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RESEARCH PAPER

Production and shelf life evaluation of three different formulations of *Beauveria bassiana* in terms of multimetal removal

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Abstract The present work describes a novel attempt to produce three different stable formulations (myco-granules, myco-tablets and myco-capsule) of *Beauveria bassiana* fungi targeted against multimetal (Cu, Cr, Cd, Ni, Zn and Pb) containing synthetic wastewater. Locally available low cost substrate (rice flour) was used for the production of these formulations. Shelf life of the above the formulations stored at 30 °C temperature was evaluated in terms of viability, biomass production and multimetal removal over the period of one year at a regular interval of 4 months. It was observed that myco-granules and myco-capsules were more stable formulations as compared to myco-tables in control conditions as well as with multimetal. In the initial phase of studies, maximum multimetal removal (93%) were observed with myco-granules and myco-capsules followed by myco-tablet (83.5%). The multimetal removal ability of all three formulation decreased by 37.6%, 53.1% and 48.5% for myco-granules, myco-capsules and myco-tablet, respectively, after 12 months of storage. Further, morphological changes caused by the multimetal toxicity were analyzed using SEM, AFM and FTIR. The developed formulations have the potential of remediating multimetal containing wastewater. Further, its prolonged shelf life at ambient temperature highlighted its superiority over conventional means of microbial storage.

Introduction

Multiple heavy metals present in the industrial effluents are difficult to remediate using conventional treatments and result in serious health hazards (Akbari, Abdurahman, Yunus, Fayaz, & Alara, 2018; Sahoo & Patra, 2018; Wang, Ma, & Yang, 2018). Specialized microbial cultures that possess tolerance against cocktails of heavy metals and other contaminates (Asad, Mohammad, Dastgheib, & Amoozegar, 2014; Dey, Gola, & Mishra, 2016; Mishra, Singh, & Pande, 2014; Panda, Kanjilal, & Das, 2018; Shah, 2014; Sonune et al., 2018) can be used for metal bioremediation but a suitable storage and delivery system for bioaugmentation

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of waste streams with selected cultures is required. The present methods of culture preservation and storage include the lyophilization of the microorganisms or refrigeration on culture media slants which are often expensive techniques and involve the utilization of sophisticated instruments and controlled storage conditions (Kaushik, Mishra, Malik, & Sharma, 2015). Moreover, reactivation of the culture requires the input of various nutrient supplements for the growth of the microbes.

The commercial availability of the microbial culture can be made through inexpensive techniques such as solid type formulation and a liquid type formulation. However, such formulations are rarely available for wastewater applications. Moreover, each of such formulations suffer from drawbacks as solid type microbial culture has a relatively long preservation time but relatively long activation time. On the other hand, the liquid type microbial culture has a relatively short activation time but a relatively short preservation time. To ensure an effective implementation of potential micro-organism to remediate these hazardous pollutants at large scale (industrial scale), mass production of these microorganisms at low cost with ease of storage and transportation is required.

Several methods have been investigated by the researchers to develop a microbial formulations using low cost substrate such as sugar beet pulp waste and waste sludge (Bradley, Kearns, Wood, & Black, 1996; Vidyarthi, Tyagi, Valero, & Surampalli, 2002). However, the shelf life of these microbial formulations was limited to three months only even at low temperature, due to inherent decomposition of the substrate used. Very few microbial formulation discuss the remediation of heavy metal or other contaminant from waste water. Lamar, Dietrich, and Glaser (1995) and Aust, Bumpus, and Tien (1990) have patented their technology for lignin degrading and halogenated hydrocarbons remediating fungi, respectively. Another study by Abraham and Silambarasan (2014) used saw dust, soil and 5% molasses (15:5:1) for mass production of bacterial and fungal consortium. This powdered consortium was able to degrade endosulfan. The shelf life of the consortium was checked upto 12 weeks only. From the above studies, we can conclude that development of microbial formulation requires specific conditions with optimized nutrient requirement but long shelf life is still a challenge and need more research. Moreover, most of the existing products are powdered formulations, which are difficult to handle and dose. Recently, our group reported production of myco-granules targeted for multiple industrial applications including dye and heavy metal sequestration and xylanase production using low cost substrates (Kaushik et al., 2015). In spite of good shelf life and activity, it was realized that in order to make the transportation easy and to make it user friendly, more structured formulations need to be developed in the form of tablets or capsules. This shall greatly ease the handling and dosing problems. Researchers have been producing tablets for pharmaceutical usage utilizing various binders such as carnauba wax (Uhumwangho, Okor, & Adogah, 2009), ginger starch (Onyishi et al., 2008) and cassava and cocoyam starch (Uhumwangho, Okor, & Eichie, 2006). It is known that the choice of a particular binding agent depends on the binding force required to form granules and its compatibility with the other ingredients particularly the active substance. Also, the compression pressure at which the tablets are formed and the binder effects the parameters influencing the tablet characteristics which may be analyzed in terms of hardness, friability, dispersion rate and disintegration time (Eichie & Kudehinbu, 2009; Uhumwangho & Okor, 2004). However, this process may adversely affect the viability of the microbes/spores. Previously, (Adholeya, Tiwari, & Singh, 2005) had demonstrated the production of tablets of mycorrhizal fungi for field application. Nevertheless, no tableted or capsule product has been reported for multimetal removal.

Beauveria bassiana has been used successfully against many insect/pests under both laboratory and field conditions. Different formulations of *B. bassiana* have been tested against houseflies (bait, encapsulation, and emulsion), white-flies (oil, talc and crude), and other agricultural pests (Mishra, Kumar, & Malik, 2013; Prithiva, Ganapathy, & Jeyarani, 2017; Saeed et al., 2017). Hence treating wastewater using *B. bassiana* would be advantageous due to its insecticidal properties. Recently, our group revealed excellent potential of *B. bassiana* for bioremediation of heavy metals when present individually as well as multimetal mixture. The present study was performed to formulate three different structured formulations (myco-granules, myco-tablets and myco-capsules) of *B. bassiana* followed by evaluation of the shelf life and heavy metal removal efficiency of the same.

