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Influence of humic acid, iron and copper on microbial degradation of fungicide Carbendazim

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ABSTRACT

Carbendazim (CBZ) is a systemic fungicide utilized for the control fungal diseases in forestry and agricultural crops. Its severe toxicity and residual nature mandated its removal and biodegradation by biological, eco-friendly and sustainable methods. Towards this direction, we searched for CBZ utilizing bacterial strains from the agricultural soils. Based on morphological, biochemical and molecular characterisation the isolates were identified as *Streptomyces* sp. CB1, *Bacillus subtilis* CB2, *Pseudomonas aeruginosa* CB3 and *Rhizobium leguminosarum* CB4. The effect of CBZ on isolates in the presence and absence of Fe(II), Cu(II) and humic acid was determined. It was found that humic acid supplementation resulted in enhanced and extended growth of bacterial isolates. Among the four isolates, *P. aeruginosa* CB3 exhibited highest CBZ degradation followed capability by *B. subtilis* CB2, *Streptomyces* sp. CB1 and *R. leguminosarum* CB4. Supplementation with Cu(II) ions resulted in higher degradation of CBZ. However, humic acid was only moderately effective, whereas Fe(II) failed to show any appreciable increase in CBZ degradation by bacterial isolates. Our study suggests that CBZ adapted bacterial strains can offer a biologically and sustainable solution for bioremediation of contaminated agricultural fields and reservoir sites.

1. Introduction

Carbendazim (CBZ) is a systemic carbamate fungicide, introduced in 1973 and first time registered with USEPA in 1974 (Fang et al., 2010; Pohanish, 2014). Presently, its annual consumption is more than 2000 metric tons which makes it more popular fungicide (Singh et al., 2016). It is widely used in pre and post harvest applications to control the wide class of insects, bugs and microorganisms (Singh et al., 2016). Maximum use of CBZ is for the fungal diseases food and crops such as beet, banana, cereals, fodder rape seed, mango, oranges, pomes, pineapples, strawberries, medicinal herbs turf grasses and ornamental plants (Tortella et al., 2013). Its various combinations with other pesticides have been reported and better activities claimed by various authors e.g. combination CBZ + mancozeb is recommended to control fungal disease in mangoes and sunflower (Devi et al., 2015; Singh et al., 2016). In India, it is registered for 18 crops including apples, beans, brinjal, barley, mango, cucurbits, cotton, grapes, groundnut, jute, peas cluster, paddy, rose, sugar beet, wheat, walnut and tapioca (Bhushan et al., 2013). Recently, its use has been approved in the leather and paint industry to increase the quality and life time of the material (Selmanoglu et al., 2001).

WHO and European Commission classify CBZ in the hazardous list of chemicals and categorized in the list of top most endocrine disruption chemicals (Singh et al., 2016). Because of its massive application and high mobility, CBZ has often been detected in both aquatic and terrestrial ecosystems at concentration above the permissible limits (Singh et al., 2016). Its monotonous applications lead to contamination and accumulation with long lasting impact on animal health, human health and soil sustainability. The increasing concern of CBZ contamination, along with its toxicological properties has prompted researchers to strive biodegradation options for CBZ contaminated sites (Singh et al., 2016). Unlike other carbamate, CBZ is often exclusively and preferentially used as carbon and nitrogen sources for microbial population (Singh et al., 2016).

Several bacterial and fungal species have been reported for biodegradation of CBZ in both soil and aquatic ecosystems. The major species

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are: *Azospirillum, Aeromonas, Alternaria, Bacillus, Brevibacillus, Nocardioides, Pseudomonas, Ralstonia, Rhodococcus, Sphingomonas, Streptomyces* and *Trichoderma* (Zhang et al. 2005; Xu et al., 2006; Fang et al., 2010; Lin et al., 2011; Xiao et al., 2013; Singh et al., 2016). The mineralization of CBZ is achieved by hydroxylation of the parent molecule through C–N bond resulting in the formation of metabolites such as 2-aminobenzimidazole, benzimidazole and 2-hydroxybenzimidazole. The enzymes intricate in this step of hydroxylation have extensive substrate specificity and can perform a variety of reactions on different substrates.

