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Degradation and detoxification of Methylene Blue dye adsorbed on water hyacinth in semi continuous anaerobic-aerobic bioreactors by novel microbial consortium-SB

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Abstract

Combinatorial adsorption-biodegradation treatment of textile wastewater provides a cost effective and ecofriendly alternative to conventional physicochemical treatment methods. Water hyacinth plant powder (WHPP) was used for adsorption of cationic dye Methylene Blue. Adsorption of Methylene Blue on WHPP was found to follow pseudo second order kinetics. The toxicity, chemical oxygen demand (COD), biological oxygen demand (BOD), alkalinity and hardness of filtrate were significantly reduced after adsorption of Methylene Blue on WHPP. The decolorization of dye adsorbed on WHPP was performed in semi continuous anaerobic sludge bioreactor and aerobic trickling bed bioreactor. The percentage of dye removal was comparatively higher in single anaerobic treatment while significant reduction in COD, BOD, alkalinity, hardness and toxicity were observed in semi continuous aerobic treatment (flow rate 80 mL h^{-1}) than in anaerobic treatment. Degradation of Methylene Blue after semi continuous anaerobic-aerobic treatment was confirmed by HPLC analysis.

Keywords- Decolorization, Methylene Blue decolorization, Water hyacinth plant powder, Anaerobic sludge bioreactor, Trickling bed bioreactor, Nuclear and chromosomal aberration analysis

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1. Introduction

The textile industries have huge economic importance by virtue of their involvement in industrial output and employment generation. During textile processing number of dyes, sizing materials and auxiliary chemicals are used. Loss of dyes in textile processing varies from 2 to 50%. If the effluent generated during textile processing is not treated properly before its disposal into water bodies, it can cause severe ecological harms.

While comparing different available technologies for textile effluent treatment, adsorption is found to be an economical, rapid and widespread method of color removal [1]. Activated carbon, coal and fly ash are some examples of adsorbents [2] which have been previously used for removal of dyes from effluent. Natural adsorbents have been reported as effective and cheaper textile dye adsorbents. Wheat straw, water hyacinth, corn cob shreds and wood chips are some natural textile dye adsorbents reported earlier [3, 4].

Though adsorptive removal of textile dyes is a reasonable process, this process does not completely eliminate these harmful textile dyes from the environment [5]. In contrast, combination of physical and biological method, i.e. adsorption and biodegradation of adsorbed dyes assures safe, economic and ecofriendly way of complete removal of textile dyes from the environment [6]. During degradation of dyes using biological processes, conventional aerobic treatments do not always achieve satisfactory color removal [7], on the other hand, though anaerobic treatments results in higher decolorization of textile dyes, formation of aromatic amines is a major problem of this treatment [8]. To overcome these problems, sequential anaerobic-aerobic treatment for decolorization of textile dyes and simulated effluent have been reported [8, 9, 10].

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In the present study, combinatorial adsorption and biodegradation methods were used to treat heterocyclic aromatic dye Methylene Blue. Dried powder of water hyacinth plant was used as a natural adsorbent for adsorption of Methylene Blue. Optimization of Methylene Blue (781 mM) adsorption capacity of water hyacinth plant powder (WHPP) was performed. Adsorbed Methylene Blue dye was treated biologically using individual cultures of *Saccharomyces cerevisiae* MTCC 463 (*S. cerevisiae*), isolated *Bacillus* sp. STIS and developed consortium of *S. cerevisiae* and isolated *Bacillus* sp. STIS (consortium-SB) in anaerobic and aerobic conditions for its complete removal from environment. Scale up of the adsorption-biodegradation process has been done in continuous anaerobic sludge bioreactor (ASBR) and aerobic trickling bed bioreactor (TBBR). In anaerobic phase, per cent dye removal at different operation times (h) and organic loading rates (OLR) was detected, while in aerobic phase per cent dye removal at different flow rates was checked. Complete removal of Methylene Blue was achieved in three major steps *viz*. adsorption by WHPP, anaerobic decolorization of adsorbed dye and complete decolorization of remaining desorbed dye from WHPP in aerobic phase. Toxicity of filtrate, products formed after anaerobic decolorization and anaerobic-aerobic decolorization were evaluated by nuclear and chromosomal aberration assay of root cells of *Allium cepa* and phytotoxicity test using *Phaseolus mungo* and *Sorghum vulgare*. Though there are many reports on adsorption of dye on water hyacinth [11, 12, 13]; degradation of adsorbed dye is also important factor from environmental point of view. This study is mainly focused on degradation of Methylene Blue adsorbed on water hyacinth along with toxicity studies of filtrate obtained after adsorption and products formed after anaerobic and anaerobic-aerobic treatment.

2. Materials and Methods

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2.1 Materials

All chemicals used for this study were highly pure and of analytical grade. Methylene Blue dye was obtained from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Methanol was obtained from S D Fine-Chem Ltd., India. Microbiological media such as nutrient broth, peptone, yeast extract and meat extract were obtained from HiMedia Laboratories Pvt. Ltd., Mumbai, India.

Water hyacinth plants (collected from Rankala Lake, Kolhapur, India) were keenly washed with tap water and dried in sunlight. After complete drying, whole plants were ground finely and sieved using Micro-Mesh sieves (Industrial Netting, USA) to obtain powder of desired particle size i.e. 0.02 mm.

2.2 Investigation and optimization of adsorption capacity of WHPP

In batch scale experiments, 1-5 gm dL^{-1} of WHPP was added to 50 mL solution of Methylene Blue (781mM) in five different 100 mL Erlenmeyer flasks. After agitation (at 120 rpm for 20 min), solution from each flask was filtered through Whatman filter paper no. 42. The filtrate obtained was centrifuged (5000 rpm for 15 min) and the intensity of supernatant was measured at maximum absorbance wavelength (664 nm) of Methylene Blue using UV-visible spectrophotometer (Hitachi U-2800, Japan). Dye adsorbed on WHPP was calculated in terms of percentage using following equation.

$$\% \text{ Adsorbed dye} = [(A_0 - A)/A_0] \times 100$$

Where, A_0 is the absorbance of dye sample before addition of WHPP and A is the absorbance of sample after adsorption of Methylene Blue on WHPP.

2.3 Investigation of adsorption kinetics during adsorption of Methylene Blue dye on WHPP

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Infrared spectrum of WHPP was obtained by using Shimadzu 8400S spectrophotometer in the mid-infrared region of 400–4000 cm^{-1} with 16-scan speed [14].

Possible kinetics mechanism behind adsorption was investigated using initial dye concentrations of (40, 80, 120, 160 and 200 mg L^{-1}) with agitation time (5, 10, 15, 20, 25 and 30 min) and pseudo-first order and pseudo-second-order kinetic models were studied. The amount of dye-adsorbed q_e (mg gm^{-1}) was determined by earlier reported equation [15].

$$q_e = \frac{(C_i - C_f)}{W} \times V \quad (1)$$

Where, C_i and C_f are the initial and final dye concentrations at equilibrium in the aqueous phase (mg L^{-1}) respectively, V is volume of dye solution (L) and W is the mass of WHPP (gm) employed.

2.4 Characterization of adsorption by density functional theory method

Characterization of adsorption was done by analyzing surface area and pore volume of adsorbent i.e. WHPP before and after adsorption of Methylene Blue using density functional theory (DFT) method (Instrument - Quantachrome NovaWin) at temperature 77.350 K using nitrogen gas as adsorbate.

