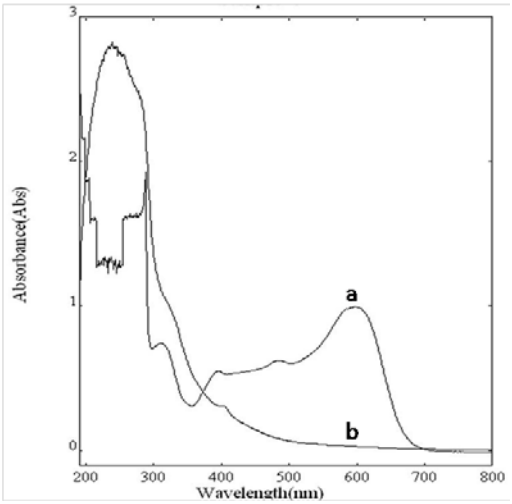

Figure 5. UV visible spectra of Black WNN during decolorization of Black WNN (100 mg/l) by *Paenibacillus alvei* MTCC 10625 [a] dye control and [b] decolorized sample

The significant difference in FTIR spectrum of Black WNN dye (Figure 6) with extracted metabolites clearly indicated the biodegradation of textile dye by *P. alvei.* Furthermore, the FTIR spectra of dye control displayed number of absorbance bands at 3432 cm^{-1}, 2924 cm^{-1}, 1595 cm^{-1}, 1496 cm^{-1}, 1413 cm^{-1}, 1226-1131 cm^{-1}, 1000 cm^{-1}, 892-845 cm^{-1}, 738-636 cm^{-1}, 637-520 cm^{-1} indicative of –NH ($–NH_2$), C-H, -N=N-, -C=N, -C-H, C-C (aromatic ring) and S=O stretching, C-N (aromatic ring), C-N (aliphatic), -C=O, $-NO_2$ and C-Cl (C-Br). On comparing the spectra with decolorized sample, the absorbance bands at 2924.15 cm^{-1} enlarged corresponding to C-H stretch and new absorbance bands at 3409.50 cm^{-1}, 2853.28 cm^{-1}, 1746.44-1664.42 cm^{-1}, 1463.46 cm^{-1}, 1261.36 cm^{-1}, 1096-1023 cm^{-1}, 802.37cm^{-1} and 720.63-703.63 cm^{-1} appeared that associated with –OH, -C-H, -C=O, $-CH_3$, C-O, cyclo-hexane ring and -C-N stretch (aromatic ring). The change in spectra obtained after bacterial treatment confirms the complete breakdown of dye molecule and associated shift in peaks of functional groups in comparison to dye control. Similar findings were reported after bacterial degradation of Orange II [39], Reactive Violet 5 [50], Reactive Blue 19 [51], Reactive Black B [52] and Reactive azo dyes [53].

HPTLC profile of degraded metabolites showed different degradation pattern with different R_f values in decolorized dye samples as compared to control Black WNN dye (0.72) and metabolites formed by *P. alvei* (0.54, 0.63, 0.69 and 0.72) indicated the biodegradation of Black WNN dye (Figure 7 A & Figure 7B). Change in R_f value of control and treated Black WNN dye samples in HPTLC profile confirms the transformation of parent compound into simpler ones. Similar findings were reported after bacterial degradation of Reactive Violet 5 [50], Reactive Blue 19 [3], Blue HERD [47], Reactive Black B [52] and Reactive azo dyes [53].

