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Research paper

Vermiremoval of methylene blue using *Eisenia fetida*: A potential strategy for bioremediation of synthetic dye-containing effluents

Manash Deep Dey[a], Subhasish Das[b], Rupesh Kumar[a], Robin Doley[a], Satya Sundar Bhattacharya[b], Rupak Mukhopadhyay[a,*]

[a] Department of Molecular Biology and Biotechnology, Tezpur University, P.O.- Napaam, Assam 784028, India
[b] Soil and Agro Bio-engineering Lab, Department of Environmental Science, Tezpur University, P.O.-Napaam, Assam 784028, India

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ABSTRACT

Synthetic dyes from industrial effluents pose significant threats on aquatic and human lives due to its toxic nature. We report removal of synthetic dye Methylene blue (MB) by vermicomposting using earthworm species, *Eisenia fetida*. The dye adsorbed in sugarcane bagasse (SB) was used as the substrate for vermicomposting. Physico-chemical characterization of compost parameters indicated high quality of vermicomposting. The dye removal from the composted samples was studied by RP-HPLC analysis. In vermicomposted samples, removal of MB was found to be 61% and 98% after 30 and 60 days, respectively. The dye removal was an outcome of combined activities of earthworms and microbes. The dye-stress caused changes in body color and tissue microstructure in earthworms along with lower reproduction rate. However, the worms reverted back to normalcy after removal from vermicomposting and regained reproduction efficacy within 60 days. This study could pave the way for an eco-friendly, inexpensive and promising bioremediation strategy to counter soil contamination due to synthetic dyes effluents.

1. Introduction

Textile industry is growing rapidly with a total production of 88.5 million tons per year (Engelhardt, 2009; Yacout and El-Kawi, 2015). The production share of developing nations presently stands at 58.6%, which is expected to rise in order to meet the rising demands (Yacout and Hassouna, 2016). The effluents produced by this industry contain synthetic dyes among other chemicals which demand a rigorous effluent management system in place. Other industries such as tannery, pharmaceuticals and food also produced dye-containing effluents. The annual production of dye as effluents is accounted to be 7×10^5 tones worldwide (Akhtar et al., 2005) and more than 10,000 dyes are used (Dey et al., 2015).

A regulatory framework has been set by countries around the world to check the concentration of toxic dyes in the industrial waste water prior to dispensing them into the various environmental matrices (Bhattacharyya and Sharma, 2004; Panda et al., 2009). However, majority of the dyes are recalcitrant in nature and therefore difficult to remove. Commonly adopted technologies e.g. adsorption, electrochemical and photochemical degradation, flocculation, reverse osmosis, membrane separation, and ozonation are highly expensive (Dey et al., 2015; Panda et al., 2009; Ulson de Souza et al., 2007). Post adsorption, these adsorbents are generally dumped in landfills, aggravating the risk of severe environmental contamination in the long run. Adsorption along with biodegradation holds immense potential to address the effluent pollution problem in textile sectors (Wanyonyi et al., 2013; Zhang et al., 2013).

Methylene blue, a basic dye with wide range of applications has been reported to have neurotoxic effects on the central nervous system at clinically relevant concentrations (Vutskits et al., 2008). It has been noted to cause skin necrosis, skin telangiectasis and inflammatory alterations (Bleicher et al., 2009). Hence, removal of trace amounts of methylene blue becomes essential to avoid bioaccumulation of the dye to clinically relevant concentrations.

Vermicomposting helps in rapid conversion of slowly biodegradable wastes into valuable materials through combined action of earthworm and microorganisms (Ali et al., 2015). It is proposed to be a suitable methodology for remediation of industrial waste products such as municipal solid waste, paper mill bamboo sludge and biogas plant slurry (Sahariah et al., 2015; Sahariah et al., 2014; Sangwan et al., 2010a, 2011; Singh et al., 2011). Vermicomposting of press mud, a waste generated from sugar industry was reported for production of good quality

* Corresponding author.
E-mail address: mrupak@tezu.ernet.in (R. Mukhopadhyay).

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fertilizers (Sangwan et al., 2010b). The major advantage of using earthworms lies in their efficiency to convert waste material into stabilized forms and detoxification of the waste through accumulation of toxic metals in their body (Goswami et al., 2014). Interestingly, a recent report has shown how earthworms can modify the soil ambience to cope up with high-polyphenol exposure (Liebeke et al., 2015). Moreover, earthworms are capable of producing fluorescent quantum dots using their metal detoxification pathway (Sturzenbaum et al., 2013). All these studies indicate towards unexplored qualities of earthworms which can be utilized to remediate toxic wastes. However, there is a dearth of information in the area of dye degradation potential of earthworms.

To the best of our knowledge, an attempt has been made for the first time to understand the efficacy of *Eisenia fetida* for time-dependent removal of sugarcane bagasse (SB)-adsorbed methylene blue (MB) by vermicomposting method. The physico-chemical characterization confirmed high quality vermicomposting. Dye removal from the vermicompost mixture has been established using RP-HPLC. The physiological impact of MB uptake in earthworms and their reversal to normal state has also been studied.