2.5 Microorganisms and culture conditions

For aerobic as well as anaerobic decolorization of Methylene Blue, facultative anaerobic bacteria and yeast were selected. Bacterial cultures were isolated from no-till soil (soil depth-around 100 mm) collected from textile waste disposal site of Shriram textile industry, Solapur, India. Soil sample (200 mg) was added to 250 mL Erlenmeyer flasks containing 100 mL of nutrient broth with different concentrations (ranging from 100 to 800 mM) of Methylene Blue in

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each flask. After 48 h of incubation, 1 mL of culture from these flasks was transferred into fresh nutrient broth containing the same concentrations of dye respectively for enrichment of the soil micro-flora. After complete decolorization, diluted sample (10^{-4} fold) from each flask was transferred to nutrient agar plates augmented with Methylene Blue (concentrations ranging from 700-800 mM). Colonies showing decolorized zones were selected and isolated using streak plate method. Decolorization efficiency of isolated bacteria (Iso-2, Iso-5 and Iso-7) was checked individually for Methylene Blue in nutrient broth. The isolate (Iso-5) best suited for decolorization was identified using 16S rRNA sequence analysis. The nucleotide sequence alignment of the sequence was performed at Blast-n site at NCBI server(http://www.ncbi.nlm.nih.HYPERLINK‘‘http://www.ncbi.nlm.nih.gov/BLAST’’gHYPERLINK‘‘http://www.ncbi.nlm.nih.gov/BLAST’’ov/BLAST). The alignment of the sequences was done using CLUSTALW program V1.82 at European bioinformatics site (http://www.ebi.ac.uk/clustalw). The bootstrap consensus tree inferred from 1000 replicates and was considered to represent the evolutionary history of the analyzed taxa. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates. MEGA 4 software was used to construct phylogenetic tree using aligned sequence (Fig. 1). The sequence was developed manually after crosschecking with the raw data to eliminate uncertainties and then submitted in GenBank databases and was identified as *Bacillus circulans*.

The 16S rRNA gene sequence of the isolated *Bacillus* sp. STIS was submitted to the GenBank database under accession number KR029828. Stock culture of isolated *Bacillus* sp. STIS was maintained on nutrient agar slants at 4 ^{o}C. This species has been reported to be facultative anaerobic microorganism [16].

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The microbial culture of *S. cerevisiae* was obtained from the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India and pure culture was maintained on malt yeast agar slants (gm L^{-1}; malt extract 3, yeast extract 3, peptone 5, and glucose 10) at 4 ^{o}C. *S. cerevisiae* is known to be a facultative anaerobe and hence was selected for the study. Degradation of Malachite green using *S. cerevisiae* under anaerobic conditions has been reported earlier [17].

Consortium of *S. cerevisiae* and *Bacillus* sp. STIS was prepared aseptically by transferring the surface culture biomass of *S. cerevisiae* grown in 100 mL malt extract medium for 24 h at 30 ^{o}C to the 24 h old culture of *Bacillus* sp. STIS grown at 30 ^{o}C in 100 mL nutrient broth.

2.6 Flask level decolorization experiments

To assess decolorization potential of individual strains, 1.5 mL inoculum from pre grown culture of *Bacillus* sp. STIS and surface culture biomass of *S. cerevisiae* (approximately 1 gm) grown for 24 h at 30 ^{o}C in their respective medium was added in the 400 mL aspirator bottle holding 781 mM Methylene Blue adsorbed on 3 gm WHPP. Nitrogen gas was sperged to remove oxygen from bottle and maintain anaerobic conditions [18]. Similar procedure was followed to evaluate anaerobic decolorization capacity of consortium-SB. Remaining dye after anaerobic decolorization was desorbed from WHPP using method described by Mallampati et al [19]. For desorption, samples were prepared by adding 0.3 gm WHPP used for adsorption and anaerobic decolorization to 10 mL of double distilled water. After continuous stirring for different time periods, samples were analyzed at different pH values (4, 7 and 10) and at different time intervals (5, 10, 15 and 20 min). The Methylene Blue desorbed was measured with a UV-visible

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spectrophotometer. To check reproducibility of results obtained, each experiment was repeated twice under identical conditions.

The remaining desorbed dye sample from WHPP was treated aerobically using suspended as well as immobilized cultures of *Bacillus* sp. STIS and *S. cerevisiae*. To study decolorization of suspended cells, 1.5 mL inoculates from pre grown culture of *Bacillus* sp. STIS and surface culture biomass of *S. cerevisiae* (approximately 1 gm) were added to desorbed Methylene Blue dye sample and decolorization performance was checked up to 24 h at 30 ^{o}C. Similar experiments were carried out using consortium-SB.

2.7 Immobilization of consortium-SB on coconut leaf sheath

Dried coconut leaf sheath has high mechanical strength and fiber-mesh support. The arrangement and sturdiness of fibers in coconut leaf sheath qualifies it as a suitable matrix for immobilization. For immobilization of consortium-SB, outer coarse fibers were separated from the sheath and inner compact mat of fibers was used. Dried coconut leaf sheath (CLS) was cut into pieces (2 cm x 2 cm in size) and soaked initially in boiling water for 30 min and then in distilled water for 24 h. The moist pieces following sterilization (at 121 ^{o}C for 15 min) were oven dried at 70 ^{o}C and stored in desicator for further use. 24 h old inoculum of consortium-SB was then added to 10 pieces of CLS used as an immobilization matrix and further incubated (30 h) for growth and entrapment of the cells (30 ^{o}C). CLS pieces were then removed from the flasks and thoroughly washed with fresh culture medium to remove the unentrapped cells, and transferred into 250 mL Erlenmeyer flask containing 100 mL fresh nutrient medium+Methylene Blue (781 mM) solution for the decolorization experiments. All steps were done under aseptic conditions. Culture flasks containing CLS pieces in the medium without inoculum served as the

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control. Microscopic examination revealed that microbial cells were entrapped in the CLS within 48 h (data not shown).

To determine per cent decolorization using individual culture, consortium-SB and CLS immobilized consortium-SB, aliquots (2 mL) of the culture broths were sub sampled after regular time intervals. For separation of cell mass, the aliquots were centrifuged (3000×g for 15 min). Decolorization of Methylene Blue was measured by calculating change in the absorbance of supernatant at its maximum absorption wavelength (664 nm). All decolorization experiments were carried out in triplicates.

2.8 Large scale process optimization for adsorption of Methylene Blue on WHPP

To check large scale adsorption efficiency of WHPP, the method described previously was applied using 1 L of Methylene Blue (781 mM) containing distilled water and 30 gm of WHPP (section 2.2). The toxicity of filtrate obtained was checked using nuclear and chromosomal aberration analyses and phytotoxicity studies. Different environmental parameters (COD, BOD, alkalinity and hardness) have also been checked.

Methylene Blue adsorbed WHPP was used for decolorization experiments.

2.9 Decolorization of Methylene Blue adsorbed on WHPP by semi-continuous anaerobic-aerobic bioreactor

Methylene Blue adsorbed on WHPP was treated in semi continuous anaerobic-aerobic conditions to achieve its complete decolorization and degradation. Anaerobic sludge bioreactor and aerobic trickling bed bioreactor were developed to treat Methylene Blue.

2.9.1 Development of ASBR for decolorization study

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The lab-scale bioreactor for anaerobic decolorization of Methylene Blue adsorbed on WHPP was designed using 1 L glass aspirator bottle. The bottle dimensions were: external diameter 107 mm; total height 200 mm; total volume 1220 ml; liquid volume 1 L and ground size neck 29/32 NS. Three different aspirator bottles (A, B and C) were used to carry out anaerobic decolorization experiments at different hydraulic retention times (HRTs). Each bottle was filled with 30 gm of WHPP on which Methylene Blue was adsorbed, and then sterilized. 100 mL culture of consortium-SB was added to each aspirator bottle. Anaerobic conditions were maintained as mentioned earlier (section 2.6). Manual mixing of reactor contents have been done after every 6 h by manual shaking for adequate mixing of the reactor content as well as hydraulic conditions [20].