Low cost granulated formulation of *Beauveria bassiana* were produced using rice flour as a substrate material. The bioremediation potential of the myco-granules has been estimated in term of metal removal capacity after one year of storage. Storage at ambient temperature without losing its viability as well as multimetal removal ability is the novel feature of the present formulation. Further, the process involved in the production of these formulation easy very simple, require minimal handling with ease of dosing in the industrial processes, especially for the unskilled masses in small scale industries.

Material and methods

Fungal spores of multi-metal tolerant fungal strain *Beauveria bassiana* were immobilized on organic base to form fungal formulations (Kaushik et al., 2015). Three different types of formulations, myco-granules, myco-tablets and myco-capsule were prepared and characterized. All the three formulations were tested and compared for their viability, multiple heavy metal removal efficiency and shelf life during one year of storage at 30 °C. The results were supported by SEM, AFM and EDX analysis.

Test organism and substrate material

Beauveria bassiana (MTCC4580) procured from IMT (Institute of Microbial Technology, India) was used owning to multimetal uptake capacity of the strain described in previous study(Gola, Dey, & Bhattacharya, 2016). The culture was maintained on PDA (potato dextrose agar) slants and stored at 4 °C. Rice flour homogenized through sieving (<74 μm) was used as a carrier for developing the granulated formulation.

The rice flour was characterized for carbon and nitrogen content using CHN analyzer.

Metal solution preparation

A stock solution of 1000 mg/L was prepared for each heavy metal. Metal stock solution of Pb was prepared using distilled water with HNO_3. Whereas, stock solution of other heavy metal (Cu, Zn, Cd, Cr and Ni) was prepared by dissolving the particular salt of heavy metal ($CuSO_4 \cdot H_2O$, $ZnSO_4 \cdot 7H_2O$, $K_2Cr_2O_7$, $Ni(NO_3)_2$, and $3CdSO_4 \cdot 8H_2O$ of Merck) in Milli Q water. The desired concentration (5 mg/L) of each heavy metal was obtained from stock solutions prior to the experiment.

Development of fungal formulation (myco-granules, myco-tablets and myco-capsules)

Myco-granules of *Beauveria bassiana* were produced using a previously disclosed methodology (Indian Patent Application no. 2590/DEL/2012). Briefly, the fungal strain was inoculated (spore count: 6×10^6) and incubated on sterilized organic base substrate cum carrier (rice flour) in a flask. Moisture content of the flask was adjusted to 60% using sterilized doubled distilled water and the flasks were incubated at 30 °C for 5 days (Kaushik et al., 2015). After 5 days, content of the flask was taken out with the help of spatula on sterilized filter paper and dried at 45 °C for 12 h.

Granulation, tabletting and capsulation techniques

Granulation

The dried biomass from the filter paper was grinded using mortar pestle, screened through sieve having the pore size of 105 μm and again kept for drying at 45 °C for 2 h to obtain myco-granules. The moisture content of the granules obtained after drying was nearly 2%. At an interval of 4 months, the myco-granules were tested for viability and metal removal efficiency. Myco-granules were further used to develop a tableted product called myco-tablet and an encapsulated formulation called myco-capsule.

Tabletting

For myco-tables, myco-granules were pressed using automated press machine (REMI 2P) at pre-optimized compression pressure using a patented process (2590/DEL/2012). At an interval of 4 months, the myco-tablets were tested for viability and metal removal efficiency. Myco-tablets were characterized for different parameters (hardness, dispersion time, weight uniformity, friability and disintegration time) using the standard methodology (Herbert, Lieberman, & Lachman, 1991).

Capsulation

Myco-capsules were produced by packing (filling) myco-granules (0.1 g) inside pre-fabricated gelatin capsules under sterile conditions in a biosafety cabinet using a patented process. The dispersion time of myco-capsules was tested and these were stored at 30 °C for a period of 12 months. At an interval of 4 months, the myco-capsules were tested for viability and metal removal efficiency.

Physical characterization of tablets and capsules

Weigh uniformity test: Twenty myco-tablets were weighed individually and mean weight with standard deviations was calculated.

Hardness test: Ten myco-tablets were selected and checked for hardness through Monsanto hardness tester. All tablets were placed in tester (between spindle and anvil) and the calibrated length was adjusted to zero value. The knob was then screwed to apply a diametric compression force (kg/cm units) and the point on the calibrated length at which the tablet broke was recorded. A mean hardness with standard deviation was calculated for all ten tablets.

Friability test: Ten myco-tablets were selected and tested for friability. The initial weight of myco-tablets was recorded collectively. Tablets were placed in the Friabilator. Friabilator was operated for 4 min at 100 rev/min. The tablets were dusted and reweighed. The percentage loss in weight was calculated for each tablet.

Disintegration time: Disintegration time was estimated through disintegration test apparatus (Herbert et al., 1991). One tablet each was kept in the all the six tubes. A disc to each tube was placed over the tablet and the apparatus was switched on. The tubes travelled in upward and downward motion in the disintegration medium (phosphate saline buffer, pH 8) maintained at 30 °C. The time taken for all the six tablets to break down and pass through the mesh at the bottom of the tube was noted. The tablets pass the test if all the six tablets disintegrate within the prescribed time (i.e. 15 min).

Dispersion time: This test was done for both the myco-tablet and myco-capsule. Tablet or capsule was added to 100 mL of water and time required for complete dispersion was measured. To test the uniformity of dispersion, three randomly selected tablets/capsules from each formulation were placed in 100 mL of water and stirred gently until completely dispersed.

