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ORIGINAL ARTICLE

Biodegradation of carbendazim by a potent novel *Chryseobacterium* sp. JAS14 and plant growth promoting *Aeromonas caviae* JAS15 with subsequent toxicity analysis

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Abstract
In the present study, carbendazim (MBC) degrading bacterial strains were isolated and identified as *Chryseobacterium* sp. JAS14 and *Aeromonas caviae* JAS15. Both the strains completely degraded 200 mg l^{-1} of MBC in the aqueous medium and soil within 4–9 days of incubation. In an aqueous medium, the degradation process was characterized by a rate constant of 53.16 day^{-1} and 42.60 day^{-1}, following zero order model and DT_{50} was 1.8 days and 2.34 days for *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15, respectively. A *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15 inoculated into the soil without the addition of nutrients showed the degradation rate constant of 27.30 day^{-1} and 23.87 day^{-1}, and DT_{50} was 3.66 days and 4.18 days, respectively. The metabolites during MBC biodegradation by *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15 were identified as 2-aminobenzimidazole, 2-hydroxybenzimidazole, 1, 2 diaminobenzene and catechol. To our knowledge, this is the first study of the detailed biodegradation pathway of MBC by *Chryseobacterium* sp. JAS14 was proposed. Phytotoxicity and cytogenotoxicity assays showed that the toxicity of the MBC reduced after biodegradation by *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15. In addition, *A. caviae* JAS15 possess important plant growth promoting traits under normal and MBC stress condition. These results suggest the *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15 could be used as a bioresource for the reclamation of MBC contaminated soil.

Keywords Biodegradation · Carbendazim · Kinetic parameters · Toxicity · Plant growth promotion

Introduction

Carbendazim (methyl 2-benzimidazole carbamate; MBC) is a systemic fungicide, which is widely used to control a broad range of fungal diseases on arable crops, fruits, vegetables and herbs (Bai et al. 2017; Singh et al. 2016, 2019; Xiao et al. 2013). It is also the hydrolytic product and active component of some systemic fungicides such as benomyl and thiophanate-methyl (Cuppen et al. 2000; McCarroll et al. 2002). The half-life of carbendazim in soil varies from several days to 12 months depending upon the soil type (Singh et al. 2016). Repeated applications of MBC results in high residual concentration in the soil, leading to detrimental effects on plants. MBC is taken up by plants through the roots, seeds or leaves and, afterwards, it is transferred to the whole plant (De la Huebra et al. 2000).

The increasing concern of MBC contamination, along with its toxicological properties has prompted researchers to strive for biodegradation options for MBC contaminated sites (Singh et al. 2016). Biodegradation has received increasing attention as a reliable, ecologically and economically attractive technique for cleaning the polluted environments. Recently, several microbial species have been reported to degrade MBC, including *Klebsiella*, *Flavobacterium*, and *Stenotrophomonas* (Alvarado-Gutierrez et al. 2020), *Pseudomonas* (Pandey et al. 2010; Sun et al. 2014), *Rhodococcus* sp. D-1 (Bai et al. 2017) and *Rhodococcus erythropolis* JAS13 (Abraham and Silambarasan 2018). However, to date, very few reports on MBC-degrading bacterial strains that can also promote plant growth have

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been published. Thus, the objectives of this study was to: (1) isolate and characterize potent MBC tolerant bacterial strains from agricultural soil, (2) study the biodegradation efficiency of MBC by selected rhizospheric bacterial strains, and (3) assess their plant growth promoting traits under MBC stress condition.

Materials and methods

Chemicals

MBC (99.2% purity) was obtained from Sigma-Aldrich (St. Louis, Mo, USA). The commercial grade MBC 50% WP (Wettable powder) was used in the present study which was purchased from Saraswati Agro Chemicals (Jammu) Pvt. Ltd., India. All other chemicals and reagents used in this study were of high purity and analytical grade.

Isolation and identification of MBC degrading bacterial strain

Isolation of the bacterial strain capable of degrading MBC was done by enrichment culture technique. The soil sample was taken from the brinjal field in Vellore district, India (12.93° N 79.13° E) with known history of previous MBC applications. The composition of the minimal salt medium (MSM) employed in isolating bacterial strain was as follows (g l^{-1}): Na_2HPO_4 5.8, KH_2PO_4 3, NaCl 0.5, NH_4Cl 1, $MgSO_4$ 0.25 and pH 6.8–7.0 (Abraham and Silambarasan 2018). The initial acclimatization was conducted in a sterile 250 ml Erlenmeyer flask containing 50 ml of the medium, 5 g of soil and MBC at a concentration of 25 mg l^{-1}. The culture was stirred continuously in an orbital shaker at 100 rpm at a temperature of 28 ± 2 °C. After 7 days of incubation, soil particles were allowed to settle and 5 ml of soil suspension was transferred into flasks containing fresh medium supplemented with the 25 mg l^{-1} of MBC and incubated for a further 7 days. After three rounds of enrichment, the culture was serially diluted with sterilized distilled water and 100 µl (10^{-6} dilution) of suspensions were plated on MSM plates containing 25 mg l^{-1} MBC and 2% agar. After 3 days of incubation at 28 ± 2 °C, microbial colonies were visibly observed. The colonies were purified and tested for their MBC degrading capability in liquid medium and in soil.

The different bacterial isolates were screened for higher tolerance of MBC. A series of 250 ml Erlenmeyer flasks containing 100 ml of MSM was spiked with increasing concentration of MBC at 25 to 200 mg l^{-1}. The flasks were inoculated with 1 ml of bacterial culture and incubated at 28 ± 2 °C on a rotary shaker at 100 rpm for 2 days. After incubation 1 ml of sample was serially diluted with sterile distilled water and plated on MSM agar plates containing MBC and incubated at 28 ± 2 °C for 2 days. The plates were examined and colonies were counted. The tolerance of MBC was noted as the complete inhibition of bacterial growth.

The identification of bacterial isolates were done by 16S rRNA gene sequence analysis. Genomic DNA was extracted using the AMpurE Bacterial gDNA Mini Spin kit (Amnion Biosciences Pvt. Ltd. Bangalore, India). The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using standard procedures with universal forward primer 5′-CWG RCC TAN CAC ATG SAA GTC-3′ and reverse primer 5′-GRC GGW GTG TAC NAG GC-3′. The amplified PCR product was sequenced using ABI3730xl genetic analyzer (Amnion Biosciences Pvt. Ltd. Bangalore, India).