Moreover, humic substances are known to increase soil health and crop yield through the complex mechanisms (Tan and Binger, 1986; Senesi and Loffredo 1999; Wright and Lenssen, 2013; Fahramand et al., 2014). Humic acid significantly reduced the toxicity of many pesticides like azinophos-methyl, chlorpyrifos, carbofuran but enhance the toxicity of methyl parathion and carbaryl (Singh et al., 2018; Kutman et al., 2013; Kumar et al., 2018a & 2018b; Kaur et al., 2017; Kumar et al., 2016; Kumar et al., 2015a, 2015b, 2015c, & 2015d). These findings suggest that humic-pesticide interactions can alter the toxicity of agricultural chemicals (Benson and Long 1991; Shehata et al. 2014).

In this study, we have evaluated the biodegradation potential of some bacterial strains against CBZ and the influence of humic acid and metal ions (Cu(II) and Fe(II)) on bacterial biodegradation efficiency. The major reason for the selection of Cu(II) and Fe(II) was the multiple applications of both metal ions in the living system and total environment. The iron (Fe(II)) and copper (Cu(II)) are very important micronutrients of soil (Long et al., 2004; Wasim et al., 2009). These ions improve the plant health through complex mechanism. Improper distributions of these ions may alter the crop production and human health too (Long et al., 2004; Wasim et al., 2009).

2. Materials and methods

2.1. Chemicals and reagents

The CBZ (Analytical grade 98.0% pure) was purchased from Rallis India Ltd. (Mumbai, India). Minimal salt media consisted of; Magnesium sulphate, Ammonium sulphate, Di-potassium hydrogen phosphate, Calcium sulphate, Ferric chloride, Humic acid, ferrous sulphate, Copper chloride, solvents and biochemicals were purchased from Loba Chemie (Mumbai, India) and from Himedia (Mumbai, India) and all were of highest AR grade.

2.2. Isolation of microorganisms from soil samples

Soil samples were taken from vegetables growing agricultural fields of eight different villages of Kapurthala, Punjab, India with a long history of repeated CBZ applications (East Longitude 75.38 E, and Latitude 31.38 E, Elevation height 225 m). List of eight villages of district Kapurthala of state Punjab (India) as: Noorpurdona, Dhariwal Dona, Kharsona, Dhadwandi, Tiba, Mothanwal, Talwandi Chodrian and Bholana.

All soil samples were sieved using 2 mm pore sized sieve and thoroughly homogenized using a pestle and mortar. Bacterial isolates were obtained by enrichment culture technique; particular microbes were isolated on the basis of their CBZ usage capability. About 5 g soil sample was inoculated in 50 ml of Erlenmeyer flask containing mineral salts medium (0.4 g $MgSO_4.7H_2O$, 0.2 g $FeSO_4$, 0.2 g K_2HPO_4, 0.2 g $(NH_4)_2SO_4$, 0.08 g $CaSO_4$, 1000 ml distilled water; at pH 7.0) supplemented with CBZ at 1000 mg/L concentration as a sole source of carbon. The cultures were incubated at 30°C for 14 days on an rotatory shaker (150 rpm) under dark conditions. After 14 days, 1 ml culture suspension was inoculated to fresh minimal salts medium containing CBZ (1000 mg/L CB) and incubated same for 14 days. The cultures were acclimatized 5 times with CBZ and thereafter sub-cultured in the presence of CBZ containing agar. Four bacterial cultures namely, CB1, CB2, CB3 and CB4 obtained through enrichment technique were acclimatized four times before their cultivation on minimal media.