Evaluation of shelf-life and multi-metal removal capacity

The shelf life of fungal formulations (myco-granules, myco-tablet and myco-capsule) was evaluated in terms of viability or colony forming units (cfu), biomass production (in presence and absence of metals) and multimetal removal efficiency at 4 months interval. The viability of the spores in the myco-granules, myco-capsules and myco-tablet was estimated through cfu count by dissolving 1 g formulation in 100 mL distilled water. The cfu count of different formulations was estimated through serial dilution method wherein serially diluted water suspension of formulations were plated (spread plate method) on sterile petri-plates containing potato dextrose agar media and incubated at 30 °C for 3 days. The number of cfu produced was counted through colony counter. To assess the biomass production and multimetal removal efficiency, weighed amount

of formulation (0.1 g) was added into the composite media (concentration in g/L) [NH_4NO_3 (0.5); $MgSO_4{\cdot}7H_2O$ (0.1); K_2HPO_4 (0.5); NaCl (1); glucose (10); yeast extract (2.5)] in absence and presence of multimetal (30 mg/L total metal constituted by 5 mg/L each of Pb, Cu, Zn, Cd, Cr and Ni). The flasks were kept at 150 rpm and 30 °C for 120 h. Flask content was withdrawn after 120 h and centrifuged at 10,000 rpm for 10 min to obtain supernatant. Supernatant was analyzed for residual metal concentrations using the following equation:

$$\text{Removal (\%)} = \frac{\text{Total metal ion concentration (mg/L)} - \text{Residual metal ion concentration (mg/L)}}{\text{Total metal ion concentration (mg/L)}} \times 100$$

The left over biomass was dried and measured for biomass production using gravimetric method (Gola et al., 2016). For morphological investigations (SEM, EDX & AFM), biomass pellets were obtained from the flask containing 30 mg/L multimetal mixture) as well as control (without metal) after growth period of 120 h and processed according to the requirement of particular analysis.

Analytical techniques

Measurement of residual metal concentration

Ten mL of the samples from flask were taken and centrifuged at 10,000 rpm for 10 min. The supernatant liquid was digested in microwave digester (160 ± 4 °C within 10 min and 165–170 °C for 10 min for reaction). Digested samples were analyzed for metal ions using Microwave plasma-atomic emission spectrometry (Agilent 4200 MP-AES).

Biomass production

Dry weight measurement of the fungal biomass was carried out gravimetrically. The dry cell weight of the fungal biomass was measured by filtering out the contents of the flasks through pre-dried and pre-weighed Whatman No.1 filter paper and drying it overnight at 60 °C to a constant weight. The dry weight was expressed as grams of dried fungal biomass per litre of media.

Morphological analysis

To determine the change in morphological features and cell wall functional group responsible for heavy metal binding analysis such as FTIR, SEM–EDX and AFM were performed. For this study, biomass sample grown in composite media (120 h) using myco-granules amended with hexa-metal ion as well as control (unamended media) was collected at 120 h and processed as described in the following sections.

Scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM–EDX)

For SEM analysis, fungal pellets (biomass grown in control condition as well as hexa-metal mixture) were fixed via 2.0% glutaraldehyde (prepare in phosphate buffer) for overnight at 4 °C. After overnight incubation samples were washed with PBS buffer and lyophilized. Samples were gold-coated for SEM and carbon coated for EDX. The SEM and EDX observation was done under the following analytical condition: EHT = 20.0 kV, Signal A = SE1 (ZEISS EVO 50).

Atomic force microscopy (AFM)

For AFM analysis, fungal pellets were air dried on glass cover slip. AFM micrographs were recorded in contact mode with force constant 0.22–0.77 N/m and tip height 10–12 nm (Das, Das, & Guha, 2009).

Fourier transform infrared spectroscopy (FTIR)

The functional group responsible for metal uptake on *B. bassiana* were analyzed by FTIR (One spectrum-spectrum v5.0.01) using lyophilized fungal biomass as described earlier (Gola, Malik, Namburath, & Ahammad, 2017).

Result and discussion

Physical characterization of myco-tablets and myco-capsules

Three different types of formulations, i.e. myco-granules, myco-tablets and myco-capsules of *B. bassiana* are shown in Fig. 1. Myco-tablet has a diameter of 8 mm with 3 mm width and convex surface. Whereas myco-capsules has a diameter of 5 mm with 12 mm length. The myco-tablets were characterized for various parameters such as dimension, hardness, weight, friability (extent of dust produced by the tablet) and disintegration time (time taken by the tablet to get disintegrated into particles in water). The calculated values of hardness and friability were 2.3 kg/cm^2 and 2.6%, respectively (Table 1). Hardness and friability are two important physical parameter that define the mechanical strength of the myco-tablet (Jensen, 2011). Manufacturers normally employ these tests to ensure that the tablets would tolerate the rigours of handling, packaging and transportation process, and would maintain the desired properties during storage period. Hardness of a tablet is a function of how much pressure has been exerted in making it and it varies with the composition of organic base and shape (thickness and diameter) of tablets (Lieberman, Riger, & Banker, 1996). The disintegration time for myco-tables in phosphate buffer was 3.5 min. The values of these parameters for stable tablets have been laid as ideal friability (less than 5%), ideal hardness (more than 1 kg/m^2) and ideal disintegration time (within 5 min). Hence, the results (Table 1) suggest good stability of the formulated myco-tablets during storage and transportation.

Dispersion test was also conducted on myco-capsules and it was found that complete dissolution of capsule wall and release of granulated formulation into the water takes

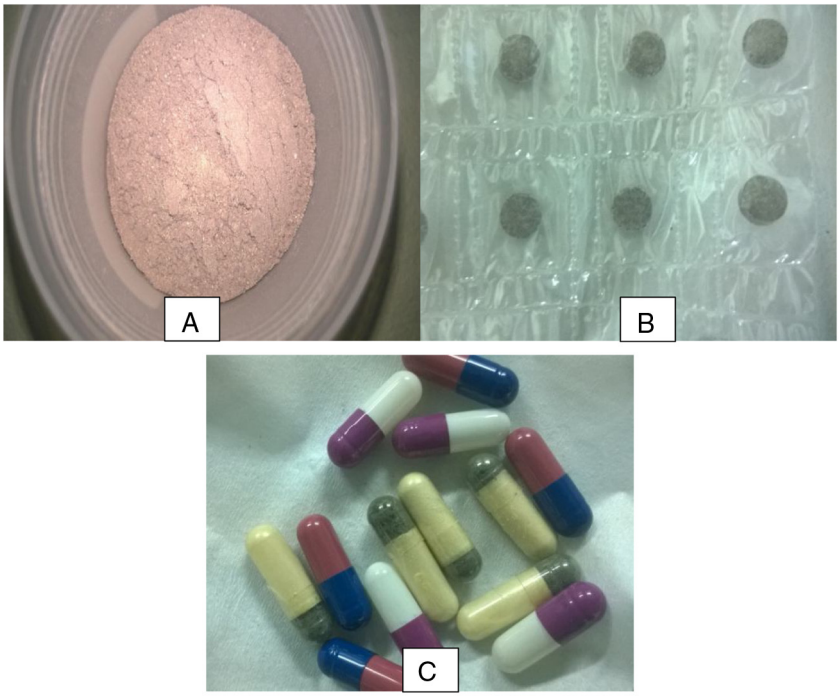


Figure 1 Different formulations of *B. bassiana:* (A) mycogranules; (B) mycotablets and (C) mycocapsules.

