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Bioremediation of Carbendazim by *Streptomyces albogriseolus*

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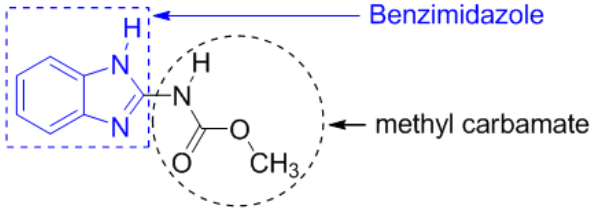
ABSTRACT
Carbendazim (methyl-1H-benzimidazol-2-ylcarbamate, or MBC) is a benzimidazole fungicide which is used to protect crops against the attack of fungi. MBC has a half-life of about 3-12 months and remain persistent in the environment which may lead to many harmful consequences. Besides chemical and photo-catalytic degradation of pesticides, microbial degradation has now been evolved as a much effective and safer way to eliminate these harmful compounds from the environment. However, in the literature very few reports are available where microbial community is involved in degrading MBC. Hence, the present study was planned to investigate the role of microbes isolated from the field soils for the bioremediation of MBC. Soil samples were collected from wheat fields of northern regions of India. Enrichment culture technique was employed to isolate the bacterium which was found to be growing at higher concentrations of MBC up to 500µg/ml. After biochemical and morphological analysis, the bacterium was identified as *Streptomyces albogriseolus*. *Streptomyces albogriseolus* was found to degrade MBC in a time-dependent manner from the initial concentration of 29 ppm to 285.67ppb and 62.73ppb in 24hrs and 48hrs respectively. LCMS-MS analysis was carried out to detect 2-aminobenzimidazole, a metabolite formed after degradation in 10 hrs of growth which eventually disappeared after 24hrs of growth. The strain *Streptomyces albogriseolus* holds a promising potential to be an efficient MBC bioremediation agent.
Keywords: Bioremediation, Carbendazim, Pesticide, *Streptomyces albogriseolus*

1. INTRODUCTION
Environmental pollution has significantly increased in many regions around the world due to extensive use of pesticides, herbicides and insecticides in agriculture. It is assumed that the use of pesticides would increase even more posing an adverse effect to human health and the environment in near future. Pesticides along with their degradative products get accumulated in top layer of soil and have become a serious threat not only to humans, but also to soil microbes especially nitrifying and ammonifying microbes. In India, alarming levels of pesticides have been reported in air, water, soil as well as in foods and biological materials [1]. Many of these pesticides are found to be toxic [2], mutagenic, carcinogenic and tumerogenic [3]. Increasing application of MBC in greenhouse production of vegetables and medicinal herbs necessitate the ways to remediate MBC contaminated soil. MBC is a systemic benzimidazole fungicide which is officially registered and used in many countries around the world to control a broad range of fungal diseases in agricultural crops [4-5].



Scheme 1. Cabendazim.

MBC was detected in fruits and leaves of plants even after they had been harvested [6-7]. Yao et al. (2010) investigated 208 litchi soil samples of Guangdong area of China and found cypermethrin (59.1%), MBC (51.0%), mancozeb (11.1%), metalaxyl (6.7%), cyhalothrin (3.4%) as prominent contaminants [8]. Similarly Shen et al (2009) detected MBC residues in the soil samples [9]. A high percentage of honey, honeybees and pollens were found to be contaminated by pesticides and fungicides like MBC [10]. A number of physico- chemical processes have been employed for the remediation of MBC from the contaminated sites. Rajeswari and Kanmani used TiO_2-based heterogeneous photocatalytic treatment combined with ozonation for MBC degradation [11]. Thermal Adsorption and Catalytic Photodegradation were also used for degradation of MBC [12]. However expensive treatment and incomplete remediation process producing metabolites which are more persistent and equally or more toxic to non-target organisms urge to look for alternative remedies [13]. Bioremediation being cost effective and ecofriendly, thus presents a promising alternative for remediation of MBC. Some microbial strains have been reported in literature capable of degrading MBC. *Pseudomonas* sp. isolated from soil was found to degrade MBC (87.1% and 99.1%) at a conc. of 1.0 and 10.0 mg/l respectively [14]. Two bacterial strains XJ-D and XJ-H identified as *Rhodococcus erythropolis* and *Azospirillum brasilense* respectively could use MBC as a sole carbon or nitrogen source [15]. *Rhodococcus jialingiae* sp. was successfully isolated from the sludge of a MBC wastewater treatment facility in Jiangsu province, China which was found to be a novel MBC-degrading actinobacterium [16].