2. Materials and methods

2.1. Materials

Sugarcane bagasse (SB) was collected from the sugarcane farms in and around the vicinity of the Tezpur University campus, Assam, India. The collected biomass was washed, dried under sunlight, ground to fine powder and stored in desiccators at room temperature. Methylene Blue (MB) (C.I. 52015) was purchased from Sigma-Aldrich, Co., USA. The absorption maximum of this dye was 668 nm as validated by the spectrophotometric scan from 200 to 1000 nm. Urine free cow dung (CD) was procured from an agricultural farm located in Tezpur, India. Non-clitellated, juvenile specimens of *E. fetida* weighing about 1–1.2 g were collected from the stock population maintained in the vermiculture unit of the Department of Environmental Science, Tezpur University, Assam (India). The earthworms were washed in deionized water and kept in dark conditions overnight on moist filter paper for gut evacuation and then used for incubation.

2.2. Composting experiment

We carried out two parallel bio-conversion experiments, *viz.* aerobic composting and vermicomposting with *E. fetida* simultaneously. A modified vermiconversion system was used to maximize the efficiency of substrate utilization as reported previously by (Das et al., 2016). We used 12 cone shaped porous earthen bioreactors (volume: 3 l, Dimension: 0.45m × 0.25m) for the biocomposting study; 3 each for the 4 treatments. Initially, the bagasse (with and without dye) was thoroughly mixed with CD in the ratio of 1:3 and earthworms were added @ 10 worm kg^{-1} substrate. Concurrently, a similar series of treatments were maintained for aerobic composting. The incubation was carried out for 60 days; under an ambient temperature range of 25–30 °C. Regular churning of the mixtures in the bioreactors was done with occasional sprinkling of water as in when required. Samples were drawn from the bioreactors on 15, 30, 45 and 60th day for physico-chemical analyses. The number and weight of adult earthworms were also monitored at mentioned time intervals to assess the growth pattern of earthworms. A gist of the treatment combinations is provided below:

Vermicomposting series		Composting series	
V1:	Bagasse only with *Eisenia fetida*	C1:	Bagasse only
V2:	Bagasse + Dye (Methylene blue) with *Eisenia fetida*	C2:	Bagasse + Dye (Methylene blue)

2.3. Physico-chemical characterization of compost

The raw and periodically collected biocomposted bagasse samples were characterized for parameters like pH, bulk density (BD), water holding capacity (WHC), total N, total organic carbon (TOC), available P and exchangeable K following standard protocols (Page et al., 1982). Microbial biomass carbon (MBC) was enumerated following the chloroform fumigation technique as per Jenkinson (1994). All samples were analyzed in triplicate and average results were recorded. The results were reproducible within ±5% error limit.

V1, V2 and C2 were subjected to scanning electron microscopy (SEM) analysis to study the changes in surface topology at different time points (0, 30 and 60 days) of the incubation period (JEOL JSM-6390LV, Tokyo, Japan).

2.4. Microbial count and biomass estimation

The microbial count (bacteria and fungi) was enumerated in the composted and vermicomposted samples following established procedures (Bhattacharya et al., 2013; Parmer and Schmidt, 1964). Briefly, 1 g vermicomposted and composted bagasse samples were suspended in 10 ml ultrapure water followed by a 15 min vortex session. Then, samples were inoculated in petriplates after serial dilution from 10^{-1} to 10^{-6}. We used Nutrient agar and malt extract media for bacterial and fungal count respectively. After incubation, the visible colonies were counted under a colony counter and the results are expressed as colony forming units (CFU g^{-1}). In addition, we calculated the total microbial biomass as per the procedure described earlier (Kim et al., 2012):

$$\text{Microbial biomass } \left(\mu\text{g ml}^{-1}\right) = \frac{(CFU + 3.60E + 06)}{1.00E + 08} \times 1.00E + 03$$

2.5. Extraction of dye from the compost and RP-HPLC analysis

To optimize extraction of MB from the sugarcane bagasse four solvents *viz.* ethanol, glacial acetic acid, chloroform and water were used. 0.5 g dye adsorbed SB was incubated with 10 ml of the extraction solvents in constant stirring condition for 2, 4, 6 or 24 h. The amount of dye extracted was estimated spectrophotometrically. This protocol was followed to extract dye from experimental vermicompost sample V2 at 0, 30 and 60 days interval. The extracts from 1 g of samples was air-dried and resuspended in 10 ml milli-Q water for RP-HPLC analysis. The amount of dye present in the extract was quantitated using C18 column (4.6 × 250 nm, Milford, Massachusetts, USA) on a Waters HPLC system at ambient temperature. Elution was carried out with 80% methanol at a flow rate of 1 ml/min. Gradient used for elution of the sample was 0.1% glacial acetic acid: 80% methanol of 5–100% over 76 min.

2.6. Histology studies

Earthworm sections (anterior gizzard, middle region and anal region) were suspended in 10% buffered formalin for 96 h prior to embedding in paraffin. 5-μm-thick sections were cut from formalin-fixed, paraffin-embedded (FFPE) whole-mount tissue block and placed on coated glass slides and heated 70 °C at 60 min. Paraffin was removed by treatment of xylene wash twice for 5 min. This was followed by tissue rehydration through multiple, graded