Table 1 Characterization of myco-tablets.

§Parameters	Result	Prescribed limits
§Dimensions	Dia: 8 mm, width: 3 mm. Convex surface	–
sWeight of the tablet	0.191 ± 0.04 g	–
sHardness (kg/cm^2)	2.3 ± 0.03	5
sFriability (%)	2.26 ± 0.05	5
sDisintegration time (min)	3.5 ± 0.08	10
sDispersion time (min)	8 ± 0.4	15

§

around 1.6 min which is a considerably less time as compared to the myco-tablets (8 min).

Viability (cfu count)

To estimate the viability of the myco-granules, myco-tablet and myco-capsules during storage period, cfu count was performed on 0, 4, 8 and 12 month of storage (Table 2). The initial cfu count (cfu/ml) of myco-granules was found to be 6.20×10^{10} suggesting that rice flour is a suitable substrate for low cost formulation production using *B. bassiana*. The carbon and nitrogen content of rice flour was found to be 45.3% and 1.2%, respectively, and C/N ratio of the substrate was 37.75. The extent of sporulation by a fungus is affected by the C/N ratio of the substrate apart from the nature of strain used (Gao et al., 2007). For example, *Metarhizium* sp. produces maximum sporulation on a substrate of 160:1 C/N ratio whereas *Paecilomyces* sp. produces maximum sporulation at 10:1 C/N ratio. During the 12 month storage, the cfu count of myco-granules decreased to 2.79×10^{10} cfu/g. Similar decrease in cfu count of myco-granules with storage time was observed for *A. lentulus* from 6.6×10^{11} g^{-1} to 9.7×10^{10} g^{-1} within 6 month of storage time (Kaushik et al., 2015).

The initial cfu count for the myco-capsuleand myco-tablet was found to be 6.10×10^{10} and 5.81×10^{10}, respectively. The results show that operations conducted during myco-tablet production had more severe adverse effect on viability of fungal spores as compared to myco-capsules. Further, it was observed that cfu count of myco-capsules followed similar trend as myco-granules and reduced up to 45% of the initial count after 12 month of storage period. Reduction in cfu count for myco-tablet was more severe as compared to myco-granules and myco-capsules, with up to 65.5% reduction in cfu count after 12 month of storage. Several studies examined the effect of shelf life on insecticidal properties as well as germination percentage of *B. bassiana*. Sy et al. (2016) examined the germination percentage of *B. bassiana* formulation based on volcanic rocks (Palo Blanco pumice rock, Puyehue volcano pumice rock and zeolite clinoptilolite) and silica gel. It was observed that germination percentage reduced by 20–50% within the 30 days of storage at 30 °C. In another study, three formulations (bait, encapsulation and emulsion) of *B. bassiana* were tested in term of shelf life and insecticidal activity. Encapsulated formulation of *B. bassiana* retained 78% conidial viability, even after storage for 12 months at 30 °C and showed 54.8% mortality of housefly larvae (Mishra et al., 2013). The viability depends on the type of substrate used during formulation development. For example, addition of glucose in formulation media improved conidial viability and shelf life of the formulated *P. frequentans* conidia up to one year (Guijarro, Melgarejo, & De Cal, 2007). In the present

Table 2 cfu count of different formulation with storage time.

cfu count/g			
Time (months)	Myco-granules	Myco-capsules	Myco-tablets
0	6.20×10^{10}	6.10×10^{10}	5.81×10^{10}
4	5.95×10^{10}	5.94×10^{10}	5.10×10^{10}
8	4.65×10^{10}	4.50×10^{10}	3.93×10^{10}
12	2.79×10^{10}	2.80×10^{10}	2.01×10^{10}

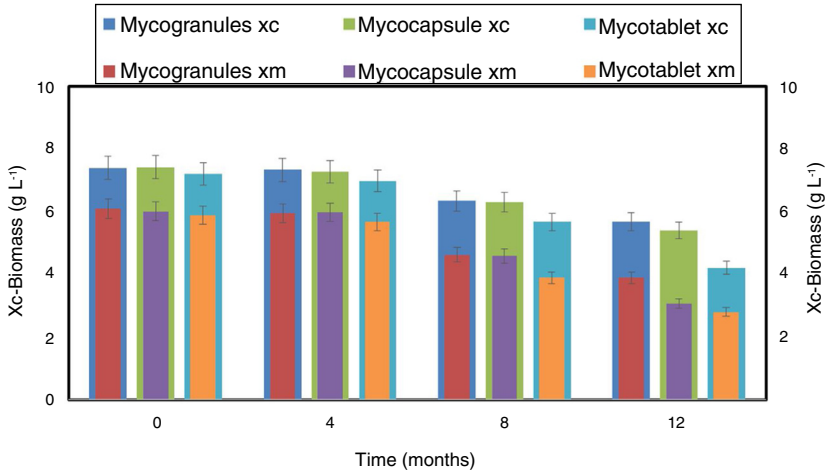


Figure 2 Comparison of biomass production by different formulation with storage time in absence (Xc) and presence (Xm) of multimetal ion.

study, such additives have not been used to minimize the process cost.