Bacterial growth and degradation of MBC

Growth of bacterial isolates in the MSM and nutrient broth were studied in terms of optical density. 100 µl of each bacterial isolates were grown separately in 20 ml of MSM, MSM with 200 mg l^{-1} of MBC, nutrient broth and nutrient broth with 200 mg l^{-1} of MBC and incubated at 30 ± 2 °C on a rotary shaker at 120 rpm. The growth of bacterial isolates was monitored regularly by measuring absorbance in a UV–Vis spectrophotometer at 600 nm. To assess the ability of the bacterial isolates to degrade MBC, the MSM was spiked with MBC as the sole carbon and energy source. All the biodegradation experiments were performed in triplicates. Biodegradation study of MBC was carried out in 250 ml Erlenmeyer flask containing 100 ml of MSM with 200 mg l^{-1} MBC and inoculated with 1 ml of bacterial culture (3×10^6 cells ml^{-1}). All the flasks were incubated at 30 ± 2 °C on a rotary shaker at 120 rpm. The samples were regularly sampled for the determination of the concentration of MBC using high performance liquid chromatography (HPLC).

Biodegradation of MBC was carried out in soil from which the bacterial isolates were obtained. The soil sample was sterilized at 121 °C for 30 min. Two treatments were carried out: (1) addition of MBC 200 mg kg^{-1} soil, bacterial isolate and nutrients (carbon, nitrogen and phosphorous), (2) addition of MBC 200 mg kg^{-1} soil, bacterial isolate without nutrients. The biodegradation experiments were conducted in separate flasks and all the experiments were performed in triplicates. 100 g of soil was taken in a 250 ml Erlenmeyer flask and 30 ml of a solution containing 3×10^6 cells ml^{-1} individual bacterial suspension, MBC 200 mg kg^{-1} soil with and without nutrients. The amount of carbon, nitrogen and phosphorus were calculated using the relationship C/N/P 100:10:1 and the sources were glucose, $(NH_4)_2SO_4$ and K_2HPO_4, respectively (Martin et al. 2007). All the flasks were incubated at 30 ± 2 °C for 10 days.

Analytical procedures

MBC in the aqueous samples were analyzed with 10 ml of sample aliquot extracted with equal volume of dichloromethane. Extracts of the organic phase were removed and dried over anhydrous sodium sulfate and then evaporated at room temperature. The residue was dissolved in 5 ml methanol, thoroughly mixed in an ultrasonic bath for 5 min and subjected to HPLC, Fourier transform infrared (FTIR) and gas chromatography-mass spectroscopy (GC–MS) analysis.

For MBC determination in soil, samples (10 g) were extracted with 15 ml of methanol:double distilled water (4:1, v/v) and then the mixture was shaken for 15 min at 200 rpm on a rotary shaker. The samples were centrifuged at 7300 rpm for 5 min, the supernatant was collected and the precipitate was treated in the same way thrice. All the supernatant was added together and 20 ml of petroleum ether was added to remove the lipophilic impurities. The mixture was kept on a rotary shaker for 20 min at 200 rpm, then the petroleum ether phase was removed and discarded, the remaining solution was dried over anhydrous sodium sulfate, and evaporated at room temperature. The residue was dissolved with 5 ml of methanol, thoroughly mixed in an ultrasonic bath for 5 min and subjected to HPLC analysis (Wang et al. 2010).

HPLC analysis was performed using Waters 1525 binary HPLC pump (Milford, USA) on a Symmetry C_{18} column (Waters 5 µm, 4.6 mm × 150 mm) with elution of methanol:water (65:35, v/v) at a flow rate of 0.8 ml min^{-1}. MBC was detected by a UV–Vis detector at 281 nm. The metabolites produced after degradation of MBC were identified by Perkin Elmer Clarus 680 gas chromatographic instrument equipped with a mass spectrometer detector (Clarus 600 model) and an Elite-5MS (30.0 m, 0.25 mm ID, 250 µm df) column. FTIR analysis of MBC and its biodegraded metabolites was carried out using IR Affinity-1 (Shimadzu) equipped with DLATGS detector. The FTIR analysis was done in the mid IR region of 400–4000 cm^{-1}.

Morphological changes in bacterial cells after the degradation process was examined by scanning electron microscopy (SEM). The bacterial cells treated with and without 200 mg l^{-1} of MBC were mounted on specimen stubs with double-sided adhesive tape and air-dried. These cells were then coated with gold in a sputter coater (Jeol, Model 1600 Ion sputter) and examined under SEM (Jeol, Model 6390).

Kinetic studies

Biodegradation of MBC in aqueous medium and soil was analyzed with a different kinetic model which includes zero order, first order, pseudo first order, second order and pseudo second order to determine the rate constant (k). The time when MBC concentration in MSM or soil was reduced by 50% (DT_{50} values) was calculated from the linear equation obtained from the regression between $C_t - C_o = kt$ (zero order model), $\ln C_t/C_o = e^{-kt}$ (first order model), $\ln C_t = -kt + \ln C_o$ (pseudo first order model), $1/C = kt + 1/C_o$ (second order model) and $t/Ct = t/Ce + 1/kCe^2$ (pseudo second order model) of the chemical data and time.

Toxicological studies

The phytotoxicity assay was carried out to assess the toxicity of MBC degraded metabolites using the method previously described by Abraham and Silambarasan (2018). The extracted metabolites of MBC were dried and dissolved in distilled water to make a final concentration of 50 mg l^{-1}. The study was carried out at room temperature using seeds of *Vigna radiata*, *Vigna unguiculata* and *Macrotyloma uniflorum.* All the experiments were conducted in triplicates. The seeds were washed with distilled water and were treated for 4 h with degraded metabolites. The control set was carried out using water at the same time. After 4 h, the seeds were placed on wet cotton in Petri plates and incubated at room temperature for 24 h in dark condition. The seeds were placed just above the surface of the sterile soil in the Petri plates and incubated at room temperature for 5 days.

Small healthy bulbs of *Allium cepa* were selected for the cytogenotoxicity study (Silambarasan and Vangnai 2016; Sipahutar et al. 2018). The mitotic index (MI) was calculated as the ratio between the number of dividing cells and total number of cells scored (1250) and expressed as percent of negative control. The data were analyzed by one-way ANOVA (analysis of variance) with Tukey–Kramer multiple comparisons test. The results were expressed as the mean ± SD (standard deviation).

Bioassays of plant growth promoting activities

The qualitative and quantitative analyses of P-solubilization with MBC free control, 50 and 100 mg l^{-1} of MBC were performed according to the method of Ramani (2011) with slight modifications. Zinc (Zn) solubilization ability of the isolates was detected by inoculating the bacterial strain in Tris-minimal salts medium plates having zinc oxide (14 mM) or zinc phosphate (5 mM) or zinc carbonate (5.2 mM) as source of insoluble inorganic zinc. The Zn solubilization was quantitated according to the method of Sharma et al. (2012) in Tris-minimal broth with different insoluble Zn was supplemented with MBC free control, 50 and 100 mg l^{-1} of MBC separately. For quantitative assay of indole-3-acetic acid (IAA), the bacterial isolates were grown in Luria Bertani (LB) broth. The 100 ml of LB broth containing 100 µg ml^{-1} of tryptophan with 50 and 100 mg l^{-1} concentration of MBC and without MBC (control) was inoculated with 1 ml of bacterial culture (10^6 cells ml^{-1}).

