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Production and shelf life evaluation of storable myco-granules for multiple environmental applications

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ABSTRACT

The present work describes a novel attempt to produce myco-granules targeted for multiple industrial applications including dye and heavy metal sequestration and xylanase production. Three low cost, locally available substrates FS1 (rice flour), FS2 (maize flour) and FS3 (wheat flour) were characterized and used for producing three myco-granules MG1, MG2 and MG3, respectively. Spore load of freshly prepared myco-granules and after 6 months of storage at 30 °C was estimated. MG1 exhibited least decrease (32%) in number of spores as compared to MG2 (40%) and MG3 (87%). Shelf-life studies conducted for different myco-granules stored at 4 °C, 30 °C and ambient temperature conditions show unaltered biomass production for the initial three months which rules out the need for controlled temperature conditions during storage. When stored at 30 °C, MG1 could remove 83% Acid Navy Blue dye, 57% Cu(II), >68% Cr(VI) (initial concentration 200 mg L^{-1} for dye and 50 mg L^{-1} for respective metals) and was able to produce 70.8 U g^{-1} crude xylanase activity through solid state fermentation after one year. The myco-granule MG1 has the potential of remediating wide varieties of wastewater containing dye and heavy metals as well as producing industrially important enzymes. Further, its prolonged shelf life under non-refrigerated conditions has highlighted its superiority over conventional means.

Introduction

In spite of the ample research on ability of specialized and efficient microbial strains for bioremediation of hazardous contaminants (Malik, 2004; Kaushik and Malik, 2009; Mishra and Malik, 2013), the actual application remains elusive due to non-availability of seed cultures. 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.

In order to ensure a successful implementation of biological system for remediation of pollutants at industrial scale, mass cultivation of microbes at lowest possible cost and its ease of storage are a pre-requisite. In this context, several low cost substrates have been attempted by the researchers for the mass production of microbes. Sugar beet pulp (Bradley et al., 1996) as well as waste products such as waste water sludge (Vidyarthi et al., 2002) has been utilized as the substrates for producing microbe based formulations. However, use of such waste products as the substrate for mass production of microbes is limited as they cannot be stored for more than three months due to the intrinsic decomposition which occurs even when stored at low temperatures (Vidyarthi et al., 2002). Therefore, there is a need to explore suitable products that can be stored for a longer duration.

Production of microbial formulations in the form of granules for use as bio-control agents is well documented (Kanjanamaneesathian et al., 1998; Zidack and Quimby, 1999; Quimby et al., 2002). Patent WO/2008/156380 (Halos and Halos, 2008), deals with the production of stable organic carrier-based microbial inoculants for crop production with a shelf life of more than 3 years. However, the microbial preparations were produced by growing *Azospirillum* spp. on complex medium, followed by lyophilization with powdered milk and supplementation with minerals and vitamins, starch and various inert substances such as talc, chalk and diatomaceous earth. Although formulated products have been reported for application as biocontrol agents (Shah et al., 2000; Dorner et al., 2003) and biofertilizers (John et al., 2010); the formulation of such products as a starting culture for use in waste water treatment and enzyme production is not yet documented.

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Few studies have been reported by Mishra et al. (2001) and Gupta et al. (2011) on the application of bacterial consortium immobilized on corn-cob for the bioremediation of oily sludge from land. Similarly, WO/2008/020818 (Tay and Jiang, 2008) describes the production of self aggregating granular microbial formulation for use in phenol degradation during wastewater treatment. Although the product does not require any support material, the shelf life is limited to approx. 6 months at 25 °C. Accinelli et al. (2010) reported the removal of selected pharmaceuticals including oseltamivir (Tamiflu) from wastewater with the help of spherical granules (diameter 3 mm) made of bioplastic Mater-Bi® type PE01S entrapping propagules of *Phanerochaete chrysosporium*. However, no shelf life studies were conducted in this regard. Another patent KR/1019960004038 (Baek et al., 1996) describes the immobilized cell formulation for wastewater treatment; however the production methodology uses complex and expensive ingredients. This clearly highlights the need of a microbial formulation which is multi-targeted in its bioremediation potential, can be developed using a low-cost and simple methodology and can be stored without refrigeration for a considerable duration of time.

The strain of *Aspergillus lentulus* isolated from textile unit effluent located at Baddi, India (Sharma, 2009) is an alkali, thermo and halo tolerant fungus (Kaushik and Malik, 2010a) with exceptional ability to remove different categories of dyes from dye mixtures and industrial effluents (Kaushik and Malik, 2013). It displays high resistance to multiple metals (Mishra and Malik, 2012) and removes nearly 100% Cr(VI) from synthetic solutions and effluents (Sharma et al., 2009, 2011). Its potential in treating textile (Kaushik and Malik, 2010b), pulp and paper (Kaushik and Malik, 2013), electroplating (Mishra and Malik, 2012) and common effluent treatment plant effluent (Sharma et al., 2011) as well as xylanase production (Kaushik et al., 2014) has already been demonstrated. In the present study, low-cost granulated formulation of *A. lentulus* has been produced using locally available substrate materials. The shelf-life of the granular formulation has been estimated at different storage conditions and its bioremediation potential has been estimated in terms of dye and heavy metal removal which are often the co-contaminants of various industrial effluents emanating from textile and pulp and paper industries. Further, the utility of granular formulation in producing xylanase enzyme which finds an extensive use in pulp and paper industry during pulp bleaching has been established.