2.3. Molecular characterization of bacterial isolates

16S ribosomal RNA (rRNA) gene analyses of the individual bacterial isolates were conducted at Samved Biotech Pvt. Ltd. (Ahmadabad, India). To authenticate the identity of all the four isolates, gene fragmentation of 1.5 kb 16 s rRNA was amplified using the total DNA of each isolates, and later on sequenced using the universal primers 1492R (ACCTTGTTACGACTT) and 27F (AGAGTTTGATCMTGGCTCAG) (Kumar et al., 2018a; Hasegawa et al., 1985; Fang et al., 2010; Jing-Liang et al., 2006). The DNA extraction from bacterial isolates, their amplification by using PCR and phylogenetic analysis were performed as given by Sun et al. (2014) and are well defined in the figures section.

2.4. Inoculum preparation for CBZ-degradation studies

Bacterial strains were cultured in minimal salts medium containing 1000 mg/L of CBZ at 30°C for 24 h in a shaker incubator at 120 rpm. Cultures were homogenized at 6500 × *g* for 5–6 min, then washed four times with fresh medium and cell counts were adjusted to 10^7 cells/ml using 0.5 McFarland standards.

2.5. Experimental setup for bacterial degradation of CBZ

Bacterial culture (100 µl) containing 10^6 cells were inoculated into 250 ml Erlenmeyer flask of minimal salts medium containing 1000 mg/L CBZ. At different time intervals (0, 3, 7, 10 and 14 days), growth parameters were quantified by assessing absorbance at 600 nm with UV spectrophotometer (Shimadzu, India) and the degradation of CBZ were determined as described earlier by Fan et al. (2012). The amount of CBZ degraded (or metabolized) by bacterial isolates was determined according to procedure described earlier by Fang et al. (2010). Un-inoculated medium with 1000 mg/L CBZ served as control. Experimental setups were designed to evaluate the effects of humic acid, Fe(II) and Cu (II) on CBZ degradation by the bacterial isolates. Each experiment was performed in triplicates to ensure efficacy.

2.6. HPLC analysis of CBZ in cultures

Samples for HPLC were sent to Herbal Health Research Consortium Private Limited Amritsar (HHRC Amritsar) and were prepared by using liquid-liquid extraction method in which 50 ml of the culture medium was completely transferred to a separating funnel and extracted 3 times using chloroform (50 ml). After that, the organic phases were collected and the samples were dehydrated in a rota-vapour condenser using anhydrous sodium sulphate and condensed to almost dryness with a light blow of N_2 steam. The contents were dissolved in methanol followed by HPLC analysis (Make up: Agilent Technologies 1200) fixed with diode array detector (DAD detector) using CBZ (98.0% pure) as a standard. The chromatographic separation was performed on an Eclipse XDB- C18 column (4.6 mm × 150 mm, particle size: 5-µm) at 25°C temperature by measuring absorbance at 281 nm with an elution of water and methanol mixture (55:45v/v) with flow rate of 0.8 ml/min (Fang et al., 2010; Kumar et al., 2017; Kumar et al, 2014; Prasad et al., 2013; Singh et al., 2017).

2.7. Statistical analysis

Biodegradation of CBZ by all the four strains (CB1, CB2, CB3 and CB4) was assessed by comparing differences in CBZ concentration between different treatments groups, each consisting of three replicates. All data were analyzed by analyses of variance (One way-ANOVA), using using statistical software GraphPad Prism ver. 5.0 to determine statistical significance in the variation of rhizobacterial inoculation