All the culture flasks were incubated at 28 ± 2 °C on a rotary shaker at 125 rpm for 24 h. IAA concentration in the supernatant was determined by the method of Gordon and Weber (1951), later modified by Brick et al. (1991). Hydrogen cyanide (Bakker and Schipper 1987) and ammonia (Dye 1962) production by bacterial isolates were also determined. All the experiments were conducted in triplicates using the same treatments. The data were subjected to statistical analysis and significant differences among the treatment means were calculated at $P \leq 0.05$ by one-way ANOVA with Dunnett's multiple comparison tests using Graphpad Prism, v5.03.

Formulation studies

Low cost powder formulations were prepared according to the method of Abraham and Silambarasan (2016). Two powder formulations were prepared as (1) sawdust-soil-5% molasses (15:5:1) and (2) sawdust-soil-nutrients (carbon, nitrogen and phosphorus) were used for mass production of bacteria. The carrier material such as fly ash was used for immobilization of bacterial isolates. The carrier material was improved by a mixture of fly ash, soil and 5% molasses in the ratio of 15:3:1 plus 75 mg cycloheximide kg^{-1} material, whereas in the other method, a mixture of fly ash, soil and nutrients C/N/P (100:10:1) in the ratio of 15:3:1 with 75 mg cycloheximide kg^{-1} material were taken. The mixture was filled in heat-resistant polythene bags and autoclaved at 121 °C for 20 min. Thereafter, 1 part stock culture was added to the bags containing carriers and mixed thoroughly for uniform distribution. 1 g of formulation was sampled weekly, before sampling, the packets were shaken thoroughly to attain uniform distribution of the propagules of the bacterial isolates in the entire formulation. The sample was serially diluted and 300 µl of 10^{-8} dilution was inoculated on nutrient agar plates and incubated at 28 ± 2 °C for 2 days. After incubation, the colonies of the bacteria were counted to determine colony forming unit (CFU) g^{-1} formulation. To test the shelf life of the formulations with regard to CFU load and contamination by other microorganisms, the packets were stored at room temperature for 24 weeks.

Results and discussions

Isolation and characterization of the MBC degrading bacterial strains

In the present study, a total of three different bacterial strains were isolated from agricultural soil by enrichment culture technique. The strains were obtained in pure culture and were initially screened for higher tolerance in liquid culture medium with increasing concentrations of MBC ranging from 25 to 200 mg l^{-1}. The assays showed that two out of the three tested strains grew at a concentration of 200 mg l^{-1} and were designated as JAS14 and JAS15. The 16S rRNA sequence of JAS14 and JAS15 strains showed greatest similarity to the reference sequences from members of genus *Chryseobacterium* and *Aeromonas*, respectively, in the GenBank database. The phylogenetic tree depicts the position of JAS14 and JAS15 strains within the genus *Chryseobacterium* and *Aeromonas* (Fig. S1). Based on these observations, the isolates were designated as *Chryseobacterium* sp. JAS14 and *Aeromonas caviae* JAS15. The nucleotide sequences of *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15 have been deposited in the NCBI (National Center for Biotechnology Information) GenBank database and the accession tnumber obtained was KF313552 and KF313551, respectively. In previous studies, some microbial strains of *Pseudomonas* sp. CBW (Fang et al. 2010), *Rhodococcus jialingiae* djl-6-2 (Wang et al. 2010), *Bacillus pumilus* NY97-1 (Zhang et al. 2009), *Burkholderia cepacia*, *Aeromonas hydrophila*, *Sphingomonas paucimobilis* (Kalwasinska et al. 2008a, b), *Rhodococcus* sp. djl-6 (Xu et al. 2006) and *Ralstonia* sp. 1-1 (Zhang et al. 2005) were reported for MBC degradation. To the best of our knowledge *Chryseobacterium* sp., have not been employed in the degradation of MBC.

Biodegradation of MBC by *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15

The growth of bacterial strains was determined in the MSM and nutrient broth supplemented with MBC by observing the optical density (OD) of the culture at 600 nm following different time intervals. In the control flask, there was no significant increase in OD with time, whereas, a continuous enhancement in the growth of *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15 were observed in the MSM supplemented with MBC and maximum growth was obtained on 8 days and 5 days, respectively (Fig. S2a and b). The bacterial growth suggests that *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15 have potency to grow on MBC as a sole carbon source and utilize it. This result is corroborated with the finding of *Pseudomonas* sp. CBW capable of utilizing MBC as the sole source of carbon and energy (Fang et al. 2010). Furthermore, MBC preferentially used as the sole carbon and nitrogen source by *Rhodococcus* sp. D-1 (Bai et al. 2017), also supporting the findings here reported. The increasing growth rate of *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15 were noticed in the nutrient broth containing MBC when compared to the control and this could be due to the addition of carbon source upon degradation of MBC in the medium (Fig. S2c and d). In the present study, different kinetic models have been used to understand the dynamics of degradation of MBC by *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15 (Tables 1, 2). The best fit of the model was obtained in the case of zero order for the

Table 1 Kinetic parameters for the degradation of MBC in aqueous medium and soil by *Chryseobacterium* sp. JAS14

Kinetic model	Parameters	Treatments		
		MSM + MBC + JAS14	S + N + MBC + JAS14	S + MBC + JAS14
Zero order	Regression equation	$C_t - C_o = -53.16t + 1.464$	$C_t - C_o = -42.11t + 0.505$	$C_t - C_o = -27.30t + 6.557$
	k (day^{-1})	53.16	42.11	27.30
	DT_{50}	1.88	2.37	3.66
	R^2	0.982	0.985	0.986
First order	Regression equation	$\ln (C_t/C_o) = -0.687t + 0.253$	$\ln (C_t/C_o) = -0.506t + 0.225$	$\ln (C_t/C_o) = -0.428t + 0.515$
	k (day^{-1})	0.687	0.506	0.428
	DT_{50}	1.0	1.36	1.61
	R^2	0.868	0.927	0.791
Pseudo first order	Regression equation	$\ln C_t = -0.687t + 5.551$	$\ln C_t = -0.506t + 5.522$	$\ln C_t = -0.428t + 5.812$
	k (day^{-1})	0.687	0.506	0.428
	DT_{50}	1.0	1.36	1.61
	R^2	0.868	0.927	0.791
Second order	Regression equation	$1/C = 0.011t - 0.001$	$1/C = 0.007t - 0.000$	$1/C = 0.014t - 0.021$
	k (day^{-1})	0.011	0.007	0.014
	DT_{50}	63.01	99.02	49.51
	R^2	0.708	0.784	0.475
Pseudo second order	Regression equation	$t/C_t = 0.039t - 0.020$	$t/C_t = 0.034t - 0.024$	$t/C_t = 0.106t - 0.190$
	k (day^{-1})	0.039	0.034	0.106
	DT_{50}	17.77	20.38	6.53
	R^2	0.729	0.770	0.475