Materials and methods

Test organism and substrate materials

An isolated fungal strain of *A. lentulus* was chosen for the present study. The culture of fungal isolate was maintained on the slants of potato dextrose agar and stored at 4 °C. For developing granulated formulation, three substrate materials were chosen; rice flour (FS1), maize flour (FS2) and wheat flour (FS3). The particle size of these materials was homogenized through sieving and <74 μm particle size was used. These substrate materials were characterized for carbon and nitrogen content using CHN analyzer (Vario EL CHNS).

Chemicals and reagents

Acid Navy Blue dye (C.I. name: Acid Blue 120) used in the present study was procured from Department of Textile Technology, IIT Delhi (India). Acid Navy Blue, an anionic azo dye, finds extensive use in textile industries where it is used for dyeing silk, woolen and nylon fabrics. Two heavy metals, Cr(VI) and Cu(II), were chosen for the present study and were used in the form of potassium dichromate [$K_2Cr_2O_7$] and copper sulphate [$CuSO_4.5H_2O$] salts, respectively. Stock solutions (10 g L^{-1}) of Acid Navy Blue dye and each of the heavy metals were prepared in distilled water. Absorption maximum for Acid Navy Blue was obtained by scanning the dye solution in the range of 400–700 nm using UV–Vis spectrometer (PerkinElmer Lambda 35). Analytical grade chemicals obtained from Merck and Himedia were used in the experiments.

Production and characterization of myco-granules

The substrate materials, FS1, FS2 and FS3 (5 g each) were kept in different flasks and sterilized by autoclaving at 121 °C and 15 lbs for 20 min. Five mL fungal spore suspension having 6.25×10^6 spores mL^{-1} was inoculated in each flask and moisture content was adjusted to 50–60% with the help of sterile distilled water. The flasks were then incubated at 30 °C for 5 days. After 5 days, contents of the flasks were taken out and dried overnight in an oven at 45 °C. The myco-granules obtained from FS1, FS2 and FS3 termed as MG1, MG2 and MG3, respectively, were transferred to sterile plastic containers and kept at different temperature conditions for further analysis. The size of the spores as well as the respective myco-granule was estimated with the help of inverted fluorescence microscope (Nikon Eclipse Ti–U) and analyzed using image analysis software NIS-ELEMENTS BR. The germination potential of the spores in the freshly prepared myco-granules and that after six months was estimated through the count of number of spores and colony forming units (cfu) in the aqueous suspension of the myco-granules. The cfu count of the myco-granules was estimated through serial dilution method wherein serially diluted water suspension of myco-granules was plated (spread plate method) on sterile Petriplates containing potato dextrose agar media and incubated at 30 °C for 4 days. The number of cfu produced was counted through colony counter. Spore count of freshly prepared myco-granules (diluted suspension of the myco-granules in water) and after six months of storage was estimated with the help of a hemocytometer visualized through phase contrast microscope (Leica DM 2500). The three substrate materials as well as their respective myco-granulations were characterized through Fourier Transform Infra-red (FTIR) spectroscopy and Scanning Electron Microscopy (SEM).

Potential applications of myco-granules

Dye removal potential

The myco-granules (MG1, MG2 and MG3) were stored at three different storage temperatures (4 °C, 30 °C and ambient temperature) for one year and evaluated for shelf life in presence of Acid Navy Blue. Under ambient conditions, maximum and minimum temperature fluctuated from 20 °C to 40 °C and 6–27 °C, respectively. Maximum and minimum relative humidity varied from 35 to 75% and 19–59%, respectively. A sample of myco-granule (0.1 g) was withdrawn every two months (till 12 months) and inoculated in 100 mL sterile composite media (glucose 10.0 g L^{-1}, yeast extract 2.5 g L^{-1}, NH_4NO_3 0.5 g L^{-1}, $MgSO_4.7H_2O$ 0.1 g L^{-1}, K_2HPO_4 0.5 g L^{-1}, NaCl 1.0 g L^{-1}, pH 6.5 ± 0.2) amended with 200 mg L^{-1} Acid Navy Blue dye. After 24 h of incubation (at 150 rpm and 30 °C), the biomass was filtered out and measured. Residual dye concentration in filtrate was measured as discussed earlier.

Metal removal potential

Two best performing myco-granules: MG1 and MG2 (stored at 30 °C) were evaluated for shelf life (biomass production) and Cu(II) and Cr(VI) removal efficiency at an interval of two months for one year. Weighed amount (0.1 g) of each formulation was added into the sterile composite media in presence of respective metal (initial