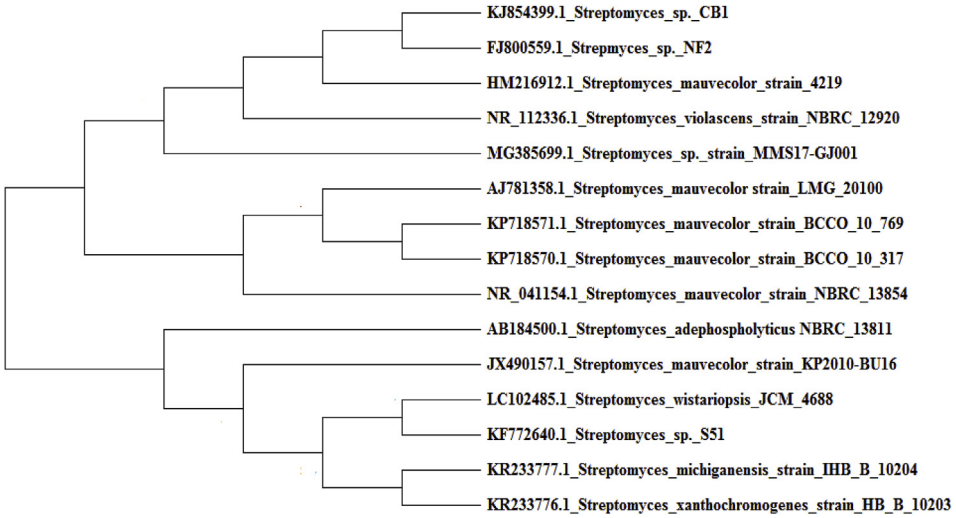

Fig. 1. Phylogenetic tree of isolate CB1 (*Actinomyces* sp.) based on 16S rRNA gene sequence analysis.

against control.

3. Results

In the search of CBZ-degrading bacteria, after in-depth study on the soil samples of eight villages based on preliminary studies, we have isolated and characterized four bacterial isolates from the soil samples originated from agricultural lands used for corn and rice farming since several crop seasons. The isolate CB1 was a Gram-positive, facultative anaerobic and filamentous bacterium with branched hyphae and exhibited 99% 16S rRNA gene sequence homology with *Streptomyces* sp (Figure - 1). Isolate CB2 was a Gram-positive bacillus having close sequence relatedness to *Bacillus subtilis* (Figure - 2). Isolates CB3 and CB4 were both Gram-negative and closely related to *Pseudomonas aeruginosa* and *Rhizobium leguminosarum*, respectively identified on the basis of 16S rRNA gene sequences (Figs. 3 and 4). The bacterial DNA sequences were submitted to GenBank with accession No. KJ854399.1, KJ854400.1, KJ854401.1 and KJ854402.1 (Figs. 1–4).

The bacterial isolates were evaluated for their ability to utilize CBZ as the sole source of carbon and energy. In 7 days samples of *Streptomyces* sp. or CB1, there was a threshold increase in optical density from 0.026 to 0.51 indicating consumption of CBZ as a source of carbon and energy (Supplementary - 1). With the addition of 1000 mg/L of Cu(II), the growth of *Streptomyces* sp. witnessed significant increase with concomitant increase in CBZ degradation as compared to CBZ alone group. A similar pattern was observed with Fe(II) and humic acid supplemented groups. In case of *B. subtilis* CB2, cell growth and CBZ decomposition rate sharply increased when supplemented with Cu(II), Fe(II) and humic acid. Comparable growth kinetics and degradation patterns were observed with *P. aeruginosa* CB3 and *R. leguminosarum* CB4.

HPLC analysis was performed and the degradation results of various combinations were compared with standard plot (Supplementary Table 1 and Fig. 2A-2D). The percentage degradation of CBZ by bacterial isolates CB1 to CB4 was determined in minimal salts media at a concentration of 1000 mg/L. Our findings have indicated the maximum CBZ degradation (73.73 ± 6.10%) by *P. aeruginosa* CB3 followed by CB2 (65.69 ± 2.82%), CB1 (59.04 ± 3.86%) and CB4