The samples were then withdrawn from ASBR A, B and C after 8, 16 and 24 h, respectively. Samples collected were further scanned against untreated Methylene Blue solution. WHPP solution (3 gm dL^{-1}) was kept as a blank. Organic loading rate (OLR) was calculated by diving chemical oxygen demand (gm mL^{-1}) by time [21]. The ASBR in which maximum decolorization was observed was repeated two times to check reproducibility of results.

The remaining dye after anaerobic decolorization was desorbed and used to check HPLC profile, status of environmental parameters (COD, BOD, alkalinity and hardness), and toxicity analyses. Remaining desorbed dye sample was autoclaved and used for aerobic decolorization.

2.9.2 Development of TBBR for aerobic decolorization of desorbed dye

The desorbed dye from ASBR was treated in TBBR. TBBR was made up of square shaped plastic box (Tarsons product private limited, India) having the length 22.5 cm, width 22.5 cm and the height was 5 cm. The box was sterilized using ethanol. Thoroughly washed, pre

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autoclaved CLS pieces (5 cm x 5 cm) were spread at the bottom of box in laminar air flow. Autoclaved silicon rubber tube was fixed to lid of box, one end of which was adjusted at the outer side of box as an inlet the other end of the tube was sealed. The tube had pores (size 0.2 mm) at a distance of 2 cm from each other to facilitate trickling of sample to be treated inside the box (Fig. S1). A 24 h old inoculum of consortium-SB (20 mL) was added to the coconut waste material placed in the TWBR in sterile conditions and reactor was incubated at 37 ^{o}C. After 24 h of incubation, the desorbed dye sample from ASBR was trickled on the immobilized culture using a peristaltic pump at different flow rates *viz.* 40, 60 and 80 mL $h^{-1.}$ Per cent decolorization was measured as described earlier (section 2.7). The decolorized samples were collected from outlet of TBBR and used for further analyses.

2.10 HPLC and environmental parameters analyses

HPLC analysis of filtrate obtained after adsorption and samples obtained after anaerobic and anaerobic-aerobic treatment were performed using C18 column (symmetry, 4.6 × 250 mm) equipped with dual wavelength PDA detector (SPD-M A, Shimadzu, Japan) in isocratic method. The methanol was used as a mobile phase at flow rate 0.75 mL min^{-1} and run time 10 min. 10 µL of sample volume was manually injected into the injector port [22].

Also, COD, BOD, alkalinity, and hardness of the same samples were checked by the method reported earlier [23].

2.11 Evaluation of toxicity

Xenobiotic compounds are known to be toxic in nature, and sometimes they form toxic metabolites after degradation [24]. Hence, evaluation of toxicity of contaminant individually or in mixture along with their metabolites is needed [9]. Toxicity of filtrate and samples of

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anaerobic and anaerobic-aerobic treatment were checked by determination of nuclear and chromosome aberrations and phytotoxicity.

2.11.1 Nuclear and chromosome aberrations assay

Nuclear and chromosomal status of *Allium cepa* (*A. cepa*) root cells were tested upon to check toxicity of Methylene blue, filtrate and degraded samples. Healthy bulbs of onions weighing 15–20 gm each, were exposed to diluted Methylene Blue dye solution (400, 500, 600 ppm), clear filtrate obtained after adsorption, treated product of anaerobic and anaerobic-aerobic treatment and distilled water in a closed chamber in the dark conditions. Temperature was maintained at 28-30 ^{o}C throughout the period and renewed water supply was provided every 24 h. Roots developed (2-3 cm in length) were pretreated with saturated aqueous solutions of 1, 4-Dichlorobenzene at 8 ^{o}C for 6 h. Pretreated root tips were hydrolyzed with 1 N HCl. After proper washing, root tips were stained and squashed with acetocarmine. 30 cells were examined for each treated sample. Suitable somatic plates were photographed with LEICA DM 2000 fluorescent microscope with attached camera at X 1000 magnification [25]. The cells and nuclei were observed under 45X to study nuclear and chromosomal aberrations.

2.11.2 Phytotoxicity evaluation

For phytotoxicity study, 10 seeds of *Sorghum vulgare* (*S. vulgare*) and *Phaseolus mungo* (*P. mungo*) were soaked in untreated Methylene Blue samples (500 ppm), filtrate obtained after adsorption of Methylene Blue, treated product of Methylene Blue (anaerobic and anaerobic-aerobic) with distilled water as a control. 10 mL of samples were added to each respective set each day and after 7 d, germination (%), length of plumule (cm) and radical length (cm) were measured [26].

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3. Results and Discussion

3.1 Investigation of required adsorbent (WHPP) dose for adsorption

Water hyacinth i.e. *Eichhornia crassipes* has an astonishing capacity to soak up and concentrate many toxic metals from aquatic environment [27]. This plant is considered as worst invasive aquatic weed due to its rapid growth and proliferation. Efforts towards controlling this weed are expensive which leads to its temporary removal. This weed has fascinated considerable attention of researchers for unlocking value of this otherwise troublesome weed.

Different concentrations of WHPP i.e. 1, 2, 3, 4 and 5 gm dL^{-1} showed 67.1, 72.7, 94.5, 94.8 and 95.7% adsorption of Methylene Blue (781 mM) respectively (Fig. S2). As 3, 4 and 5 gm dL^{-1} of WHPP showed almost similar Methylene Blue adsorption capacity, 3 gm dL^{-1} of WHPP was used for further experiments in order to reduce sludge generation problem. The almost equal Methylene Blue adsorption capacity of WHPP at 3, 4 and 5 gm dL^{-1} concentration was might be due to reduction in effective adsorption surface area and unsaturation of the adsorption sites [28]. The obtained adsorption capacity of WHPP for Methylene Blue (246 mM gm^{-1}) was significantly higher than earlier studies reporting adsorption of textile dyes using various parts of water hyacinth [29, 30]. Adsorption using individual plant parts of water hyacinth creates secondary pollution of the disposed weed which in contrast is tackled when the entire dried weed is used. Hence, the further experimentation was carried using water hyacinth plant powder.

3.2 Investigation of possible adsorption mechanism of Methylene Blue on WHPP

Water hyacinth is mainly composed of lignin, cellulose and wax. The FTIR spectrum of WHPP would therefore contain many peaks at the different absorption regions. FTIR spectrum

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of water hyacinth root powder has been reported earlier [31]. Water hyacinth roots The infrared spectrum for WHPP in the range of 4000-400 cm^{-1} showed a large number of functional groups that constitute this biomaterial (Fig. S3).The broad peak in the range of 3760−3204 cm^{-1} corresponds to stretch of alcohols and O−H bond vibration of carboxylic acids. The peak present at 2920.29 cm^{-1} corresponds to C-H stretch. Peak at 1638.18 cm^{-1} corresponds to C=C stretch, carboxylic acids between 1400-1300 cm^{-1} and C–O–C between 1200–1000 cm^{-1}. The bands at 860, 769 and 710 cm^{-1} can be assigned to ester vibrations and mono substituted aromatic rings, due to the lignin fraction in the WHPP [32].