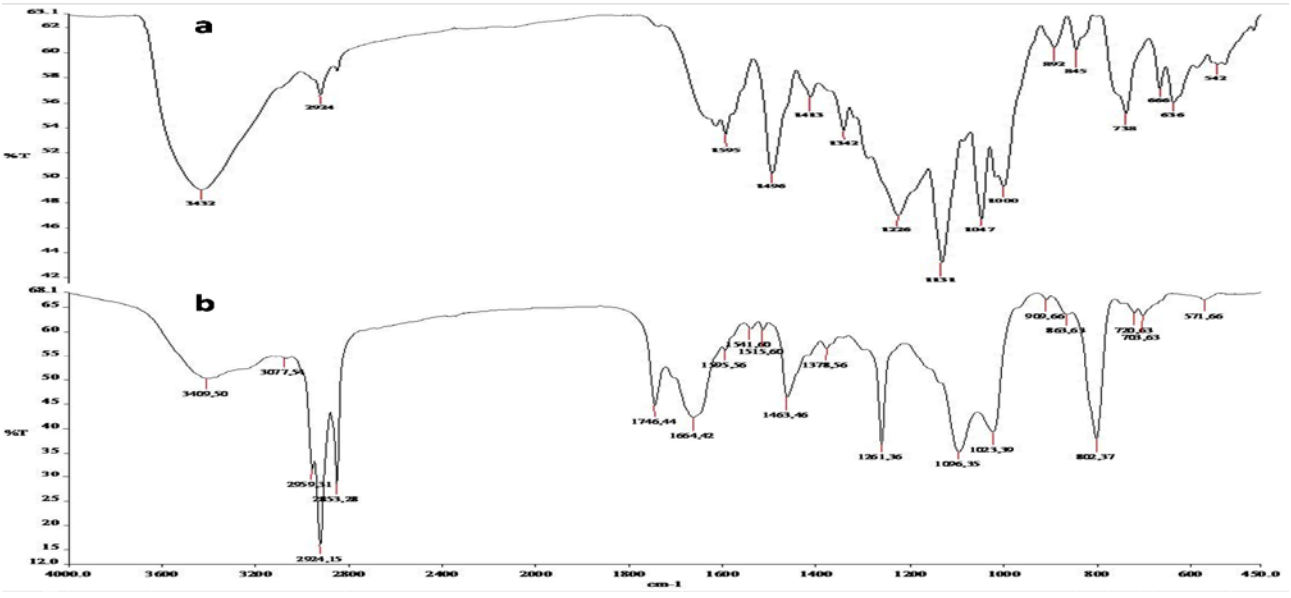

Figure 6. FTIR spectra of [a] Dye control and [b] decolorized sample after treatment with *Paenibacillus alvei* MTCC 10625

3.5. Scanning Electron Microscopy (SEM)

Microscopic observations were performed to evaluate the morphological characteristics of *P. alvei* MTCC 10625 immobilized over PUF and NM matrices during the process of decolorization of Black WNN dye in a continuous up flow column under optimal conditions. Pieces of matrices were collected from the column at the end of the decolorization process, which was studied up to 12 d. Matrices without bacterial biomass served as control (Figure 8a & Figure 8b) It is evident from the scanning electron micrographs, (Figure 8c & Figure 8d) that a contiguous layer of rod shopped bacteria affixed on the surface of matrices has the ability to decolorized xenobiotic Black WNN dye in 12 h. Development of bacterial biofilm on polyurethane foam [21,27,48] and nylon mesh [21] was evaluated by SEM analysis.

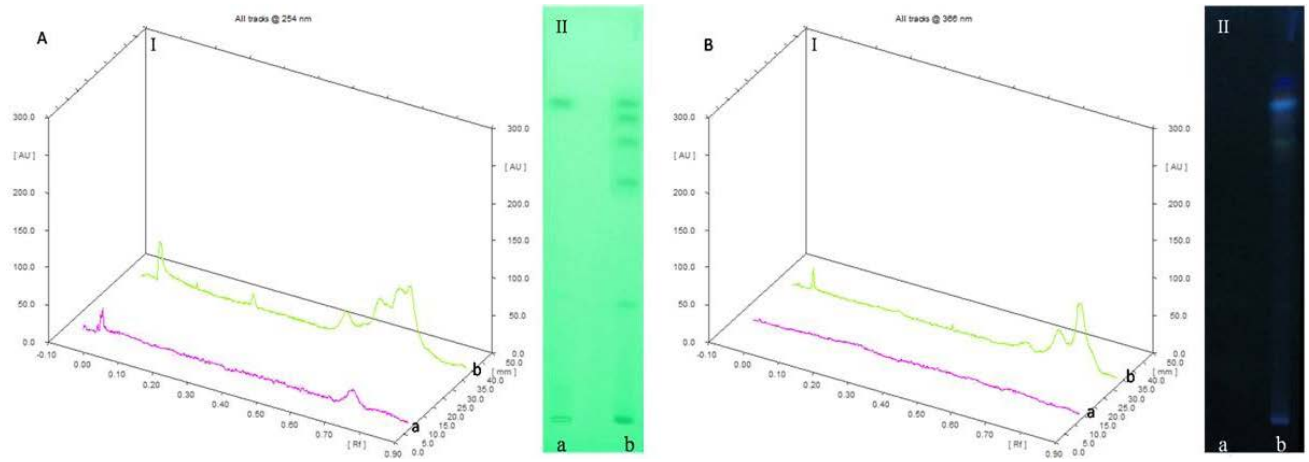

Figure 7. HPTLC 3-D chromatogram (I) and HPTLC plate (II) of control dye Black WNN [a] and its metabolites [b], obtained after decolorization by *Paenibacillus alvei* MTCC 10625 at 254 nm (A) and 366 nm (B)