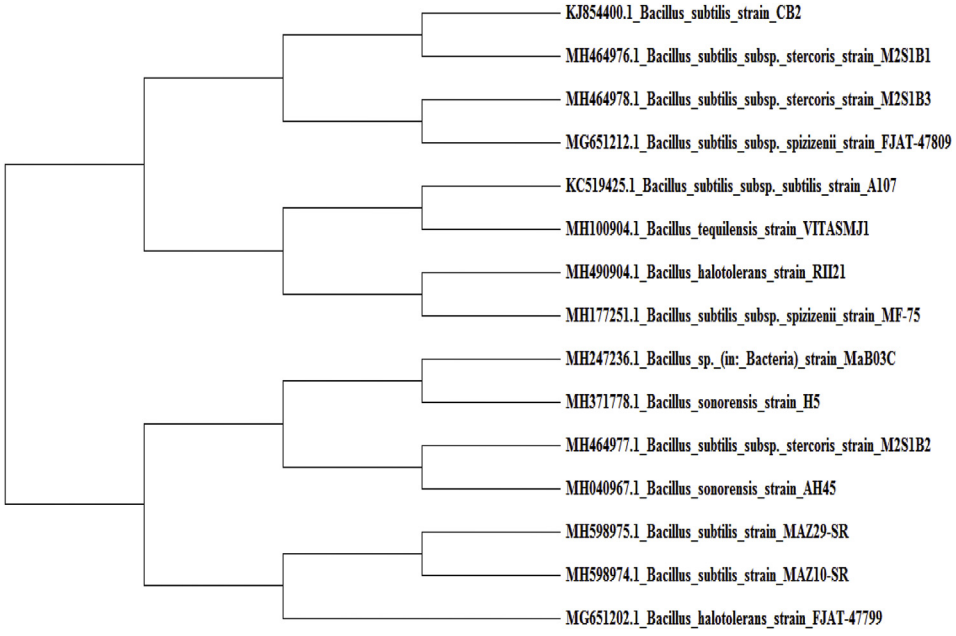

Fig. 2. Phylogenetic tree of isolate CB2 (*Bacillus subtilis*) based on 16S rRNA gene sequence analysis.

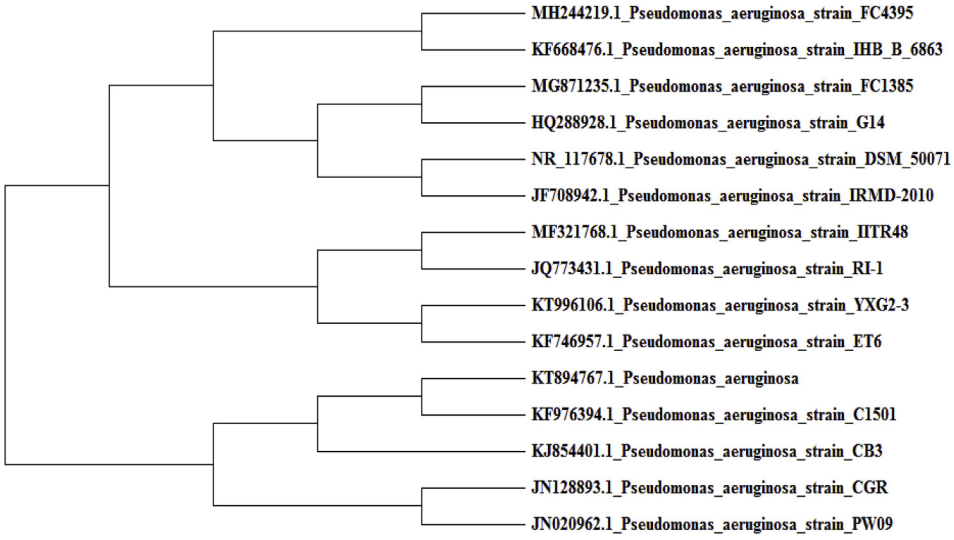

Fig. 3. Phylogenetic tree of isolate CB3 (*Pseudomonas aeruginosa*) based on 16S rRNA gene sequence analysis.