The experimental data found during adsorption study using different dye concentrations and time interval was used to investigate possible adsorption mechanism. Pseudo first-order (PFO) and pseudo-second-order (PSO) adsorption models were fit to test the experimental data. The first order rate expression of Lagergren [9] is given as:

$$\frac{dq_t}{dt} = k_1 (q_e - q_t) \quad (2)$$

Where, qe and qt are the amounts of dye adsorbed on WHPP (mg gm^{-1}) at equilibrium and at time t, respectively, and k_1 is the rate constant of this adsorption (min^{-1}). Integrating Eq. (2), after applying the initial condition of qt =0, at t = 0 gives the linear form in Eq. (3):

$$\log (q_e - q_t) = \text{Log } q_e \frac{k_1 t}{(2.303)} \quad (3)$$

The values of k_1 and q_e calculated were calculated using Sigma plot 11 software. For the second kinetic model [33], the expression is given by:

$$\frac{dq_t}{dt} = k_2 (q_e - q_t)^2 \quad (4)$$

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Where, k_2 (gm mg^{-1} min^{-1}) is the rate constant of second-order adsorption. Similarly, linear form of following expression was obtained by integrating eq. (4):

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{t}{q_e} \qquad (5)$$

The values of k_2 and q_e calculated were calculated using Sigma plot 11 software. The log (q_e–q_t) ×t plot pointed out that, Lagergren equation is not applicable for the given adsorption system and hence the process does not follow pseudo-first-order kinetics (Table 1). The values of q_e from the pseudo-second order kinetics are in agreement with experimental data, $q_{e,\ exp}$. Also, R_2 values are in the range of 0.980–0.999. Therefore pseudo second order kinetic model effectively explains the kinetics of Methylene Blue adsorption with a high correlation coefficient for all ranges of dye concentrations studied. This type of mechanism of interaction between water hyacinth and Methylene Blue was also reported earlier also [34]. The kinetics study data of dye/WHPP interaction suggest that WHPP could be explored for practical applications as an adsorbent. Due to the different nature of the adsorbent used in the present investigation from that described above, a direct comparison of the k_2 values is not possible.

3.3 Characterization of adsorption process

Seaton et al. [35] had initially proposed a DFT model for calculation of the pore size distribution from adsorption isotherms, and it has been widely accepted as a logical and multipurpose approach to calculate the pore structure parameters. The DFT surface area measurement is essential to understand the performance of the adsorbent. Adsorbent interacts with its surrounding environment via its surface and therefore, higher surface area and larger pore volume are essential criteria while selecting an adsorbent for adsorption process.

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The DFT proposed surface area and pore volume of untreated WHPP were determined as 14.426 m^2 gm^{-1} and 0.030 cc gm^{-1} respectively. After adsorption of Methylene Blue, the surface area and pore volume of WHPP were reduced to 9.747 m^2 gm^{-1} and 0.020 cc gm^{-1}, respectively (Table 2). Results obtained concluded that, surface area and pore volume of WHPP was decreased after adsorption of Methylene Blue. Cationic dye coating on WHPP might have resulted in pore blocking and further lack of free surface area of WHPP. Hence decrease in surface area and pore volume of WHPP was probably obtained as a result of adsorption of Methylene Blue.

3.4 Flask level decolorization experiments

Though removal of textile dyes by adsorption is an inexpensive process, this method is unable to remove these harmful compounds completely from the environment. Hence these hazardous compounds remain persistent in the environment [36]. Therefore combined treatment of adsorption and degradation is needed for complete removal of textile dyestuff from environment [37].

The Methylene Blue decolorization efficiency of *S. cerevisiae* and isolated *Bacillus* sp. STIS was studied individually as well as in consortium (Table 3). *S. cerevisiae* treated Methylene Blue dye showed 31.7% decolorization in anaerobic environment and 58.5% decolorization was observed after semi continuous aerobic treatment. When Methylene Blue was treated with isolated *Bacillus* sp. STIS, 20.3% decolorization was obtained in anaerobic phase and 34.8% decolorization was calculated after semi continuous aerobic phase. Decolorization of Methylene Blue using developed consortium-SB was resulted in enhanced rate of decolorization in comparison with individual microbial species. Developed consortium-SB has reduced the color of adsorbed Methylene Blue (781 mM) by 58.2% and the remaining desorbed dye was removed

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by 62.11% after semi continuous aerobic treatment using suspended cells of consortium-SB. Effective biodegradation of Reactive Red 198 by bacterial-yeast consortium has been reported by Kurade et al [38]. No antagonistic effect of one organism over the other was observed during decolorization study. Adsorption effect of consortium-SB was checked by centrifugation of decolorized product. The pellet obtained after centrifugation was colorless which confirmed that, there was no any adsorption effect of the consortium.

Suspended cells of consortium-SB were unable to decolorize desorbed dye completely. Therefore, an effort was made to immobilize cells on the bio-matrix and decolorization using CLS immobilized consortium-SB. Complete removal of the dye was observed only when such immobilization was applied.

The anaerobic process plays a vital role in removing color and also improves the biodegradability of the textile wastewater under aerobic conditions [39]. Hence, semi-continuous anaerobic-immobilized aerobic treatment was used for further experimentation as it was resulted in higher decolorization of Methylene Blue than that of individual anaerobic, aerobic and semi-continuous anaerobic-aerobic treatment.

Immobilization of the consortium-SB on CLS facilitates certain advantages over suspended cells such as improvement in catalytic steadiness of microorganisms and degradation of highly concentrated toxicants is possible. Hence, CLS can prove to be an ideal bio-matrix for immobilization of microorganisms owing to increase in decolorization capacity, low cost and eco-friendly nature.

3.5 Decolorization of Methylene Blue adsorbed on WHPP by semi-continuous anaerobic-aerobic bioreactor

3.5.1 Decolorization of adsorbed Methylene Blue in ASBR

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The ASBR reactors were operated at different operation times i.e. ASBR A for 8 h, B for 16 h, and C for 24 h showed different performances for removal of Methylene Blue dye i.e. 34.2, 51.6 and 68.1%. Also, over time OLR values decreased with increase in Methylene Blue reduction (Table 4). This could be due to the initial high concentration of readily biodegradable sources i.e. peptone and beef extract present in the media that stimulated the microorganisms to utilize them as energy sources. As a result, the organic load supplemented to the ASBR, decreased with increasing time and the Methylene Blue removal efficiency was improved. Similar results have been reported by Ong et al [18] during the biodegradation of Methylene Blue in anaerobic sludge blanket reactor. Comparison of dye removal under anaerobic conditions is very difficult because of the differences in type and concentrations of dye used (azo or others), microorganism used for decolorization and electron donor (glucose or others) used. Removal of Methylene Blue dye was optimum (58±0.02) at OLR 0.47±0.01 and SRT θ_H = 24 h. Hence, further studies were carried out using samples collected from ASBR C as the samples showed maximum color removal as compared to ASBR A and B. The samples obtained were further used for HPLC analysis, evaluation of environmental parameters and toxicity studies.

The remaining dye from ASBR C was desorbed at acidic pH (pH 4) after 10 min. This indicates that under acidic conditions, H^+ ions probably replace cationic pollutant Methylene Blue from WHPP. Similar results have been reported by Mallampati et al [19] during desorption of cationic dyes including Methylene Blue adsorbed on tomato peel.

3.5.2 Decolorization of desorbed Methylene Blue in TBBR

The desorbed Methylene Blue dye sample from ASBR was trickled on consortium-SB immobilized CLS. Desorbed Methylene Blue sample showed 89.11% decolorization at flow rate 60 mL h^{-1}. While desorbed Methylene Blue dye samples trickled at 60 and 80 mL h^{-1} flow rates

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were resulted in complete decolorization of desorbed dye. Direct interaction of CLS immobilized consortium-SB with targeted dye which was continuously trickled on CLS bed resulted in complete removal of color at above mentioned flow rate. Further increase in flow rates i. e. 100 and 120 mL h^{-1} were resulted in decreased decolorization performance (92.11 and 89.47%, respectively) of consortium SB. Thus, 80 mL h^{-1} was the highest flow rate at which complete decolorization was achieved. The sample collected at this flow rate was further used for HPLC analysis, evaluation of environmental parameters and toxicity studies. The cell-free CLS matrix showed only 8.32% of adsorption at an inlet concentration of 781 mM Methylene Blue. This observation indicates that the decolorization obtained using CLS-immobilized cells was due to microbial action and not because of adsorption by CLS. Hence, semi continuous aerobic treatment after anaerobic treatment was resulted in complete decolorization of Methylene Blue. In previous studies it has been reported that, anaerobic-aerobic sequential treatment results in higher removal of pollutant, COD and toxicity [40, 41], therefore an aerobic treatment used after an anaerobic decolorization works as a polishing treatment. The removal of intermediate compounds formed during anaerobic decolorization has been reported in combined anaerobic-aerobic system [10]. Therefore, color removal by anaerobic phase as a pre-treatment for decolorization seems to be an attractive technology for color removal from textile dyes with aerobic post treatment.