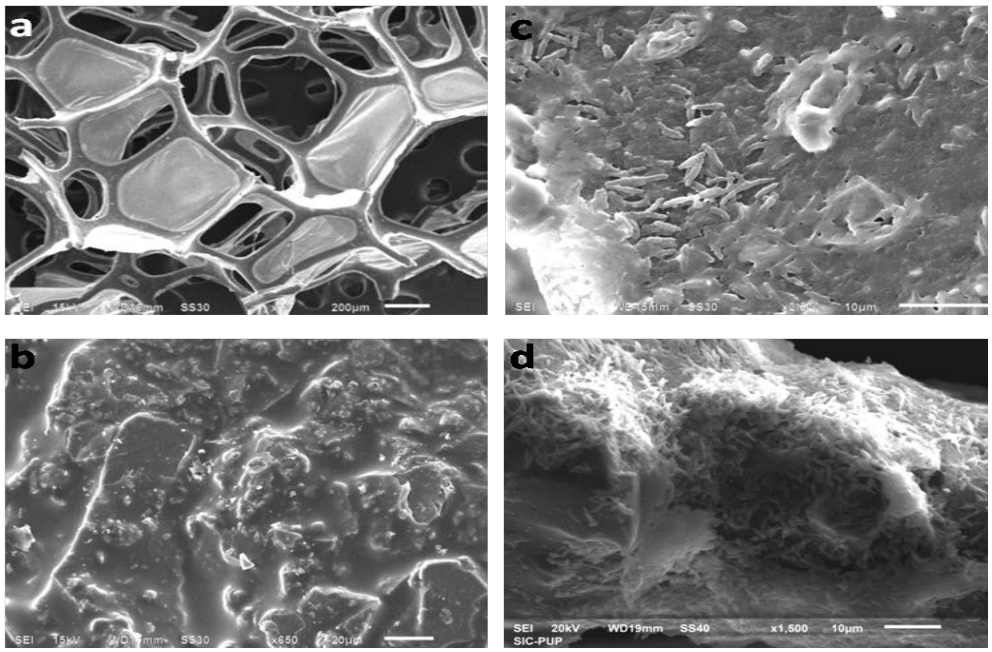

Figure 8. Scanning electron micrograph of support matrices (a) Polyurethane foam (b) Nylon mesh and (c) *P. alvei* MTCC 10625 immobilized on PUF (d) *P. alvei* immobilized on NM

3.6 Toxicological Assays

Sulfonated reactive azo dyes and their intermediate metabolites (sulfonated and non sulfonated metabolites) are major environmental pollutants released from textile industry and are high toxic [3]. Improper disposal of textile effluent, both in natural water reservoirs and/or agricultural land, affects the soil fertility and causes serious environmental and health hazard. Phytotoxicity and microbial toxicity studies were performed to check the toxic behavior of textile dyes and their metabolites as well as to explore the possible reuse of the treated solution into irrigation fields and recreation areas such as parks and playgrounds etc.

During phytotoxicity assay, it was observed that because of the toxic nature of Black WNN dye (100 mg/l) germination rate, length of plumule and radicle were short in case of *T. aestivum* and *V. radiata* seeds exposed to Black WNN dye (Figure 9A). However, insignificant difference was spotted in case of seeds exposed to distilled water and degraded metabolites (Table 1).

Table 1. Phytotoxicity analysis on *Triticum aestivum and Vigna radiata* using dye control (Black WNN dye) and decolorized samples after biodegradation with *Paenibacillus alvei* MTCC 10625.