Biomass production and heavy metal removal efficiency of formulations

The products developed in this study were investigated for multimetal removal from the synthetic wastewater. Performance of formulated products was estimated in terms of biomass production and multimetal removal efficiency at 30 mg/L hexa metal mixture at every 4 months interval for 1 year. Comparison of biomass production by formulations at different storage time in absence (Xc) and presence (Xm) of multimetal ion is shown in Fig. 2. In the absence of metals, the reduction in biomass production with the increase in storage time was less prominent especially in case of myco-granules and myco-capsules. Up to 23.2% and 27.2% reduction in biomass was observed with myco-granules and myco-capsules, after 12 month of storage. On the other hand, maximum reduction in biomass was observed with myco-tablets (41.6%) after 12 month of storage and this reduction in biomass also correlates with the cfu counts.

In presence of multi-metals, biomass production did not decrease significantly during the first four month of storage and up to 2.4%, 0.4% and 3.7% reduction in biomass was observed for myco-granules, myco-capsules and myco-tables, respectively. However, with the increase in the storage period up to 8 and 12 months, biomass production capability of the different formulation deteriorated significantly in presence of multi-metals as shown in Fig. 2. Greater decrease in biomass production (52.4%) was observed for myco-tablet as compared to the myco-granules (41.1%) and myco-capsules (49.0%), after 12 month of storage period. Possible reason for the above observation is the compression pressure at which myco-tables are formed, that negatively impacted the overall viability of the spores as also reflected through the cfu count (Table 2). The resulting biomass therefore showed poor tolerance to multi-metal stress. Fig. 3 represent the multimetal removal obtained using all the three formulations. In the initial phase of studies, maximum removal (93.1%) was observed with myco-granules followed by myco-capsules (93.0%) and myco-tablet (83.5%). Gradual decrease in the multimetal removal efficiency was observed for all the three formulations with the increase in storage period. As compared to myco-granules and myco-capsules, higher decrease in multimetal removal was observed with myco-tablets (8.3%), during first 4 months that might be attributed to the low biomass production. After 12 months, up to 55.4%, 39.9% and 35% removal was achieved with myco-granules, myco-capsules and myco-tablet, respectively.

Kaushik et al. (2015) observed similar decrease in the biomass production by myco-granules produced using *Aspergillus lentulus* immobilized on two different bases. Up to 31.1% and 40.0% decrease in biomass production was observed with maize based and rice based myco granules, respectively, in a dye removal process after one year of storage of myco-granules at ambient temperature. In another study, same fungus was immobilized on organic base and

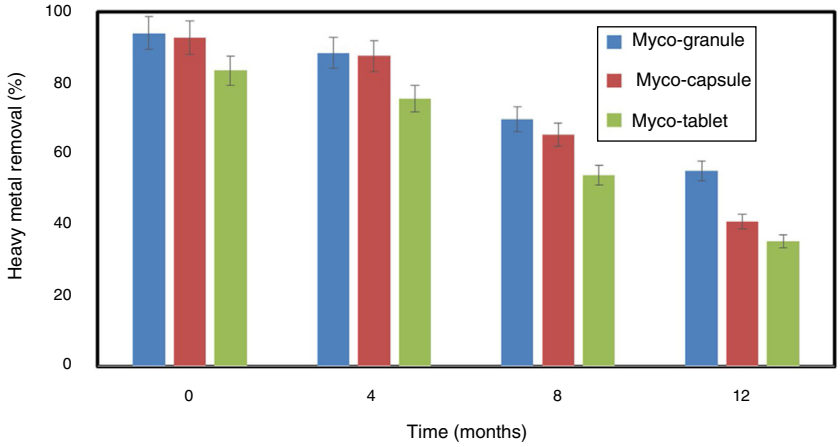


Figure 3 Multimetal removal (%) with time by myco-granules, myco-capsules and myco-tablet.

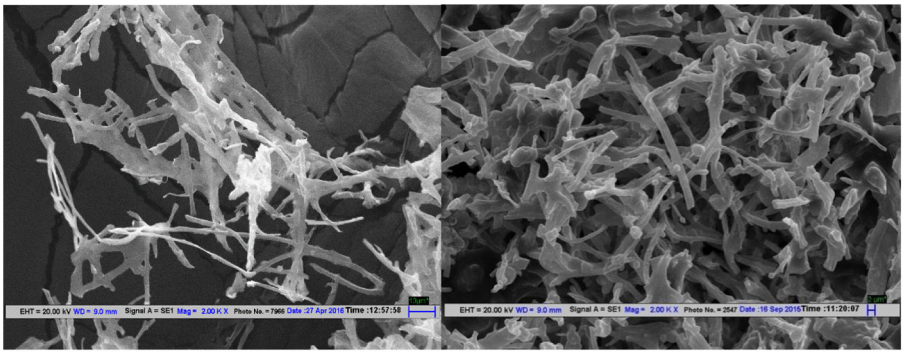


Figure 4 SEM images of fungal biomass grown under control (A) and multi heavy metal condition (B) by myco-granules after 12 month of storage at ambient temperature.

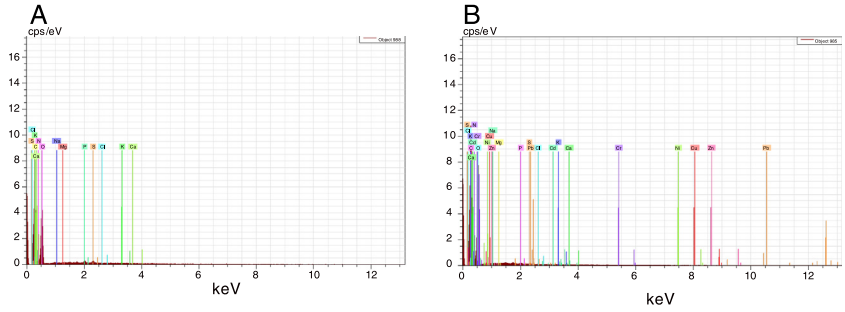


Figure 5 EDX monograph of fungal biomass grown under control (A) and multi heavy metal (B) condition by myco-granules after 12 month of storage at ambient temperature.

production of biomass was observed in presence of two different heavy metals (Cu^{2+} and Cr^{6+}). Up to 40.4% and 41.0% decrease in biomass production in the presence of Cu^{2+} and Cr^{6+}, respectively, was obtained after one year of storage at ambient temperature (Mishra, 2013). As a result, the Cr^{6+} removal ability of the formulation declined by 25.4% while Cu^{2+} removal declined by 20.3%.