2. EXPERIMENTAL SECTION

Soil samples were collected from wheat fields of various cities of Punjab and Haryana (Northern India). Micro-organisms were selected by enrichment culture technique with MBC (25µg/ml) addition to Jensen's Medium. The number of microbes was established as colony forming units/ml (CFU/ml). In order to assess the effect of MBC on growth, the isolated microbial strain was grown in media containing different concentrations of MBC (25µg/ml, 50µg/ml and 500µg/ml respectively). Cells were stained according to classical Gram's staining and Endospore staining procedures. Morphological, biochemical and physiological analysis of the isolated strain was done at Microbial Type Culture Collection, IMTECH, Chandigarh, India. Samples were collected at 10 hrs, 24hrs, and 48hrs growth of isolate in Jensen's medium containing MBC (~30ppm) at 37^{o}C. Liquid Chromatography Mass Spectrometry (LCMS-MS) analysis of the above samples was carried out to assess the degradation products or metabolites of MBC at Punjab Biotechnology Incubator, an NABL accredited Agri and Food testing lab, Mohali (Punjab) India.

3. RESULTS SECTION

3.1. Enrichment, isolation, and characterization of *Streptomyces albogriseolus.* MBC-degrading enrichment culture was established from MBC-exposed soil samples. Different dilutions of the enrichment were plated onto nutrient agar plates having a gradient of MBC concentration (25µg/ml, 50µg/ml and 500µg/ml respectively). Isolated colonies were picked and pure cultures were maintained. A spot on lawn assay was performed to evaluate the ability of the strain to grow at different MBC concentrations (Table 2). The strain exhibited growth even upto 500µg/ml. Upon biochemical analysis, strain was found to be a gram-positive, spore forming bacterium; *Streptomyces albogriseolus* did grow well under alkaline conditions (pH8-10) and was found to have starch hydrolase and catalase properties (Table 1).

3.2. Growth on MBC and metabolite identification. An initial concentration of ~30 ppm MBC was provided as a sole source of carbon in minimal medium. The initial ~26 ppm of MBC in this enrichment culture was rapidly degraded within 24 hrs. (Fig. 1). *Streptomyces albogriseolus* exhibited high MBC-degrading efficacy with ~98% of the fungicide MBC removed within 48 hrs. *Streptomyces albogriseolus* was found to reduce MBC concentration from 29.12ppm to 25.53ppm in 10hrs to 285.67ppb and 62.73ppb in 24hrs and 48hrs respectively (Fig 1). Single metabolite peak appeared in the LCMS-MS chromatogram during the *Streptomyces albogriseolus* growth after 10 hrs on MBC (Fig 2), while no other metabolites were detected in 24hrs and 48hrs growth samples. The metabolite was identified as 2-aminobenzimidazole (2-AB) which is a major MBC degradation product in soils, plants, and mammalian systems.

3.3. Discussion. MBC is known to be highly stable in the environment and only a few bacterial strains have been found to degrade this fungicide. Strains from the bacterial genus *Rhodococcus* such as *R. erythropolis* [17], *Rhodococcus qingshengii* [18-19], *Rhodococcus jialingiae* [16], *R. erythropolis* dj l-11 [20], *Brevibacillus borstelensis* [21] quite effectively degrade MBC utilizing MBC as a sole carbon and nitrogen source. In the present study we explored *Streptomyces albogriseolus* for the first time to remediate MBC. *Streptomyces albogriseolus* exhibited high MBC-degrading efficacy, with ~98% of the fungicide MBC removed within 48 hrs. The remediation efficiency was comparatively higher than *Rhodococcus erythropolis* djl-11 with 95% removal after 48 hours of duration as reported elsewhere [20]. A single metabolite 2-aminobenzimidazole (2-AB) was found which was highly unstable and may have further reduced to smaller compounds in consistent with the earlier studies reported in literature [18-20,22-23]. However in other reports the metabolite 2-hydroxybenzimidazole (2-HB) has also been found in that time period upon MBC degradation [20,23].