Sample	Germination (%)	*Triticum aestivum*	
		Plumule (cm)	Radical (cm)
Water control	100	14.53±0.50	5.60±0.36
Black WNN dye	50	3.03±0.45	0.96±0.04
Treated decolorized sample	100	12.46±0.61	4.46±0.15
		Vigna radiata	
Water control	100	12.2±1.05	3.90±0.10
Black WNN dye	40	1.5±0.10	0.89±0.01
Treated decolorized sample	90	8.8±0.20	2.60±0.10

Similar results for phytotoxicity were observed during decolorization and biodegradation of Blue HERD, reactive azo dyes and Basic Red 46 after treatment with *Lysinibacillus* sp. RGS [47], bacterial consortia [53] and *Staphylococcus epidermidis* MTCC 10623 [21], respectively. Phytotoxicity studies conducted by various researchers on agricultural crops such as *Cicer arietinum*, *Hordeum*

vulgare, Oryza sativa, Phaseolus vulgaris, Sorghum bicolor, Sorghum vulgare, Triticum aestivum, Vigna mungo, Vigna radiata and *Zea mays*; revealed high germination rate and significant growth of plants irrigated with intermediate metabolic products of dyes formed after the decolorization, as compared to control textile dyes authenticate the significance of biological treatment [54]. This study also concluded the biotransformation of toxic dye into nontoxic metabolites, which had no adverse effect on the plant growth signifying the detoxification of dye.

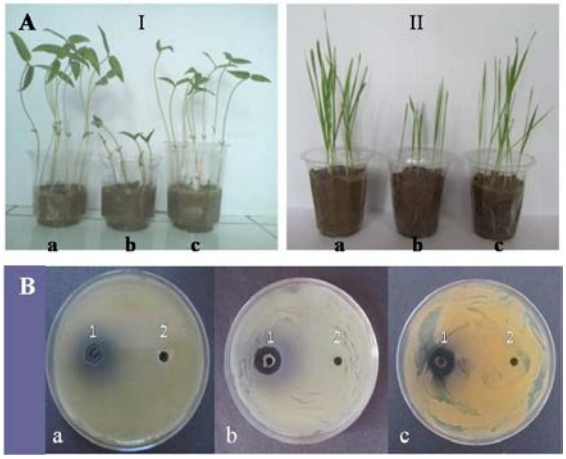

Figure 9. A. Phytotoxicity assay showing effect of a) water control b) Black WNN dye control and c) decolorized dye metabolites after treatment with *P. alvei* MTCC 10625 on [I] *Vigna radiata* and [II] *Triticum aestivum*. B. Microbial toxicity assay showing effect of 1) Black WNN dye control and 2) decolorized dye metabolites after treatment with *P. alvei* MTCC 10625 on [a] *Bacillus cereus* [b] *Bacillus subtilis* and [c] *Kocuria rosea* MTCC 5932

Further, microbial toxicity assay was executed to evaluate whether metabolites formed from the noxious waste after biotransformation had higher toxicity than the parent compound. In this study microbial toxicity test was conducted by exposing soil microorganisms (*Bacillus cereus, Bacillus subtilis* and *Kocuria rosea* MTCC 5932) to Black WNN dye and degraded metabolites to determine the potential of biological effect due to the presence of dye contaminant. Black WNN dye control had shown zone of inhibition of 1.40±0.20 cm, 1.10±0.10 cm and 1.44±0.04 cm with *Bacillus cereus, Bacillus subtilis* and *Kocuria rosea* MTCC 5932 (Figure 9B) respectively. In contrast, dye metabolites did not show any growth inhibition in all soil microorganisms. Researchers studied the toxic effect of azo dyes and their intermediate metabolites after bacterial treatment on plate assay with agriculture important cultures [5,12,16,30]. No zone of inhibition for soil microbes was observed in the degraded metabolites of Reactive Red and Green HE4BD by *Pseudomonas aeruginosa* [55] and *Proteus vulgaris* NCIM 2027 [3]. Zone of inhibition was investigated against bacterial species accessible in soil in the presence of a control azo dye and their metabolites, as some of the nitrogen and phosphate solubilizing bacteria have the stimulatory effect on plant growth [54].

4. Conclusion

Paenibacillus alvei MTCC 10625 efficiently decolorize 96% and detoxify Black WNN dye (Reactive Black 5) at pH 9. Moreover, under optimized conditions, *P. alvei* immobilized on polyurethane foam and nylon mesh decolorized 97.6% and 98.7% of Black WNN dye. FTIR, HPTLC and UV-Vis spectrophotometric analysis confirms the degradation of Black WNN dye. Further, the metabolites formed after decolorization is found to be non-toxic after the examination of microbial and phytotoxicity assay. Studies presented here demonstrate the success of immobilized *P. alvei* MTCC 10625 for the decolorization of dye in continuous column system established a fast, cost effective, eco-friendly technique for the application in bioremediation of textile effluent at commercial scale.

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Conflict of Interest

The authors have declared no conflict of interest.

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