All the above studies, investigated removal of single pollutant from synthetic wastewater, whereas present study deals with the removal of multiple metal from contaminated synthetic wastewater. Present study involved entomopatogenic fungus that can be used to treat irrigational wastewater contaminated with multiple metal. Further, none of the studies have evaluated the performance of structured products and the impact of product formulation on functionality of the strain. In the present study, it was observed that activities of the myco-capsules were comparable to that of myco-granules while mycotablets showed relatively poor performance. Further, the myco-capsules can be produced without a need for production equipment such as die, Automated Press Machine and careful control of tableting process conditions. Loss in viability is minimal during formulation of myco-capsules and thus, efficacy of metal removal using a particular mycocapsule is higher than

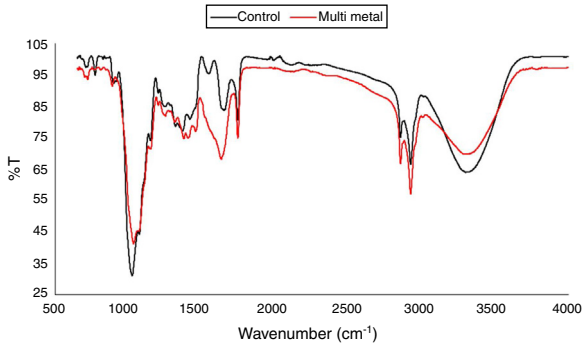


Figure 6 FTIR spectra of fungal biomass in control and presence of multi heavy metal.

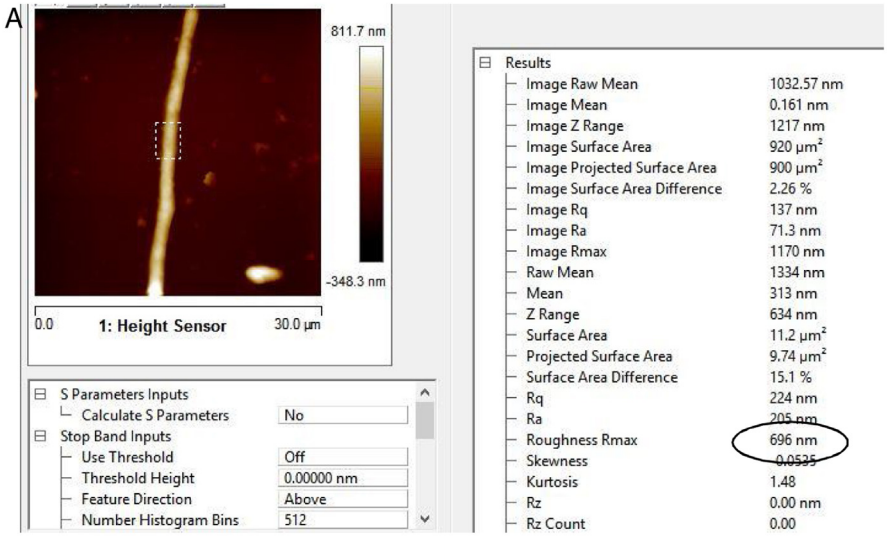
corresponding mycotablet. Further, contamination is prevented in myco-capsules as the process can be done in sterilized environment and post production the mycogranules are protected due to the encasing of the gelatin shell. Moreover, the myco-capsules can be colour coded and optimized for different organisms and therefore, can be used for specific applications. Further studies on the use of formulations with industrial or municipal wastewaters are needed in order to determine the actual performance of the product.

Morphological investigations of *B. bassiana* myco-granules

In order to substantiate the multi-metal removal by formulated *B. bassiana* after 12 months of storage, further investigations using SEM, EDX, FTIR and AFM were conducted on the biomass grown from myco-granules in absence as well as presence of multimetals.

SEM–EDX

The SEM images indicated significant changes in hyphal morphology between fungus grown in control and multimetal



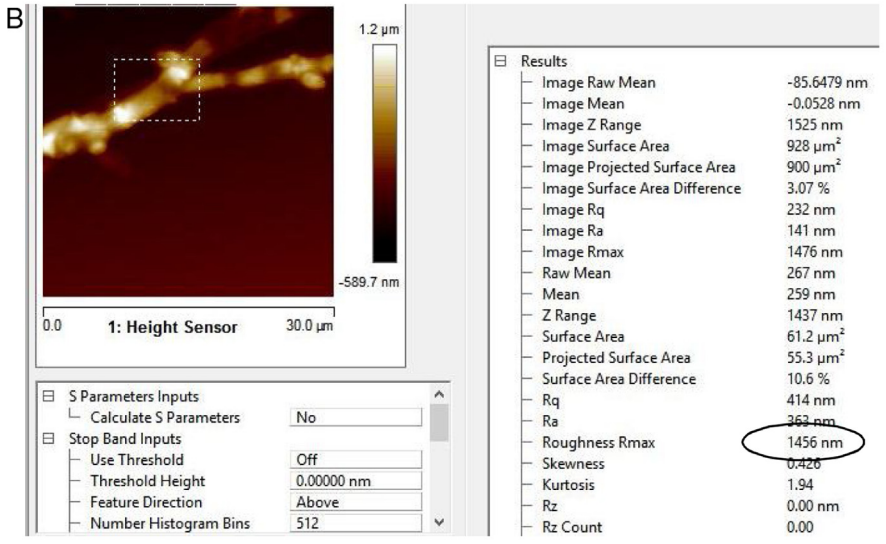


Figure 7 AFM images of fungal biomass in control (A) and multi heavy metal (B).

conditions, even after 12 month of storage when grown in composite media (Fig. 4). The hyphal morphology was clearly visible, mostly single stranded ribbon like structure, loosely packed and uniform in shape. Whereas in the presence of multimetal, hyphal morphology changed to more dense, tightly packed and aggregated structures. These changes might be due to the toxic response by fungus in the presence of multi heavy metal condition. The aggregation of hyphae act as a strategy to reduce the overall surface area hence area exposed to the multimetal ion gets reduced. Binding of heavy metals to the surface functional group present on cell wall and membrane causes loss in their integrity, hence causing aggregation and breakage of hyphae as evident from the SEM images. The EDX monograph of fungal biomass grown under control as well as in the presence of heavy metals is shown in Fig. 5. As compared to the control biomass, the peak intensity of alkali and alkaline earth metal decreased in the presence of multi heavy metal, this might be due to the binding of heavy metal to the cell surface via bio-sorption.