Pesticides function by targeting particular enzymes with essential physiological roles and thus are susceptible to enzyme-mediated degradation. The conversion of MBC to 2-AB, 2-HB and to further smaller compounds probably employs enzymes viz. transferases, isomerases, esterases, hydrolases and ligases. Pandey et al isolated a novel MBC-degrading esterase encoded by gene, *mheI* from *Nocardioides* sp. Strain SG-4G [23]. Similar studies by Zhang et al isolated another MBC-hydrolysing esterase encoded by *Mhe* gene from *R. erythropolis* djl-11 [20]. Both strains were reported to utilize enzymatic hydrolysis as the first step in the catabolism and detoxification of MBC to 2-AB and 2-HB. Further studies are still warranted to isolate MBC degrading gene from *Streptomyces albogriseolus* and decipher the exact mechanism of MBC degradation to 2-AB and other degradation compounds.

Table 1. Biochemical Characteristics observed for *Streptomyces albogriseolus*

TESTS	*Streptomyces albogriseolus*	**TESTS**	*Streptomyces albogriseolus*
Gram Staining	+	Indole	-
Motility	-	Catalase	+
Spore staining	+	Oxidase	-
Growth at 15^{o}c	-	Urea	-
Growth at 25^{o}c-40^{o}c	+	Acid production from	
Growth at pH 5.2	-	Arabinose	-
Growth at pH 8.0-10.0	+	Galactose	-
Growth on NaCl 2%-5%	+	Glucose	-
Growth on NaCl 7%	-	Mannitol	-

Growth on NaCl 10%	-	Raffinose	-
Starch hydrolysis	+	Salicin	-
Casein hydrolysis	-	Sucrose	-
Citrate utilization	-	Rhamnose	-
MR	-	Meso-inositol	-
VP	-	Fructose	-
Nitrate reduction	-		

Table 2. Effect of different concentrations of MBC on the growth of ***Streptomyces albogriseolus***

Isolate	Conc. of pesticide-MBC	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Streptomyces albogriseolus	25 µg/ml	Confluent	Confluent	26	19	15
	50 µg/ml	Confluent	Confluent	24	32	23
	500 µg/ml	Confluent	Confluent	30	22	19

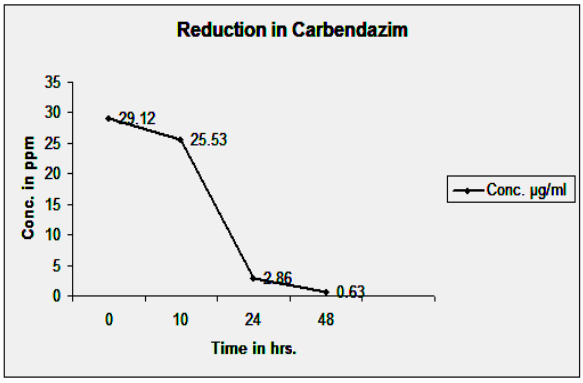


Figure 1. Reduction in MBC concentration by *Streptomyces albogriseolus* with respect to incubation time for growth.

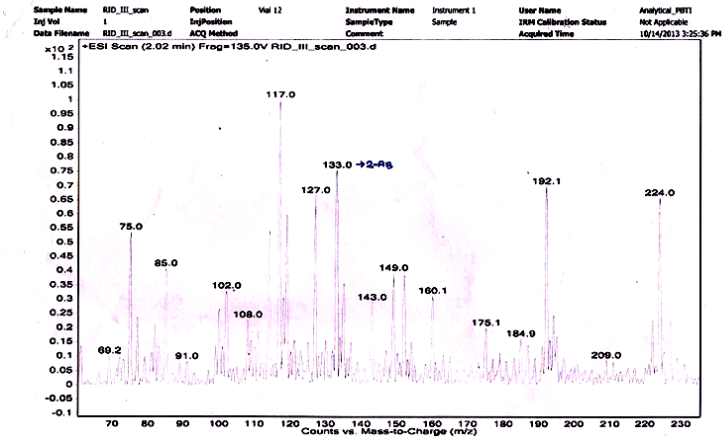


Figure 2. Single metabolite detected by LCMS-MS observed as major peak was 2-aminobenzimidazole in 10 hrs growth.

4. CONCLUSIONS

The strain *Streptomyces albogriseolus* has been reported to degrade MBC to 2-Aminobenzimidazole (2-AB) which becomes undetectable after 48 hrs. It could be possible that 2-AB could have been metabolized to further smaller compounds or even to CO_2. Further studies are in progress to delineate the mechanism of biodegradation so that this strain could be potentially used to bio-remediate MBC from contaminated sites in the near future.

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