3.6 HPLC analysis

HPLC i. e. high performance liquid chromatography is used as a separation technique for organic molecules. HPLC analysis of parent dye Methylene Blue showed a prominent peak at retention time 1.632. While HPLC profile of filtrate obtained after adsorption of Methylene Blue on WHPP showed peaks at retention times 2.130 and 2.165 (Fig. 4A and B). Disappearance of

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peak appeared for Methylene Blue in the HPLC profile of filtrate obtained confirmed removal of Methylene Blue by adsorption. Product obtained after anaerobic and semi continuous anaerobic-aerobic treatment of Methylene Blue by consortium-SB also showed vanishing of the peak for Methylene Blue. Formation of new major peaks at retention times 2.116, 3.037 and 3.339 were observed after anaerobic treatment (Fig. 4C) and 2.030, 2.488, 2.790 and 3.055 (Fig. 4D) after semi continuous anaerobic-aerobic treatment which were not seen in the control sample supporting the degradation of the dye. The differential HPLC spectra obtained after adsorption, anaerobic decolorization and anaerobic-aerobic decolorization confirmed the adsorption of Methylene Blue on WHPP and its biodegradation into metabolites after anaerobic-aerobic treatment. Confirmation of biodegradation of Reactive Red 120 by HPLC analysis has been reported earlier [42]. The general observation can be made that decolorization is a consequence of the transformation of the molecular structure of the dyes [43].

3.7 Environmental parameters analysis before and after adsorption and degradation of Methylene Blue

The COD of the industrial effluent generally depends upon the organic load of the effluent. The mineralization of the textile effluent is indicated by reduction in COD and BOD. Removal of dyes from effluent reduces TOC, COD and BOD values [44]. COD, BOD, alkalinity and hardness of filtrate obtained after adsorption of Methylene Blue were reduced by 92, 81, 51 and 40%, respectively than Methylene Blue dye sample. The per cent reduction in COD, BOD, alkalinity and hardness was higher (80, 69, 50 and 37%, respectively) after anaerobic-aerobic degradation of Methylene Blue than anaerobic degradation (57, 28, 21 and 15%, respectively). Similar results for COD removal have been reported by Kapdan et al [18] and Murali et al [45] during degradation of simulated textile wastewater and Methyl Orange, respectively. The

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simultaneous aerobic treatment might have resulted in degradation of the intermediates formed during anaerobic degradation, resulted in reduction in COD, BOD, alkalinity and hardness in anaerobic-aerobic treatment.

3.8 Toxicity analysis before and after adsorption and decolorization process

The increasing release of harmful chemicals into the environment has affected the balance of natural ecosystems and thus called the attention of many researchers and legislative organizations. Genotoxic and mutagenic effects of chemical agents induce genetic damage, which can lead to several health issues and also affect future generations, as these alterations can be inheritable. Hence it is necessary to check toxicity of textile dyes and the products formed after their degradation before releasing in water bodies.

3.8.1 Nuclear and chromosomal aberration assay

Beginning of genotoxicity studies for the environmental protection is of great significance, as it enables to recognize the impact of genotoxic substances present in water. The chromosomal aberration assay is still the preferred plant genotoxicity test of many researchers around the world for analysis of chromosome abnormality in plants exposed to environmental pollutants [46]. Roots of *A. cepa* tubers exposed to Methylene Blue dye solution, exhibited abnormalities in their cells, nuclei and also in the chromosomes also.

Nuclear aberrations are illustrated by morphological alterations in nuclei, as a result from the action of the agent tested. Usually, these alterations are observed in *A. cepa* test in the form of nuclei carrying nuclear buds, polynuclear cells and mini cells [47, 48, 49]. *A. cepa* roots exposed to Methylene Blue dye sample in the present study showed fluorescent nuclei in the root cells (Fig. 4A and B) which might be due to absorption of fluorescent dye Methylene Blue by the

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root cells. Toxicity of Methylene Blue was to the root cells was also resulted in enucleated cells (Fig. 4C), bursting of cells, abnormal position of nucleus in the cell, irregular nuclear shape, nuclear notch formation (Fig. 4D and 5E) and also comet shaped nucleus was also observed (Fig. 4F) as a result of unwinding and modification of DNA [11]. Similar abnormalities in nuclei of *A. cepa* root cells have been reported by Leme and Marin-Morales [48] when exposed to chemical agents. In case of distilled water treated *A. cepa* roots (Fig. 4G) root cells showed exact spherical nuclear shape, considered as a positive control. The cells of *A. cepa* roots exposed to the filtrate (Fig. 4H) obtained after adsorption, showed considerably similar nuclear shape as that of distilled water treated root cell's nuclei, representing less toxic nature of the filtrate. Nuclei of cells of roots exposed to semi continuous anaerobic-aerobically treated sample (Fig. 4J), showed almost similar nuclear shape as that of distilled water treated root cell nuclei (Fig. 4I). Pakrashi et al reported nuclear aberrations in *A. cepa* root cells exposed to Titanium dioxide nanoparticles [50].

Chromosomal aberrations are described by either structural change in chromosomes or in total number of chromosomes, which can occur impulsively or as a result of exposing to physical or chemical agents [49]. In case of chromosomal structure and arrangement, number of chromosomal aberrations were observed in Methylene Blue treated *A. cepa* root cells such as, deletion of chromosome (Fig. 4K (a)), acrocentric chromosomes (Fig. 4K (b)) and acentric chromosomes (Fig. 4K (c)). The chromosomal structure and arrangement in nuclei of root cells exposed to filtrate (Fig. 4L) was almost similar to chromosomal framework of root cell nuclei exposed to distilled water (Fig. 4M) i.e. positive control confirming less toxic nature of filtrate obtained after adsorption of Methylene Blue on WHPP than Methylene Blue dye sample. The another important observation during this toxicity test was, semi continuous aerobic treatment

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after anaerobic treatment have reduced the toxicity of product formed after anaerobic treatment as chromosomal framework of root cell nuclei exposed to anaerobic-aerobic treatment product (Fig. 4O) was more comparable with distilled water treated cells nuclei than anaerobically treated root cell nuclei (Fig. 4N).

A. cepa has been used to assess damages in DNA, such as chromosome aberrations and disturbances in the mitotic cycle [49]. The genotoxicity assessment using *A. cepa* is a low cost and easy test. The *A. cepa* test also allows the assessment of different endpoints. Hence, *A. cepa* test system has been widely used to assess the impacts caused by xenobiotics, characterizing an important tool for environmental monitoring studies, where satisfactory results have been reported [23, 48]. Kalyani et al [51] and Jadhav et al [52] have reported *A. cepa* as a test plant for cytotoxicity and genotoxicity assessment of textile dyes and the metabolites generated after their biological degradation.