(55.29 ± 3.52%) in the 3 days incubation samples (Figure - 5). On the other hand, longer incubation period of 7 days resulted in highest degradation in CB1 (91.65 ± 3.00%) followed by CB3 (87.35 ± 4.10%), CB2 (81.85 ± 2.90%) and CB4 (76.54 ± 2.15%). After 14 days, the degradation in CB1 (94.03 ± 2.75%) followed by CB3 (89.11 ± 3.21%), CB2 (84.77 ± 3.12%) and CB4 (79.67 ± 2.55%). The results of 14 days are approximately close to that of 10th days results means there are very small degradation.

Effect of humic acid on biodegradation results in the removal of CBZ at a slower rate after 3 days. The degradation percentage was highest in CB1 (82.18 ± 3.63%), CB3 (81.9 ± 3.54%), CB2 (67.33 ± 2.65%) and CB4 (45.36 ± 4.10%). After 7 days the degradation was highest in CB2 (98.92 ± 2.82%) as compared to CB1 (94.92 ± 3.82%), CB3 (92.12 ± 6.15%) and CB4 (61.26 ± 3.42%). After 14 days the degradation was highest in CB2 (99.03 ± 3.77%) as compared to CB1 (97.52 ± 3.74%), CB3 (94.16 ± 4.02%) and CB4 (69.98 ± 2.27%) which are similar to that of 10th day result.

Effect of Cu(II) on the degradation of CBZ is quite high as compared to un-supplemented group (Fig. 2). The degradation percentage after 3 days were highest in isolate CB3 (93.73 ± 3.40%) followed by CB1 (86.72 ± 3.50%), CB2 (85.4 ± 2.53%) and CB4 (83 ± 1.80%). However, after 7 days of incubation, maximum CBZ was degraded by *P. aeruginosa* CB3 (94.92 ± 3.76%) followed by CB2 (89.61 ± 2.66%), CB1 (87.89 ± 3.65%) and least by CB4 (76.54 ± 2.17%). After 14 days of incubation, maximum CBZ was degraded by *P. aeruginosa* CB3 (95.22 ± 3.55%) followed by CB2 (91.63 ± 2.57%), CB1 (89.98 ± 3.21%) and least by CB4 (79.25 ± 2.19%). The results are consistent with 10th day results.

Effect of Fe(II) on the degradation of CBZ was examined under the same circumstances and found that the degradation percentage was highest in CB3 (82.46 ± 4.09%) after 3 days as compared to other isolates CB2 (78.42 ± 2.89%), CB1 (75.24 ± 3.00%) and CB4 (57.85 ± 2.17%). After an incubation of 7 days the degradation percentage was highest in CB1 (95.54 ± 3.48%), CB3 (89.89 ± 3.38%), CB2 (87.85 ± 2.53%) and CB4 (79.47 ± 1.83%). After an incubation of 14 days the degradation percentage was highest in CB1