MSM minimal salt medium, *MBC* carbendazim, *S* soil, *N* nutrients

Table 2 Kinetic parameters for the degradation of MBC in aqueous medium and soil by *A. caviae* JAS15

Kinetic model	Parameters	Treatments		
		MSM + MBC + JAS15	S + N + MBC + JAS15	S + MBC + JAS15
Zero order	Regression equation	$C_t - C_o = -42.60t + 4.186$	$C_t - C_o = -29.81t + 2.052$	$C_t - C_o = -23.87t + 6.834$
	k (day^{-1})	42.60	29.81	23.87
	DT_{50}	2.34	3.35	4.18
	R^2	0.987	0.989	0.987
First order	Regression equation	$\ln (C_t/C_o) = -0.513t + 0.261$	$\ln (C_t/C_o) = -0.365t + 0.280$	$\ln (C_t/C_o) = -0.306t + 0.361$
	k (day^{-1})	0.513	0.365	0.306
	DT_{50}	1.35	1.89	2.26
	R^2	0.886	0.900	0.891
Pseudo first order	Regression equation	$\ln C_t = -0.513t + 5.558$	$\ln C_t = -0.365t + 5.577$	$\ln C_t = -0.306t + 5.658$
	k (day^{-1})	0.513	0.365	0.306
	DT_{50}	1.35	1.89	2.26
	R^2	0.886	0.900	0.892
Second order	Regression equation	$1/C = 0.008t - 0.001$	$1/C = 0.006t - 0.002$	$1/C = 0.005t - 0.005$
	k (day^{-1})	0.008	0.006	0.005
	DT_{50}	86.64	115.52	138.62
	R^2	0.689	0.699	0.699
Pseudo second order	Regression equation	$t/C_t = 0.037t - 0.028$	$t/C_t = 0.040t - 0.050$	$t/C_t = 0.048t - 0.084$
	k (day^{-1})	0.037	0.040	0.048
	DT_{50}	18.73	17.32	14.44
	R^2	0.699	0.693	0.687

MSM minimal salt medium, MBC carbendazim, S soil and N nutrients

degradation of MBC in MSM and soil. The regression coefficient R^2 value suggests that a zero order was a better fit than the other orders. The *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15 were capable of efficiently degrading MBC in the MSM which was used as the sole carbon and energy source. It was found that 100% of 200 mg l^{-1} MBC was degraded by *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15 at 4 days and 5 days of incubation, respectively (Fig. 1a). While there is no significant change in MBC concentration observed in the control. Degradation process was characterized by a rate constant of 53.16 day^{-1} and 42.60 day^{-1}, following zero order model and the DT_{50} was 1.8 days and 2.34 days for *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15, respectively. The degradation of MBC in soil spiked with and without addition of nutrients revealed that *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15 were the main components behind MBC degradation in soil. In the sterile soil supplemented with and without addition of nutrients inoculated with *Chryseobacterium* sp. JAS14 completely degraded MBC (200 mg l^{-1}) in 5 days and 8 days, respectively, (Fig. 1b) and the rate constants were 42.11 day^{-1} and 27.30 day^{-1}, and DT_{50} was 2.37 days and 3.66 days, respectively (Table 1). However, in sterile soil amended with and without nutrients, *A. caviae* JAS15 degraded 100% of MBC within 7 days and 9 days of incubation, respectively, (Fig. 1c) and the rate constants were 29.81 day^{-1}, 23.87 day^{-1} and the DT_{50} was 3.35 days, 4.18 days, respectively (Table 2). To the best of our knowledge, this is the first report on degradation of MBC by *Chryseobacterium* sp. JAS14. The results are in agreement with our previous report of *R. erythropolis* JAS13 was capable of degraded 100% of MBC (150 mg l^{-1}) in the liquid medium, whereas complete removal of MBC was observed in soil amended with and without addition of nutrients was characterized by a