3.8.2 Phytotoxicity evaluation

Textile dyes are toxic to plants [42]. Disposal of untreated dye wastewater in water bodies might cause serious environmental risk. If such type of water is used for agriculture purpose, it imparts toxic effects on the germination as well as growth of several plant species which are important from ecological point of view [53, 54]. The comparative sensitivity towards the dye Methylene Blue and its anaerobic and anaerobic-aerobic degradation products by consortium-SB in relation to *P. mungo* and *S. vulgare* were studied (Table 5). In case of seeds of *P. mungo* and *S. vulgare* exposed to distilled water (control), the mean of shoot length and root length of *P. mungo* were 20.2±0.03 and 8.7±0.0 cm, respectively, and in case of *S. vulgare*, 17.8±0.01 and 8.9±0.1 cm, respectively. The germination of seeds of *P. mungo* (20%) and *S. vulgare* (20%) were inhibited as well as shoot and root length were drastically affected when

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exposed to Methylene Blue (500 ppm). In contrast, shoot and root length were found as 16.5±0.02 and 5.2±0.1 in *P. mungo*, and 12.5±0.03 and 6.1±0.09 cm in *S. vulgare* with 80 and 70% germination, respectively when treated with filtrate obtained after adsorption of Methylene Blue by WHPP. Seeds of *P. mungo* and *S. vulgare* exposed to metabolites formed after anaerobic degradation showed 60% germination of *P. mungo* seeds with 11.5±0.01 and 4.3±0.02 cm shoot and root growth, respectively while in case of *S. vulgare*, 10.3±0.01 and 7.5±0.03 cm root and shoot length were observed with 50% seed germination. The results obtained for seeds exposed to degradation product of anaerobic-aerobic degradation were interesting. For *P. mungo*, 90% seeds were germinated with 19.7±0.03 and 7.2±0.09 cm shoot and root growth in 7 d. *S. vulgare* seeds showed 80% germination with 14.2±0.09 and 7.8±0.01cm shoot and root length, respectively. Seed germination, root and shoot length of *P. mungo* and *S. vulgare* exposed to anaerobic and anaerobic-aerobic treatment products were found higher than that of exposed to Methylene Blue where inhibition in all these parameters was observed. Reduction in germination percentage, shoot and root length of *P. mungo* and *S. vulgare* in presence of dye and their subsequent increase after the decolorization and degradation of dye by using biological treatment has been reported earlier [14, 26]. Also, metabolites extracted after anaerobic-aerobic treatment were found to be less toxic than metabolites extracted after anaerobic treatment as resulted in high germination rate as well as significant root and shoot length of *P. mungo* and *S. vulgare*. Hence, phytotoxicity studies revealed that the biodegradation of the Methylene Blue by the consortium-SB also led to detoxification of the pollutant.

Many a times, the filtrate obtained after adsorption of pollutant may still be toxic; hence it is necessary to evaluate the toxicity of filtrate obtained after adsorption. The toxicity evaluation using both the methods i.e. nuclear and chromosomal aberration analysis and

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phytotoxicity studies confirmed that, the filtrate obtained after adsorption process was less toxic in nature. Hence, the filtrate obtained after adsorption of Methylene Blue on WHPP, qualifies it for its secondary use for agriculture and other purposes. Methylene Blue toxicity removal in anaerobic-aerobic treatment was comparatively higher than in aerobic treatment. This observation suggests that, the toxic intermediates formed in anaerobic treatment get degraded in semi continuous aerobic treatment. The toxicity test outcome recommends that consortium-SB is safe for treatment of textile wastewater and thus enhances its feasibility in practical applications.

4. Conclusion

Methylene Blue adsorption capacity of dried powder of water hyacinth plant was optimized. Adsorbed Methylene Blue dye was successfully treated in semi continuous anaerobic-aerobic reactors for complete removal of dye from environment. Methylene Blue decolorization rate was higher in anaerobic phase while significant reduction in COD, BOD, alkalinity, hardness and dye toxicity were found in semi continuous aerobic treatment. Hence, degradation and detoxification of Methylene Blue by anaerobic-aerobic treatment instead of using only anaerobic or aerobic process for makes the treatment more applicable. The combinatorial physical and biological treatment system i.e. adsorption and anaerobic-aerobic treatment suggests an efficient and ecofriendly solution to treat textile dyestuff.

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Figure captions

Fig. 1. Phylogenetic analysis of isolated *Bacillus* sp. STIS.

Fig. 2. COD, BOD, alkalinity and hardness reduction in filtrate obtained after adsorption of Methylene Blue on water hyacinth plant powder, anaerobic degradation of Methylene Blue and Aerobic degradation of Methylene Blue.

Fig. 3. HPLC analysis of (A) control dye Methylene Blue, (B) supernatant obtained after adsorption of Methylene Blue, (C) degradation product of anaerobic bioreactor and (D) degradation product of semi-continuous aerobic bioreactor.

Fig. 4. Nuclear and chromosomal aberrations in *Allium cepa* root cells exposed to (A, B, C, D, E) Methylene Blue dye, (F) comet observed in Methylene Blue treated sample, (G) distilled water, (H) nuclear structure of *Allium cepa* root cells exposed to filtrate obtained after adsorption of Methylene Blue, (I) sample obtained after anaerobic degradation, (J) sample obtained after semi continuous anaerobic-aerobic treatment. Chromosomal arrangement in *Allium cepa* root cells exposed to (K) Methylene Blue dye; (K[a]) deletion of chromosome, (K [b]) acrocentric chromosome, (K [c]) acentric chromosome formation, (L) distilled water, (M) filtrate obtained after adsorption, (N) product of anaerobic degradation, (O) product of anaerobic-aerobic semi continuous treatment product.

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Tables

Table 1 Kinetic parameters for the removal of Methylene Blue dye at different dye concentrations (40, 80, 120, 160 and 200 mg L^{-1} with dosage of 30.0 gm L^{-1} of water hyacinth plant powder (WHPP)

Methylene Blue (mg l^{-1})	$q_{e\ exp}$ (mg gm^{-1})	Pseudo-first-order			Pseudo-second-order		
		$q_{e\ cal}$ (mg gm^{-1})	k_1 (min $^{-1}$)	R^2	$q_{e\ cal}$ (mg gm^{-1})	k_2 (min $^{-1}$)	R^2
40	1.07	1.89	0.251	0.868	1.14	0.102	0.988
80	2.17	2.57	0257	0.906	2.20	0.099	0.990
120	3.40	1.77	0.273	0.953	3.81	0.087	0.992
160	4.94	5.87	0.215	0.960	5.02	0.065	0.995
200	5.56	6.68	0.290	0.825	5.81	0.026	0.998

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Table 2 Surface area and pore volume analysis by DFT method at temperature 77.350 K

	Surface area (m^2 gm^{-1})	Pore volume (cc gm^{-1})
Untreated water hyacinth plant powder	14.426	0.030
Methylene Blue adsorbed water hyacinth plant powder	9.747	0.020

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Table 3 Decolorization of adsorbed Methylene Blue (0.781 mM L^{-1}) by consortium-SB, *Saccharomyces cereviacae* MTCC 463 and *Bacillus* sp. STIS

Methylene Blue adsorbed on water hyacinth	λ max	% adsorption	% Decolorization by consortium-SB		% Decolorization by consortium-SB		% Decolorization by *Bacillus* sp. STIS		% Decolorization by *Saccharomyces cereviacae* MTCC 463	
Methylene Blue	660	94.5	Anaerobic	Anaerobic-aerobic	Anaerobic	Anaerobic-immobilized aerobic	Anaerobic	Anaerobic-aerobic	Anaerobic	Anaerobic-aerobic
			58.2	62.11	58.2	CD	20.3	34.8	31.7	58.5

CD- complete decolorization

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Table 4 Operation parameters for anaerobic sludge bioreactor (ASBR)

ASBR	Dye concentration ($mM\ L^{-1}$)	HRT (θ_H)	Organic loading rate (OLR) COD ($gm\ d^{-1}$)	Methylene Blue Removal (%)
1	781	8	1.82 ± 0.01	34.2
2	781	16	1.03 ± 0.03	51.6
3	781	24	0.47 ± 0.01	68

Values are mean of three experiments ± SEM

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Table 5 Phytotoxicity evaluation of Methylene Blue (500 ppm), (A) supernatant obtained after adsorption of Methylene Blue on water hyacinth, (B) after anaerobic degradation of Methylene Blue dye and (C) after anaerobic-aerobic degradation of Methylene Blue dye in comparison with distilled water.