FTIR

FTIR spectra of lyophilized fungal biomass grown under control and in the presence of multi heavy metal were obtained in the range of 650–4000 cm^{-1} (Fig. 6). The FTIR spectrum provides the probable information about the surface functional groups which are responsible in binding and interaction with the heavy metals in term of shifts, stretching and masking of particular wavelengths formed by the functional groups. Wavelength shifts of following groups occurred in the presence of heavy metal: carboxylic group (O–H stretch), alkanes (C–H stretch), aldehydes (H–C=O: C–H stretch), aromatic amines (C–N stretch), aliphatic amines (C–N stretch) and 1°, 2° amines (N–H wag). Involvement of these functional groups in binding of various heavy metal such as Cd, Cr, Pb, Cu, Ni and Zn were studied with various micro-organism (Gola et al., 2016). Moreover, in the presence of multi heavy metal two new peaks at 1460 cm^{-1} and 1316.66 cm^{-1} appeared indicating towards the participation of these functional groups in the binding of heavy metals. Whereas complete masking of peak 1546.66 for nitro compound (N–O asymmetric stretch), was observed in the presence of multi heavy metals.

AFM

The AFM analysis of the fungal hyphae grown under control as well as in the presence of multi heavy metals condition is shown in Fig. 7. The Rmax values correspond to the surface roughness of the cell surface. increase in the cell surface roughness of the fungal hyphae grown in presence of multi heavy metal (Rmax 1456 nm) was observed as compared to the control one (Rmax 696 nm). Hence, about 2 fold increase in surface roughness was observed in presence of multi heavy metal. This might be attributed to the rupturing of cell surface due to the multi heavy metal toxicity. This increase in Rmax value in the presence of multi heavy metal correlate with the hyphal morphology observed via SEM images in the presence of multi heavy metal. A similar increase in the roughness values was obtained with fungus *Aspergillus* sp. and bacteria *E. coli* in presence of Cr and Ni, respectively (Das, Mukherjee, & Guha, 2008; Jing, Shiying, Lina, & Ning, 2007).

Conclusion

To solve the problem of storability and transportation, three different formulations (myco-granules, myco-capsules and myco-tables) were developed by immobilizing the fungal spores on an organic base. Multimetal removal ability with the increase in shelf life up to 12 month clearly indicated that the performance of myco-granules (55.4%) was superior as compared to myco-capsule (39.9%) and myco-tablet (35.0%). The developed formulations do not require any specific storage temperature and can be used as a source of inoculum for the treatment of metal contaminated wastewater. Further, performance of developed formulations can be tested with actual wastewater, so that they can be commercialized as cheap bioremediation technology.

Conflict of interest

The authors declare no conflict of interest.

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References

Abraham, J., & Silambarasan, S. (2014). Biomineralization and formulation of endosulfan degrading bacterial and fungal consortiums. *Pesticide Biochemistry and Physiology*, *116*, 24–31. http://dx.doi.org/10.1016/j.pestbp.2014.09.006

Adholeya, A., Tiwari, P., & Singh, R. (2005). Large-scale inoculum production of arbuscular mycorrhizal fungi on root organs and inoculation strategies. *In Vitro Culture of Mycorrhizas*, *4*, 315–338. http://dx.doi.org/10.1007/3-540-27331-X_17

Akbari, S., Abdurahman, N. H., Yunus, R. M., Fayaz, F., & Alara, O. R. (2018). Biosurfactants—A new frontier for social and environmental safety: A mini review. *Biotechnology Research and Innovation*, *2*, 81–90.

Asad, S., Mohammad, S., Dastgheib, M., & Amoozegar, M. A. (2014). Optimization for decolorization of azo dye Remazol Black B by a Halomonas strain using the Taguchi approach. *Progress in Biological Sciences*, *4*, 33–42.

Aust, S. D., Bumpus, J. A., & Tien, M. (1990). Methods for the degradation of environmentally persistent organic compounds using shite rot fungi. Google Patents.

Bradley, C., Kearns, R., Wood, P., & Black, W. (1996). *Bioremediation method using a high nitrogen-containing culture of white rot fungi on sugar beet pulp. US Pat 5,486,474.*

Das, S. K., Das, A. R., & Guha, A. K. (2009). Structural and nanomechanical properties of *Termitomyces clypeatus* cell wall and its

interaction with chromium(VI). *Journal of Physical Chemistry B, 113*, 1485–1492, 10.1021/jp808760f.

Das, S. K., Mukherjee, M., & Guha, A. K. (2008). Interaction of chromium with resistant strain *Aspergillus versicolor*: Investigation with atomic force microscopy and other physical studies. *Langmuir, 24*, 8643–8650, 10.1021/la800958u.

Dey, P., Gola, D., Mishra, A., et al. (2016). Comparative performance evaluation of multi-metal resistant fungal strains for simultaneous removal of multiple hazardous metals. *Journal of Hazardous Material*, *318* http://dx.doi.org/10.1016/j.jhazmat.2016.07.025

Eichie, F. E., & Kudehinbu, A. O. (2009). Effect of particle size of granules on some mechanical properties of paracetamol tablets. *African Journal of Biotechnology*, *8*, 5913–5916, 10.5897/AJB09.859.

Gao, Li, Man H., Sun., Xing Z., Liu., & Yong, S. (2007). Che.Effects of carbon concentration and carbon to nitrogen ratio on the growth and sporulation of several biocontrol fungi. *Mycological research*, *111*, 87–92.