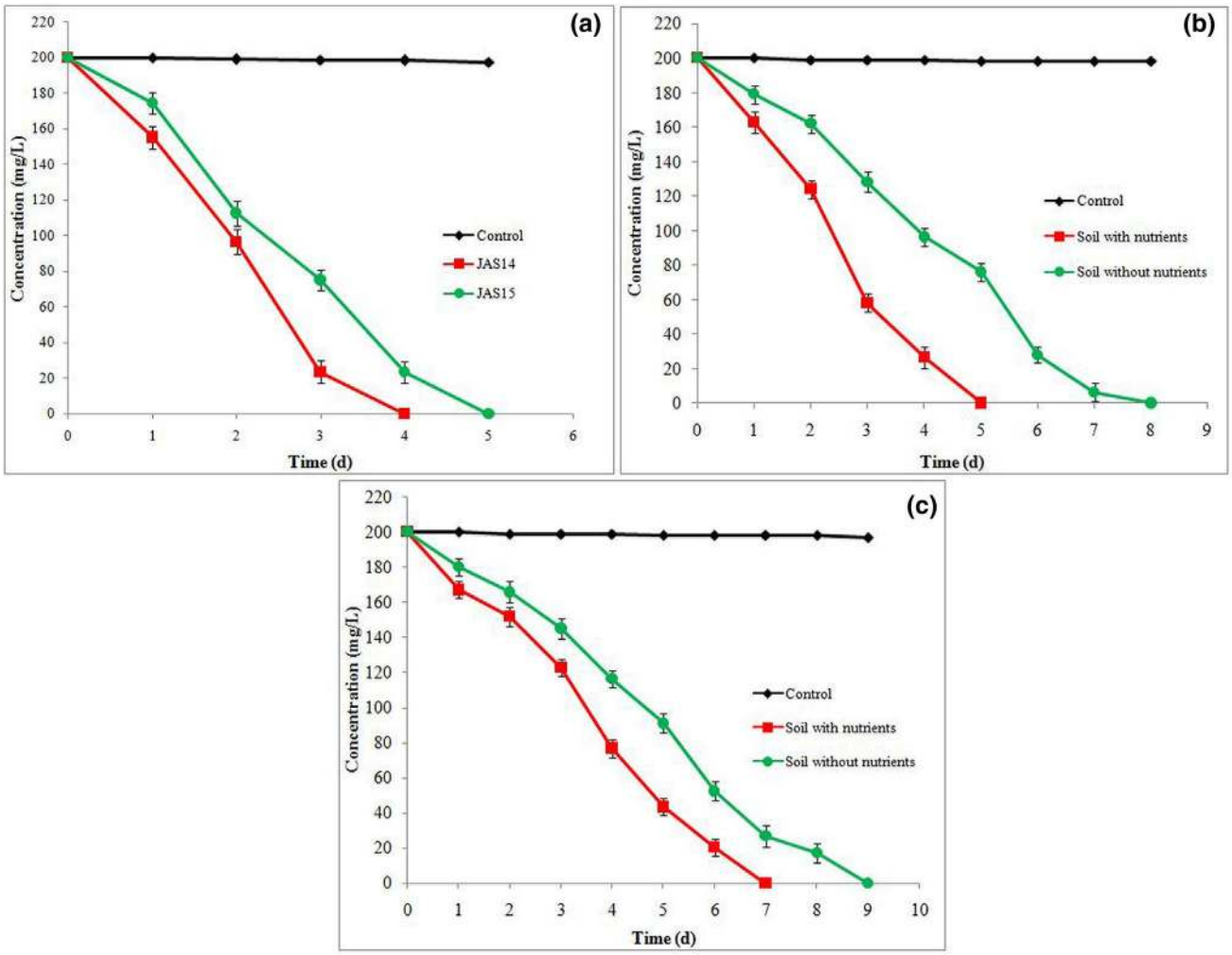


Fig. 1 **a** Biodegradation of 200 mg l^{-1} MBC in the aqueous medium by *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15. **b** Degradation of 200 mg kg^{-1} MBC in the soil by *Chryseobacterium* sp. JAS14 and **c** degradation of 200 mg kg^{-1} MBC in the soil by *A. caviae* JAS15. MBC removal was determined at different time intervals and the data are mean ± standard deviation from triplicate treatments

rate constant of 29.61 day^{-1} and 25.77 day^{-1}, and DT 50 was 2.53 days and 2.91 days, respectively (Abraham and Silambarasan 2018). Zhang et al. (2009) reported that the degradation rate of MBC by *Bacillus pumilus* NY97-1 was measured to be 42.44–90.07% with its increasing concentrations ranging from 10 to 300 mg l^{-1} and temperature. About 87.1 and 99.1% of MBC at concentrations of 1.0 and 10.0 mg l^{-1} in mineral salts medium were removed by the *Pseudomonas* sp. CBW after incubation for 3 days, respectively (Fang et al. 2010). Wang et al. (2010) reported that about 94% of 100 mg l^{-1} MBC in the aqueous medium was degraded by *Rhodococcus jialingiae* djl-6-2 within 60 h of incubation. The *Rhodococcus* sp. D-1 was effectively degraded 98.20% of 200 ppm MBC in 3 days (Bai et al. 2017). Recently, Singh et al. (2019) also reported the MBC degradation of 91.65%, 87.35%, 81.85% and 76.54% by *Streptomyces* sp. CB1, *Pseudomonas aeruginosa* CB3, *Bacillus subtilis* CB2 and *Rhizobium leguminosarum* CB4, respectively, at an initial concentration of 1000 mg l^{-1} after 7 days. The results reported here indicate that MBC is being degraded and detoxified rapidly by *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15 without supplementation of other carbon sources. Such characteristics are desirable in microorganisms to be used for bioremediation purposes. It is evident in the study that the inoculation of *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15 degraded MBC (200 mg kg^{-1}) in the soil even without the addition of nutrients.

The degradation products of MBC in the culture extracts were analyzed by GC–MS. The degradation of MBC by *Chryseobacterium* sp. JAS14 produced four metabolites were identified with mass ion at *m/z* of 133 [$(M-1H)^-$, where $M=134$], 135, 109 [$(M+1H)^+$, where $M=108$] and 111 [$(M+1H)^+$, where $M=110$] which corresponds to 2-aminobenzimidazole (2-AB) (Fig. S3a), 2-hydroxybenzimidazole (2-HB) (Fig. S3b), 1, 2-diaminobenzene (Fig. S3c) and catechol (Fig. S3d), respectively. Upon degradation four metabolites were produced by *A. caviae* JAS15. A molecular ions at *m/z* 133 [$(M-1H)^-$, where $M=134$], 135, 107 [$(M-1H)^-$, where $M=108$] and 111 [$(M+1H)^+$, where $M=110$] was observed and identified as 2-AB (Fig. S3e), 2-HB (Fig. S3f), 1, 2-diaminobenzene (Fig. S3g) and catechol (Fig. S3h), respectively, by mass spectrometry. According to the analysis of metabolites produced during the degradation of MBC, the schematic pathway has been proposed (Fig. S4). The earlier reports on microbial degradation of MBC indicated that the microbes might not be able to open the ring structure of MBC, and only utilize its side-chain as growth substrates (Xu et al. 2006). However, in the present study it has been confirmed that *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15 were could open the ring structure of MBC as well as utilize it as the sole carbon and energy source. In the present investigation, MBC was converted to 2-AB and then rapidly to 2-HB by *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15. The 2-HB is further converted by ring-cleavage through degradation of 1,2-diaminobenzene and catechol, and probably even to carbon dioxide. However, in earlier studies on the biodegradation of MBC reported 2-AB and 2-HB which are the major metabolites that strongly support our observations (Bai et al. 2017; Fang et al. 2010; Wang et al. 2010).

FTIR spectrum of the control is presented in Fig. 2a. The FTIR spectrum of the degraded samples showed significant changes in the position of peaks (Figs. 2b, 3c). The two C=C stretch bands appeared in the degraded samples at 1631 cm^{-1} and 1641 cm^{-1} (Abraham and Silambarasan 2018). The OH bending in the control spectrum at 1400 cm^{-1} was observed as shifted in the degraded samples (1402 cm^{-1}). The peak pattern shifts as well as few new peaks appeared are corresponds to the biodegradation of MBC by *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15. The morphology of *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15, and its interaction with MBC degradation were studied by SEM analysis. However, when exposed to 200 mg l^{-1} of MBC, the shape of cells was distorted and there was marked deviation from the cells in control (Fig. 3). Similar results were observed with *Sphingobacterium* sp. JAS3, *Ochrobactrum* sp. JAS2 and *Alcaligenes* sp. JAS1 cells when treated with insecticides (Abraham and Silambarasan 2013, 2016; Silambarasan and Abraham 2013).

Toxicological studies

The phytotoxicity assay was carried out to determine the adverse impact of the environmental contaminants and the metabolites on plants (Silambarasan and Vangnai 2016). Three agriculturally valuable plants such as *V. radiata*, *V. unguiculata* and *M. uniflorum* were used for phytotoxicity assessment of MBC degraded metabolites on the seedlings. The parameters analyzed were percentage of seed germination, shoot length and root length (Table 3). The shoot length was not much reduced in MBC degraded samples in case of all the plants. In all tested parameters, MBC degraded metabolites were found to have almost negligible effect on all the plants which is indicative of less toxic nature of biodegradation metabolites.