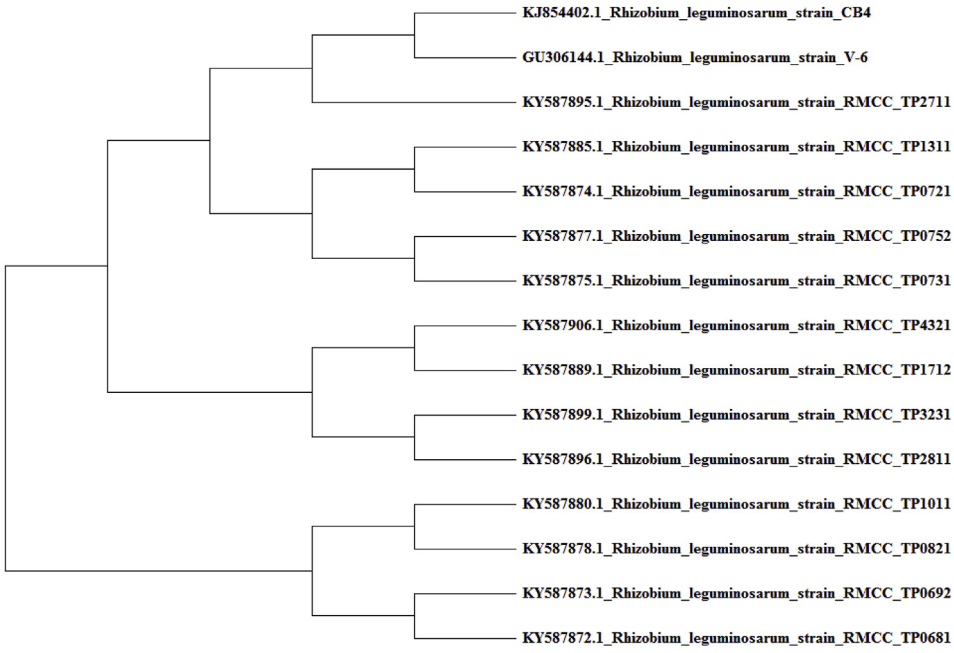

Fig. 4. Phylogenetic tree of isolate CB3 (*Actinomyces* sp.) based on 16S rRNA gene sequence analysis.

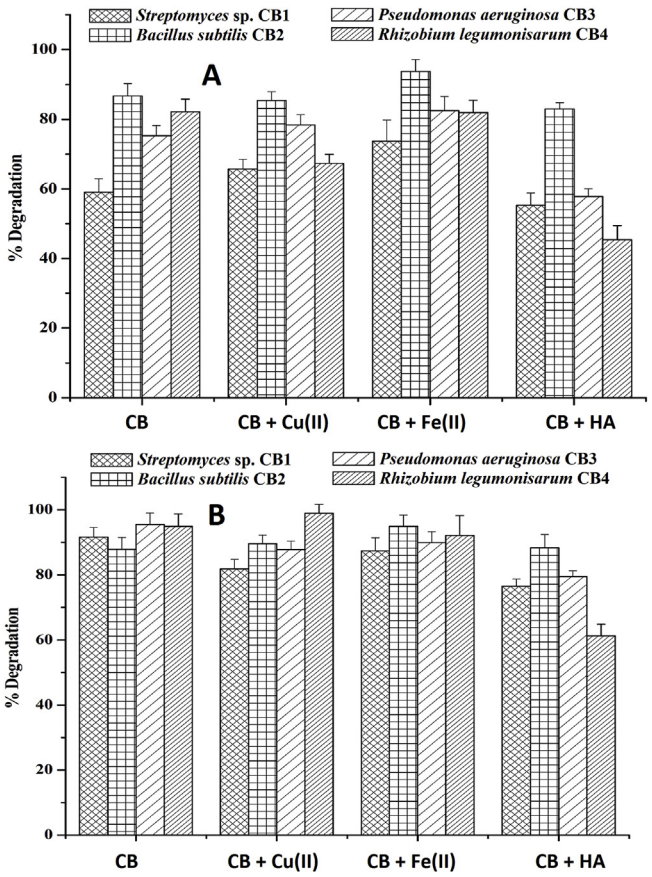

Fig. 5. Based on HPLC analysis the degradation (%) of CBZ by bacterial isolates inoculated either singly or co-supplemented with humic acid (HA), Cu(II) and Fe(II) on day 3 (A) and day 7 (B) post-inoculation.

(96.51 ± 3.22%), CB3 (91.24 ± 3.64%), CB2 (90.21 ± 3.22%) and CB4 (81.24 ± 2.77%). The observed results are consistent with 10th day results.