Parameters	*Phaseolus mungo*					*Sorghum vulgare*				
	Distilled Water	Methylene Blue	A	B	C	Distilled Water	Methylene Blue	A	B	C
Germination (%)	100	20	80	60	90	100	20	70	50	80
Shoot length (cm)	20.2 ±0.03	5.02 ±0.02**	16.5 ±0.02***	11.5 ±0.01**	19.7 ±0.03***	17.8±0.01	6.7 ±0.03	12.5 ±0.03**	10.3 ±0.01*	14.2 ±0.09***
Root length (cm)	8.7 ±0.01	2.1 ±0.09*	5.2 ±0.1	4.3 ±0.02**	7.2 ±0.09***	8.9±0.1	2.1 ±0.01	6.1 ±0.09**	7.5 ±0.03***	7.8 ±0.01**

Values are mean of three experiments ± SEM

Significantly different from control (0 h) at *P<0.05, **P<0.01 and ***P<0.001 by one-way ANOVA with Tukey–Kramer Comparison test

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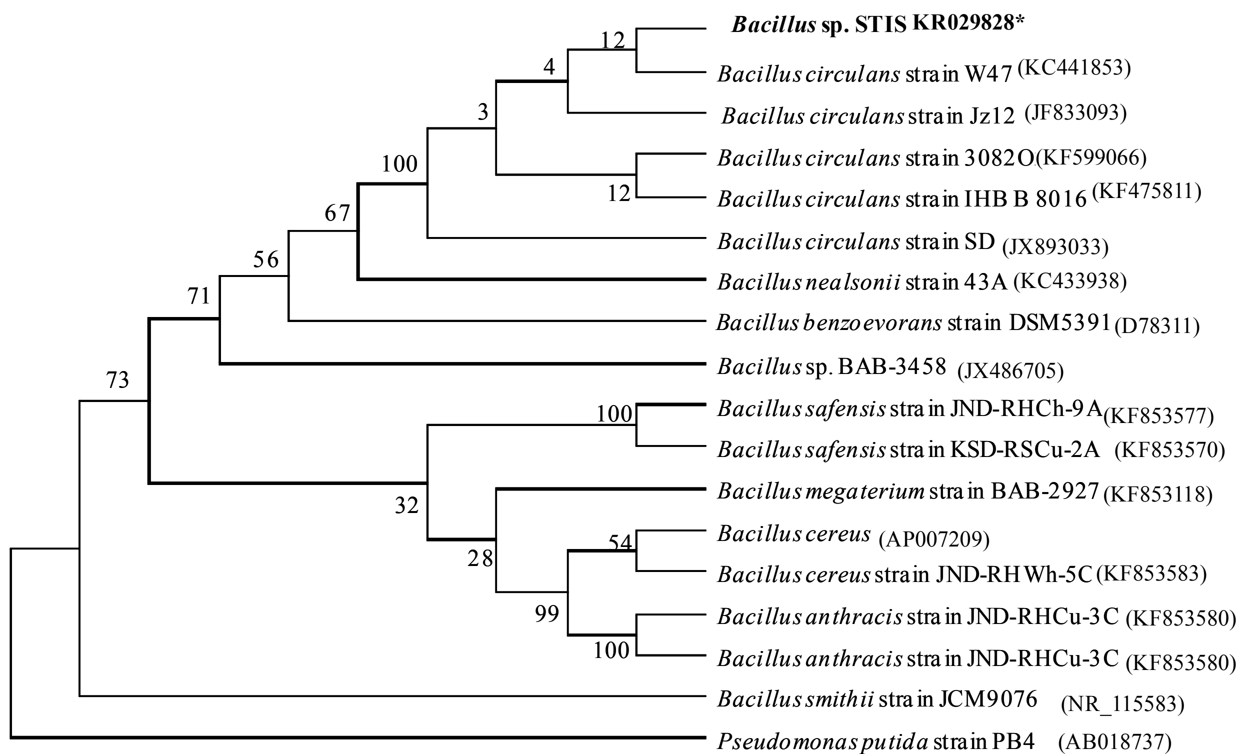

Fig. 1.

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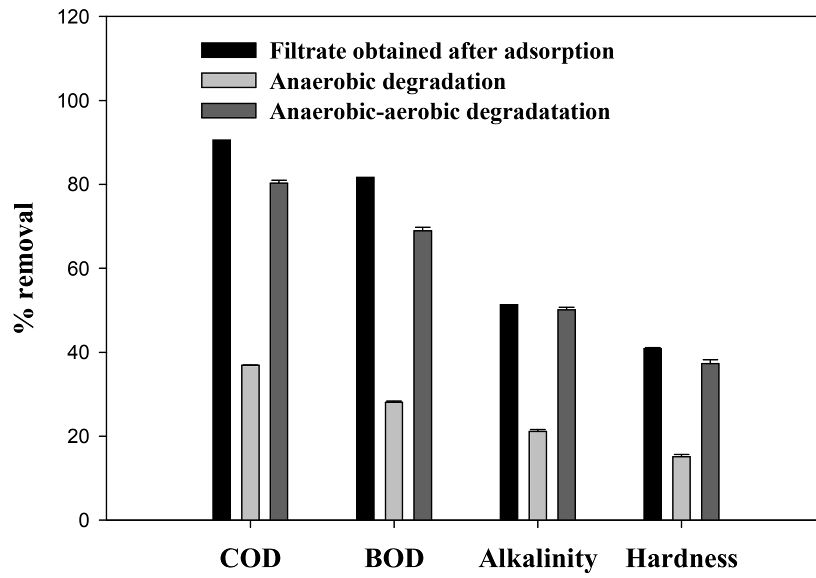

Fig. 2.

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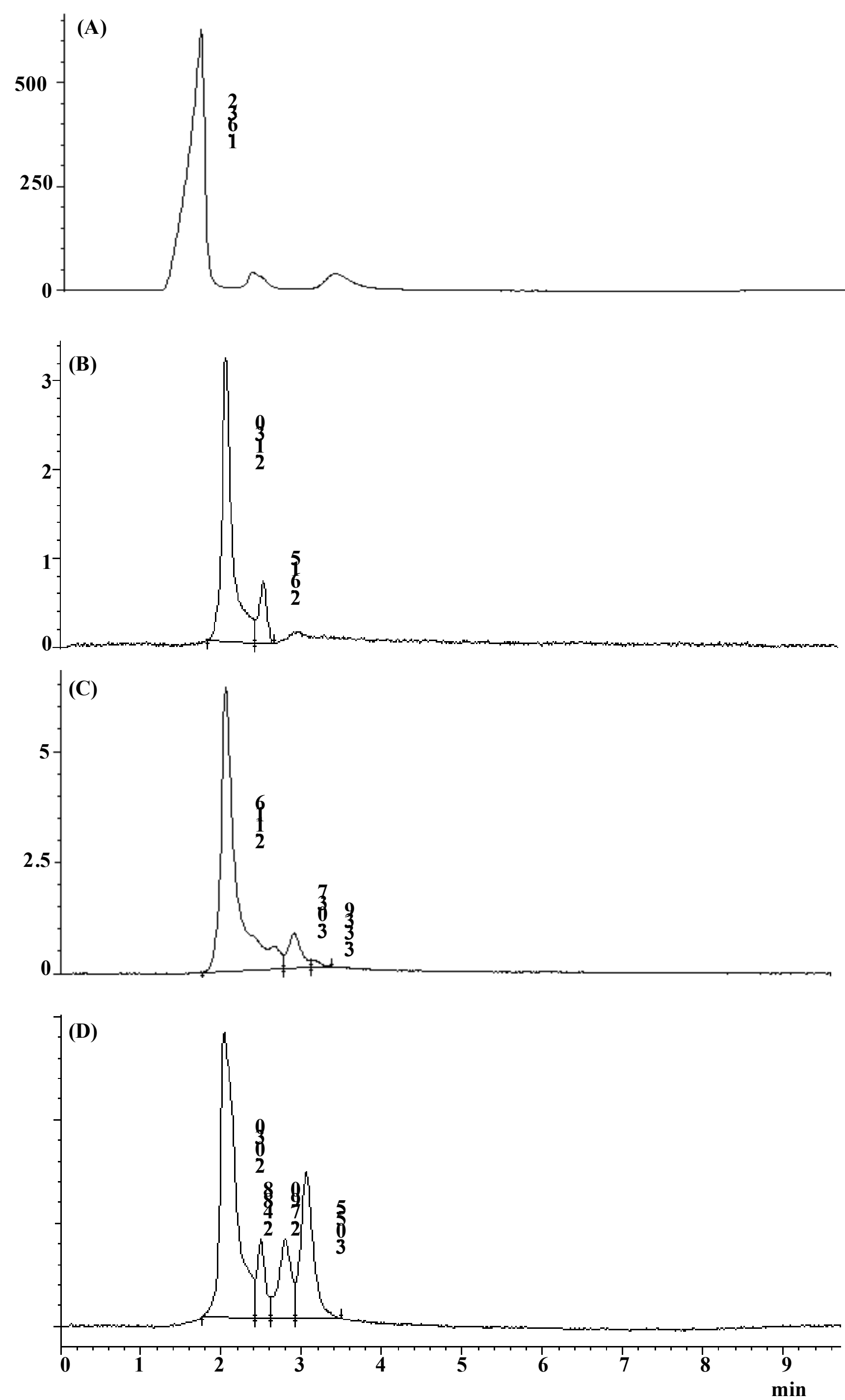