Allium plant root tip test was carried out for in situ monitoring the cytogenotoxicity of the MBC degraded metabolites. The cytotoxicity level of degraded metabolites was determined based on the increase or decrease in the MI, which is being used as a parameter of cytotoxicity in studies of an environmental biomonitoring (Carita and Marin-Morales 2008). The MI for examined cells from MBC degraded samples was shown in Table 4. The negative control, *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15 degraded MBC metabolites exhibited MI of 14.82%, 10.21% and 9.94%, respectively, while cells which were exposed to

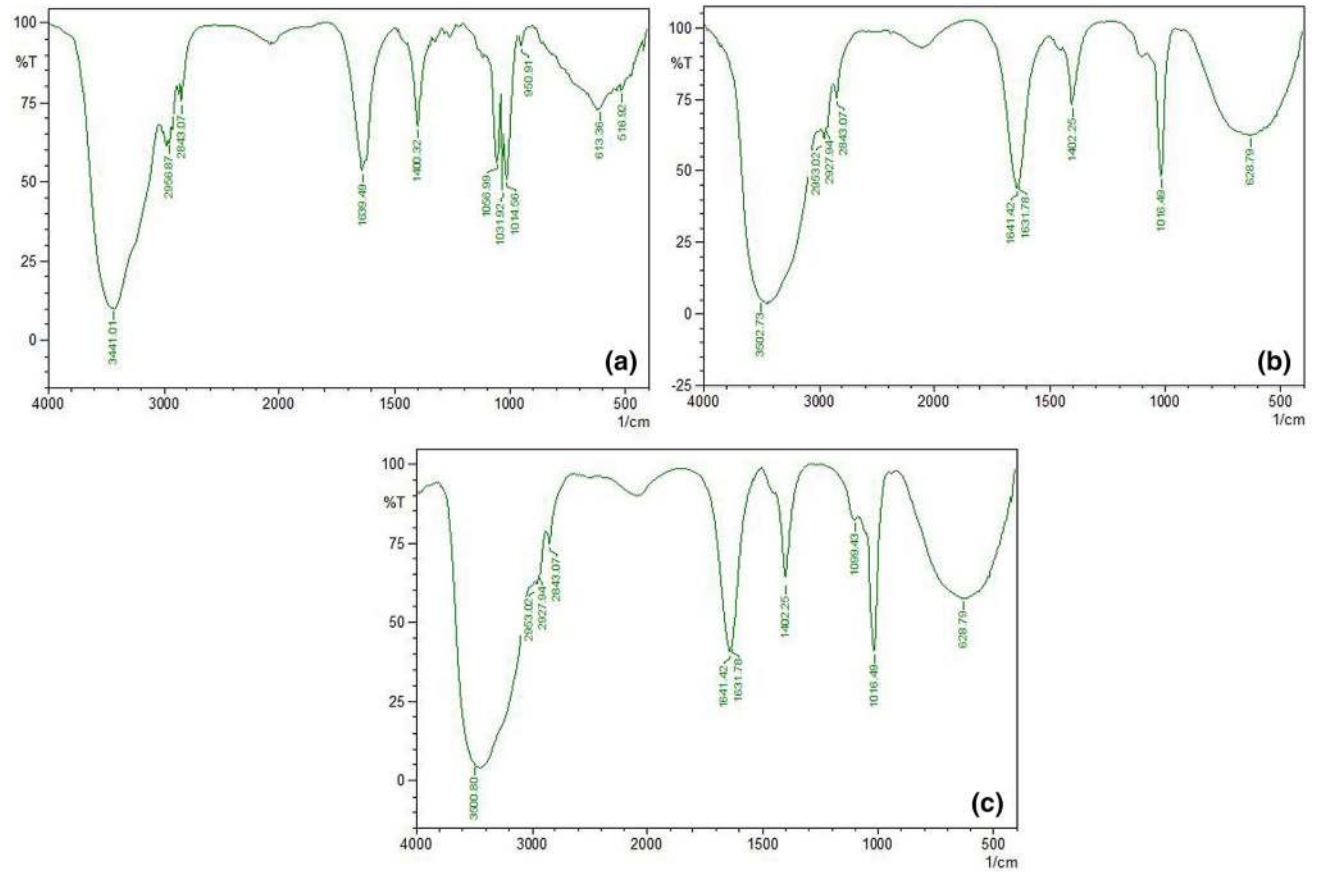


Fig. 2 **a** FTIR spectra of MBC at standard condition, **b** FTIR spectra for the degradation of MBC in aqueous medium by *Chryseobacterium* sp. JAS14 and **c** FTIR spectra for the degradation of MBC in aqueous medium by *A. caviae* JAS15

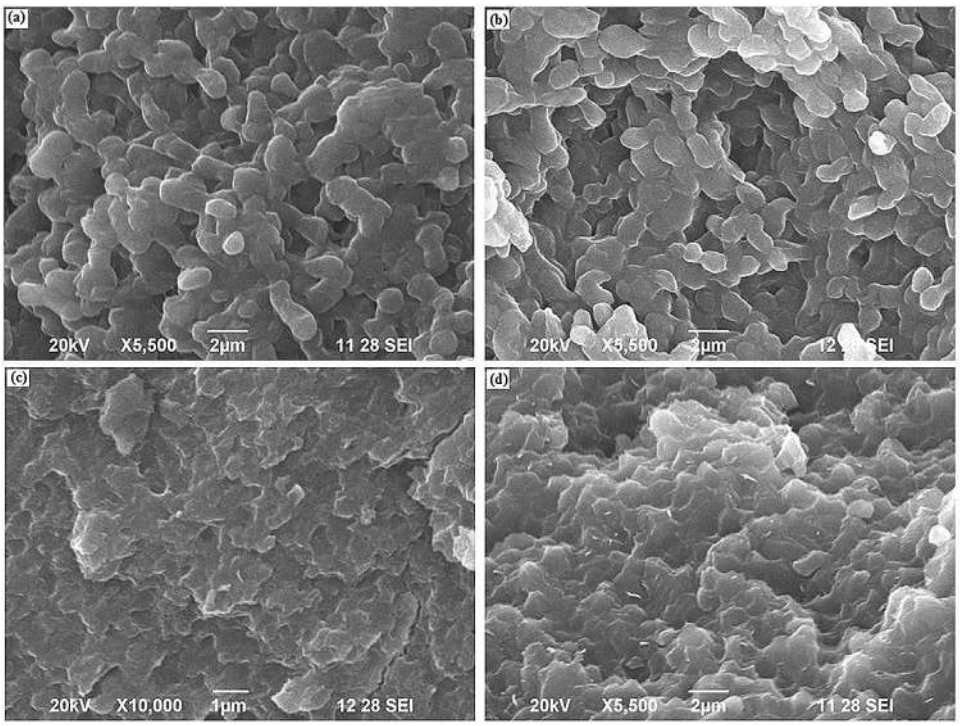


Fig. 3 SEM images of *Chryseobacterium* sp. JAS14 (**a**) and *A. caviae* JAS15 (**b**) cells were not treated with MBC. *Chryseobacterium* sp. JAS14 (**c**) and *A. caviae* JAS15 (**d**) cells were treated with 200 mg l^{-1} MBC