Gola, D., Dey, P., Bhattacharya, A., Mishra, A., Malik, A., Namburath, M., et al. (2016). Multiple heavy metal removal using an entomopathogenic fungi *Beauveria bassiana*. *Bioresource Technology*, *218*, 388–396. http://dx.doi.org/10.1016/j.biortech.2016.06.096

Gola, D., Malik, A., Namburath, M., & Ahammad, S. Z. (2017). Removal of industrial dyes and heavy metals by *Beauveria bassiana*: FTIR, SEM, TEM and AFM investigations with Pb(II). *Environmental Science Pollution Research*, http://dx.doi.org/10.1007/s11356-017-0246-1

Guijarro, B., Melgarejo, P., & De Cal, A. (2007). Effect of stabilizers on the shelf-life of *Penicillium frequentans* conidia and their efficacy as a biological agent against peach brown rot. *International Journal of Food Microbiology*, *113*, 117–124. http://dx.doi.org/10.1016/j.ijfoodmicro.2006.06.024

Herbert, A., Lieberman, L., & Lachman, L. (1991). Pharmaceutical dosage forms. Tablet series. *Medicat Lozenges*, 339–467.

Onyishi, V. I. (2008). he role of ginger starch as a binder in acetaminophen tablets. *Scientific Research and Essays*, *3*, 46–50.

Jensen, M. P. (2011). The pain stethoscope: A clinician's guide to measuring pain. *Springer Science & Business Media*.

Jing, W., Shiying, H. E., Lina, X. U., & Ning, G. U. (2007). Transmission electron microscopy and atomic force microscopy characterization of nickel deposition on bacterial cells. *Chinese Science Bulletin*, *52*, 2919–2924. http://dx.doi.org/10.1007/s11434-007-0390-y

Kaushik, P., Mishra, A., Malik, A., & Sharma, S. (2015). Production and shelf life evaluation of storable mycogranules for multiple environmental applications. *International Biodeterioration and Biodegradation*, *100*, 70–78. http://dx.doi.org/10.1016/j.ibiod.2015.02.011

Lamar, R., Dietrich, D., & Glaser, J. (1995). *Solid phase bioremediation methods using lignin-degrading fungi*. US Pat 5,476,788.

Lieberman, H., Riger, K., & Banker, G. (1996). *Pharmaceutical dosage forms: Disperse systems*.

Mishra, A. Development of biological system employing microbial consortium for pollutant removal from mixed waste stream. PhD Thesis, Submitted to IIT Delhi, 2013.

Mishra, S., Kumar, P., & Malik, A. (2013). Preparation, characterization, and insecticidal activity evaluation of three different formulations of *Beauveria bassiana* against *Musca domestica*. *Parasitology Research*, *112*, 3485–3495. http://dx.doi.org/10.1007/s00436-013-3529-6

Mishra, S., Singh, S. N., & Pande, V. (2014). Bacteria induced degradation of fluoranthene in minimal salt medium mediated by catabolic enzymes in vitro condition. *Bioresource Technology*, *164*, 299–308. http://dx.doi.org/10.1016/j.biortech.2014.04.076

Panda, J., Kanjilal, T., & Das, S. (2018). Optimized biodegradation of carcinogenic fungicide Carbendazim by *Bacillus licheniformis* JTC-3 from agro-effluent. *Biotechnology Research and Innovation*, *2*(1), 45–57.

Prithiva, J. N., Ganapathy, N., & Jeyarani, S. (2017). Efficacy of different formulations of *Beauveria bassiana* (Bb 112) against *Bemisia tabaci* on tomato. *Journal of Entomology and Zoology Studies*, *5*, 1239–1243.

Saeed, M. B. E. E. E. M., Laing, M. D., Miller, R. M., & Bancole, B. (2017). Ovicidal, larvicidal and insecticidal activity of strains of *Beauveria bassiana* (Balsamo) Vuillemin against the cigarette beetle, *Lasioderma serricorne* Fabricius (Coleoptera: Anobiidae), on rice grain. *Journal of Stored Product Research*, *74*, 78–86. http://dx.doi.org/10.1016/j.jspr.2017.10.001

Sahoo, M. M., & Patra, K. C. (2018). Development of a functional water quality index: A case study in Brahmani River Basin, India. *International Journal of Environmental Science and Technology*, 1–14. http://dx.doi.org/10.1007/s13762-018-1933-2

Shah, M. P. (2014). Eco-friendly treatment of acid red by an application of *Pseudomonas* spp. *International Journal of Environmental Bioremediation & Biodegradation*, *2*, 62–68. http://dx.doi.org/10.12691/ijebb-2-2-3

Sonune, N., & Garode, A. (2018). Isolation, characterization and identification of extracellular enzyme producer *Bacillus licheniformis* from municipal wastewater and evaluation of their biodegradability. *Biotechnology Research and Innovation*, *2*(1), 37–44.

Sy, V. E., Schalamuk, S., Scorsetti, A. C., & Botto, I. L. (2016). Volcanic materials as carriers for the formulation of mycoinsecticides using the fungus *Beauveria bassiana*. *Ciencia e Investigación Agraria*, *43*, 10. http://dx.doi.org/10.4067/S0718-16202016000200010

Uhumwangho, M. U., & Okor, R. S. (2004). Anomalous effect of compression pressure on the brittle fracture tendency of α-cellulose tablets. *International Journal of Pharmacology*, *284*, 69–74. http://dx.doi.org/10.1016/j.ijpharm.2004.07.002

Uhumwangho, M. U., Okor, R. S., & Eichie, F. E. C. A. (2006). Influence of some starch binders on the brittle fracture tendency of paracetamol tablets. *Journal of Biotechnology*, *5*, 1950–1953.

Uhumwangho, M. U., Okor, R. S., & Adogah, J. T. (2009). Potential of carnuba wax in ameliorating brittle fracture during tableting. *Pakistan Journal of Pharmaceutical Sciences*, *22*, 58–61.

Vidyarthi, A., Tyagi, R., Valero, J., & Surampalli, R. (2002). Studies on the production of *B. thuringiensis* based biopesticides using wastewater sludge as a raw material. *Water Research*, *35*, 3807–3816.

Wang, L., Ma, L., & Yang, Z. (2018). Spatial variation and risk assessment of heavy metals in paddy rice from Hunan Province, Southern China. *International Journal of Environmental Science and Technology*, *15*, 1561–1572. http://dx.doi.org/10.1007/s13762-017-1504-y