Fig. 3.

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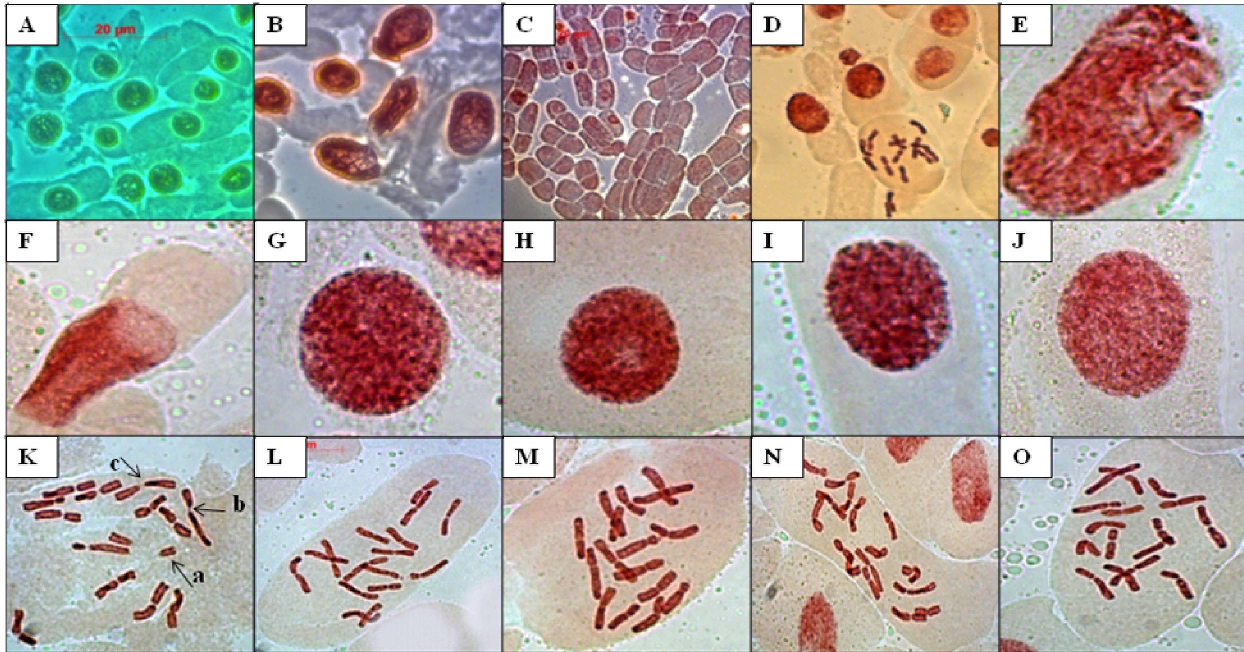

Fig. 4.

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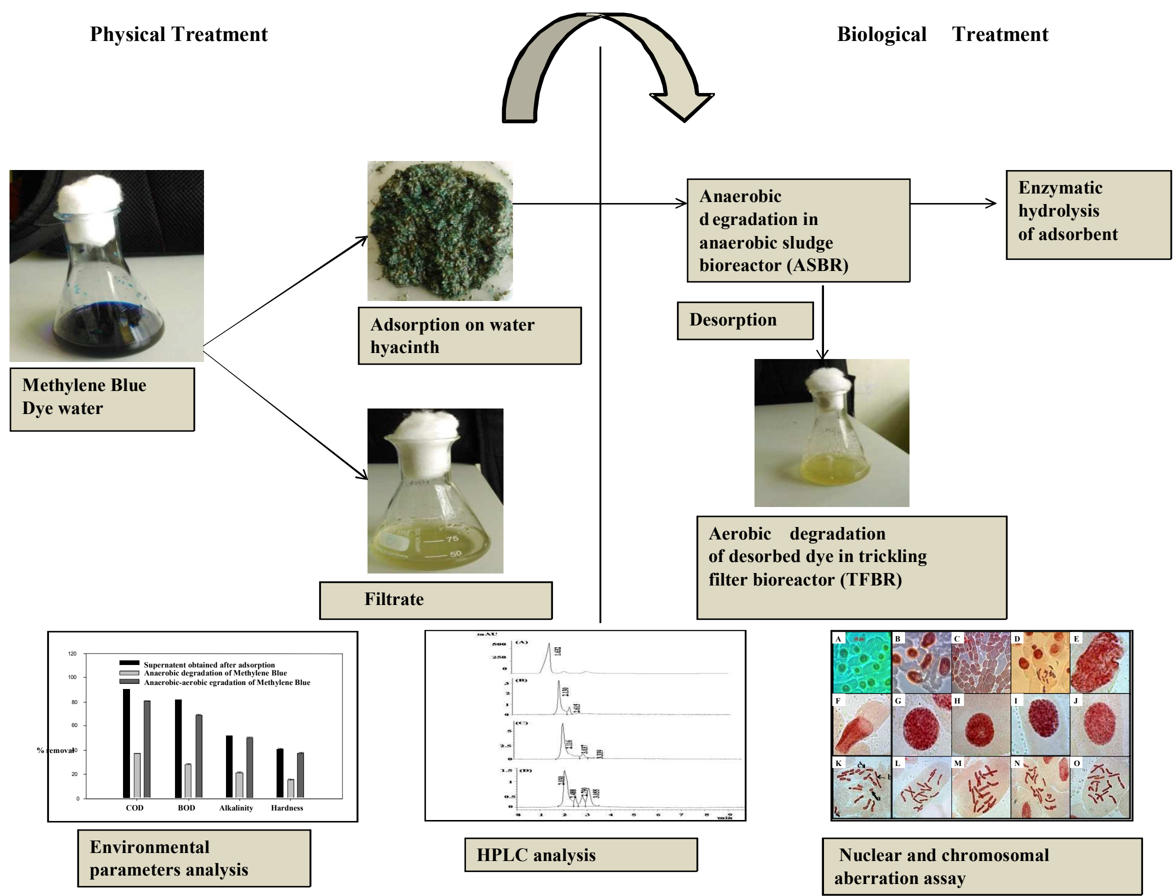

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