Table 3 Phytotoxicity study of MBC degraded metabolites

Parameters	*Vigna radiata*			*Vigna unguiculata*			*Macrotyloma uniflorum*		
	Control	EM[a]	EM[b]	Control	EM[a]	EM[b]	Control	EM[a]	EM[b]
Germination (%)	100	100	100	100	100	100	100	100	100
Shoot (cm)	19.1 ± 0.28	17.53 ± 0.4**	19.43 ± 0.35	21.43 ± 0.25	17.46 ± 0.37***	19.73 ± 0.2***	12.23 ± 0.15	11.73 ± 0.15*	11.6 ± 0.26*
Root (cm)	14.3 ± 0.3	6.43 ± 0.32***	8.5 ± 0.3***	14.6 ± 0.36	11.76 ± 0.15***	13.43 ± 0.35**	13.46 ± 0.15	7.73 ± 0.15***	13.2 ± 0.2
Shoot fresh weight (mg)	297 ± 1	272.66 ± 1.52***	297.33 ± 1.52	547.33 ± 0.57	533.66 ± 2.08***	540 ± 1.73**	194.66 ± 1.52	171.33 ± 1.52***	189.66 ± 1.52*
Root fresh weight (mg)	51.66 ± 0.57	32.33 ± 1.52***	28.33 ± 0.57***	92.66 ± 1.52	85.66 ± 2.08**	89.33 ± 1.52	79.33 ± 1.52	55.33 ± 1.52***	72.66 ± 0.57**

Values are mean of three experiments, SD (±), significantly different from the control at $*P<0.05$, $**P<0.01$, $***P<0.001$ by ANOVA with Tukey–Kramer comparison test

[a]EM (seeds are treated with extracted metabolites obtained from MBC degradation by JAS14 strain)

[b]EM (seeds are treated with extracted metabolites obtained from MBC degradation by JAS15 strain)

Table 4 Effect of MBC degraded metabolites on mitotic index and chromosome aberration in *A. cepa* root meristematic cells

Treatments	Number of dividing cells	Mitotic index (MI) %	Chromosome aberrations					
			MA	MC	ML	AB	AL	BC
Negative control	185.33 ± 1.15	14.82	−	−	+	−	−	−
Positive control	13.66 ± 0.57*	1.09	+	+	+	+	−	+
EM[a]	127.66 ± 1.52*	10.21	−	−	−	−	+	−
EM[b]	124.33 ± 1.15*	9.94	+	−	−	−	−	−

Each value represents the mean ± SD of three replicates per treatment

Chromosome aberrations: *MA* metaphase aberration, *MC* metaphase cluster, *ML* metaphase lagging chromosome, *AB* anaphase bridge, *AL* anaphase lagging chromosome, *BC* binucleated cell

$*P<0.001$, by One-way ANOVA with Tukey–Kramer comparison test. MI was calculated as: (number of dividing cells/total number of cells observed) × 100

[a]EM (Extracted metabolites obtained from MBC degradation by JAS14 strain)

[b]EM (Extracted metabolites obtained from MBC degradation by JAS15 strain)

H_2O_2 as positive control had the lowest average (1.09%). Earlier reports have shown that a decreased mitotic index of meristematic cells of *A. cepa* may be considered a reliable method to determine the presence of cytotoxic compounds in the environment (Chakraborty et al. 2009). The meristematic root tips treated with distilled water showed normal cell physiology (Fig. S5a–d). The H_2O_2-treated cells induced the strongest abnormalities with higher chromosomal aberration. However, root tips treated with MBC degraded metabolites showed less chromosomal damage in the cell stages of *A. cepa*.

Plant growth promoting traits of *A. caviae* JAS15

Among the two isolates, *A. caviae* JAS15 possessed plant growth-promoting traits both in normal and MBC stressed media. *A. caviae* JAS15 isolate formed clear halos around the spot of inoculation on Pikovskaya's medium. The medium containing different sources of inorganic phosphates with bromophenol blue changed its color from blue to yellow due to the growth of *A. caviae* JAS15 decreasing the pH in the medium. *A. caviae* JAS15 solubilized three inorganic phosphates such as DCP (dicalcium phosphate), TCP (tricalcium phosphate) and ZP (Zinc phosphate), and the measured zone was 12 mm, 15 mm and 13 mm, respectively. The P-solubilizing capability of *A. caviae* JAS15 under MBC stress was evaluated quantitatively using Pikovskaya's broth medium (Table 5). The maximum solubilization of DCP was 278.33, 140.33 and 112.66 µg ml^{-1} with control, 50 and 100 mg l^{-1} of MBC in liquid medium, respectively. The maximum amount of TCP solubilization was found to be 324, 146.33 and 134 µg ml^{-1} obtained with control, 50 and 100 mg l^{-1} of MBC in the culture medium, respectively. Maximum ZP solubilization by *A. caviae* JAS15 in the control, 50 and 100 mg l^{-1} of MBC spiked medium were found to be 319.66, 132.66 and 117.33 µg ml^{-1}, respectively. Among different

Table 5 Phosphate and zinc solubilization under the presence of MBC in liquid broth by *A. caviae* JAS15