The results presented here indicated that more than 98% of the CBZ can be degraded and detoxified rapidly by isolate CB2 with supplementation of 100 mg/L of humic acid in 7 days. In other isolates, the degradation percentage was low as compared to CB2. On 7th day, the degradation percentage was increased in all the isolates with the supplementation of Cu(II), Fe(II) and humic acid. Moreover, the Cu(II) detoxifies the CBZ in all the isolates within three days. The effect of Fe (II) was found similar to with of the 1000 mg/L CBZ group in most of the isolates. More than 98% of the CBZ was removed from the samples by the addition of humic acid by isolate CB2 after incubation for 7 days. The results of 3rd day indicate the degradation was enhanced by the addition of Cu(II) and Fe(II) has shown no effect on degradation rate. The degradation results of CBZ with and without Fe(II), Cu(II) and humic acid were maximum on 7th as well 10th day and constant thereafter means maximum CBZ was utilized within 10 days.

4. Discussion

Our result indicates that CBZ can be degraded and detoxified rapidly by bacterial strains. In previous studies, some species of *Rhodococcus* (Holtman and Kobayashi 1997 Jing-Liang et al., 2006; Xu et al., 2006; Xu et al., 2007; Wang et al., 2010a,b; Zhang et al., 2013; Xiao et al., 2013) *Pseudomonas* (Kalwasinska et al. 2008; Fang et al., 2010; Pandey et al., 2010; Sun et al., 2014) and *Bacillus* (Salunkhe et al. 2014) have shown degradation of CBZ under environmental and experimental conditions. They cleaved methyl carbamate side chain of CBZ parent structure leading to the generation of 2-amino-benzimidazole, benzimidazole and 2-hydroxybenzimidazole derivatives (Singh et al., 2016). Encouragingly, the newly isolated strains endowed with superior bioremediation of CBZ up to a concentration of 1000 mg/L under experimental conditions.

The chemical control agents (insecticides, herbicides, fungicides) when applied to crops, interact with soil humates, clays and essential metal ions resulting in low mobility and less accessibility for microbial degradation (Long et al., 2004; Wasim et al., 2009; Beddington 2010; Cáceres et al., 2010). Pesticides may contain one or more than one coordination sites, and they can interact with metal ions of soil's (bounded metal ions or free metal ions), soils oxides, organic matter, etc. (Bhati et al., 2019; Kapoor et al., 2019; Kumar and Singh, 2018; Singh et al., 2019a&b; Sidhu et al., 2019). The interactions of the pesticides in soil at the molecular level are central to their bioavailability, bioaccumulation, and transport in the environment (Huyee and Keiter 2009; Kutman et al., 2013). There are limited studies on CBZ interactions with metal ions and soil humic acid. Since CBZ contains NH, CO and CH_3 coordinating sites, it is expected that it is involved in interaction with soil metal ions and soil humic contents.

Metal ions and humic acid are known to form complex with CBZ and enhances its degradation as per as our assumption Cu(II) exhibited maximum degradation of CBZ as compared to control and other samples because Cu has ability to decompose CBZ through chemical decomposition due to its paramagnetic nature and complex formation ability as compared to other metal ions, whereas after 10 days, humic acid plays an essential role in the removal of CBZ from the samples. Hence, the addition of Fe(II), Cu(II) and humic acid enhances the capability of bacterial isolates to exert degradation of CBZ. Mechanistically, it is assumed that Fe(II), Cu(II) and humic acid blocks the active sites of CBZ and thus enhances its availability for bacterial catabolism. As we know, humic acid is a bulky molecule, initially CBZ (CBZ) gets interacted with it and make complex suitable for decomposition by microorganisms. The humic acid interacts with CBZ and thus, can reduce toxicity of CBZ due to hydrogen and Vander Waals interactions. As the all four bacterial strains exhibited the ability to survive and grow on CBZ alone without the need for any supplements, it makes them ideally suitable for bioremediation under natural conditions. Therefore, the isolated strains definitely play a possible bioremediation role in the areas contaminated by CBZ. Our study confirmed that the isolated strains were capable to remove CBZ residues under various conditions by a high percentage.

Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflicts of interest

All authors declare that no conflicts of interest exist.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101196.

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