Microorganism	Treatments	3 day		6 day		9 day		12 day	
		P/Zn solubilization (μg ml^{-1})	pH	P/Zn solubilization (μg ml^{-1})	pH	P/Zn solubilization (μg ml^{-1})	pH	P/Zn solubilization (μg ml^{-1})	pH
P-Solubilization by *A. caviae* JAS15	DCP + Control	257.66 ± 2.08	4.6	278.33 ± 1.52	4.3	233 ± 2	4.7	203.33 ± 2.51	4.7
	DCP + MBC[A]	63.66 ± 1.52[a]	5.1	98 ± 2.64[a]	5.1	140.33 ± 2.08[a]	4.6	118 ± 2.64[a]	4.6
	DCP + MBC[B]	38 ± 2[a]	5.6	63.66 ± 1.52[a]	5.3	112.66 ± 3.05[a]	4.8	99.33 ± 2.51[a]	5.1
	TCP + Control	306.66 ± 1.52	3.9	324 ± 2.64	3.3	287.66 ± 0.57	3.7	267 ± 2.64	4.1
	TCP + MBC[A]	72.66 ± 2.51[a]	5.8	126.33 ± 1.52[a]	5.3	146.33 ± 2.08[a]	5.1	144 ± 2.64[a]	5.0
	TCP + MBC[B]	60.66 ± 1.52[a]	5.9	114 ± 1.73[a]	5.6	134 ± 1[a]	5.3	122.33 ± 2.51[a]	5.2
	ZP + Control	273.33 ± 2.08	4.3	296.66 ± 1.52	4.1	319.66 ± 2.08	3.8	296 ± 1.73	3.9
	ZP + MBC[A]	68 ± 1.73[a]	5.8	105 ± 2[a]	5.5	132.66 ± 1.52[a]	4.6	127.33 ± 2.51[a]	5.1
	ZP + MBC[B]	48.33 ± 1.52[a]	3.6	92.66 ± 0.57[a]	3.5	117.33 ± 1.15[a]	3.2	102 ± 1[a]	3.3
Zn-Solubilization by *A. caviae* JAS15	ZO + Control	243.33 ± 1.15	4.2	329.33 ± 2.08	3.8	383.33 ± 1.52	3.4	330 ± 1	3.7
	ZO + MBC[A]	83.66 ± 0.57[a]	5.4	144.33 ± 1.52[a]	5.1	155.66 ± 1.15[a]	4.8	136 ± 2[a]	4.9
	ZO + MBC[B]	53.33 ± 1.52[a]	5.7	83.33 ± 2.08[a]	5.3	109.33 ± 1.52[a]	5.1	95.66 ± 1.52[a]	5.1
	ZP + Control	167.66 ± 1.52	4.6	277.33 ± 2.08	4.3	307.33 ± 2.51	3.8	279.33 ± 1.15	4.4
	ZP + MBC[A]	66.66 ± 2.08[a]	5.8	114 ± 1[a]	5.5	136.33 ± 1.52[a]	5.3	115.66 ± 1.15[a]	5.4
	ZP + MBC[B]	43.66 ± 2.51[a]	5.9	58.66 ± 0.57[a]	5.8	107 ± 2[a]	5.6	87.33 ± 1.52[a]	5.7
	ZC + Control	190.33 ± 1.52	4.3	317 ± 1.73	3.5	345 ± 1	3.4	303.66 ± 2.08	4.1
	ZC + MBC[A]	77.33 ± 0.57[a]	5.3	129.33 ± 1.52[a]	5.2	146 ± 2[a]	4.6	121.66 ± 2.51[a]	5.2
	ZC + MBC[B]	51.33 ± 1.15[a]	5.6	77.66 ± 1.52[a]	5.5	119 ± 1.73[a]	5.2	95.33 ± 2.08[a]	5.4

Each value represents the mean ± SD of three replicates per treatment. In the same column according to Dunnett's multiple comparison test significant differences at $P \leq 0.05$ levels over control are indicated by different letters

DCP dicalcium phosphate, *TCP* tricalcium phosphate, *ZP* zinc phosphate, *ZO* zinc oxide, *ZC* zinc carbonate

[A]MBC (50 mg l^{-1} of carbendazim)

[B]MBC (100 mg l^{-1} of carbendazim)

nutrients essential to plant, deficiency of soil P is one of the most important aspects that is involved in the growth and development of plants. In general, rhizospheric bacteria promote plant growth by different mechanisms. One of the important mechanisms is the solubilization of phosphate in the rhizosphere through which the growing plant receives the soluble form of phosphate (Ahemad and Kibret 2014). In our study, *A. caviae* JAS15 was capable of solubilizing various types of inorganic phosphate considerably in the presence of recommended and even at higher dosage of fungicide.

Zn is an essential micronutrient for plant growth and it is a vital constituent for various metabolic enzymes. The poor mobility of Zn in plants suggests the need for a constant supply of soluble Zn for optimum growth. Therefore, Zn supplied to plants in the form of fertilizers like $ZnSO_4$ was converted into insoluble forms depending upon the soil types and soil chemical reactions (Saravanan et al. 2007). However, it is plausible that exploitation of Zn solubilizing bacteria may aid in overcoming Zn deficiency in the soils. In our study, *A. caviae* JAS15 solubilized insoluble form of Zn compounds even in the presence of higher dose of fungicide. The solubilization potential of *A. caviae* JAS15 towards insoluble metal compounds was examined on the Tris minimal salts agar media, where solubilization could be visualized as a clear halo in the otherwise turbid agar medium. *A. caviae* JAS15 produced a clear halo zone on ZO (Zinc oxide) and ZC (Zinc carbonate) with a solubilization diameter of 16 mm and 13 mm, respectively, which was found to be significantly greater as compared with the other ZP amended agar medium (12 mm). In addition, the amount of Zn solubilized in liquid medium was also evaluated under control, 50 and 100 mg l^{-1} of MBC (Table 5). *A. caviae* JAS15 solubilized the Zn compounds added to the media and produced 383.33, 307.33, 345 μg ml^{-1} Zn from control flask supplemented with ZO, ZP and ZC, respectively. The amount of Zn solubilized in liquid medium decreased with 50 and 100 mg l^{-1} concentrations of fungicide. *A. caviae* JAS15 in culture broth spiked with 50 mg l^{-1} of MBC, solubilized the maximum amount of Zn compounds from ZO, ZP, ZC at 155.66, 136.33, 146 μg ml^{-1},respectively. *A. caviae* JAS15 produced 109.33, 107 and 119 μg ml^{-1} of soluble Zn in the liquid medium containing ZO, ZP and ZC with 100 mg l^{-1} of MBC, respectively.

The major mechanism of mineral phosphate and Zn solubilizing property is by the secretion of organic acids synthesized by microorganisms (Patel et al. 2011). The ability of microorganism to solubilize P complexes has been attributed to the process of acidification, chelation, exchange reactions and production of organic acids (Gulati et al., 2010). The mineral P and Zn solubilizing property results in a drop in pH, which has been associated with their ability to secrete low-molecular-weight organic acids such as gluconic, 2-ketogluconic, oxalic, citric, acetic, malic, succinic and tartaric acids and so forth (Busato et al. 2012; Saravanan et al. 2007). Similar trend was recorded in our study with a decrease in the pH of liquid medium during P and Zn solubilization (Table 5).

In the absence of fungicide, *A. caviae* JAS15 produced a maximum amount of 37.33 µg ml^{-1} IAA, which however decreased progressively with increasing concentration of fungicide. The quantity of IAA released by the *A. caviae* JAS15 with the graded increment of 50 and 100 mg l^{-1} of MBC in the culture medium was 26.66 and 14.66 µg ml^{-1}, respectively. The IAA produced by rhizobacteria is known to promote root growth by directly stimulating plant cell elongation or cell division (Ahmed and Khan 2012). In our study, substantial IAA was produced by the *A. caviae* JAS15 even at the presence of higher dose (100 mg l^{-1}) of fungicide.

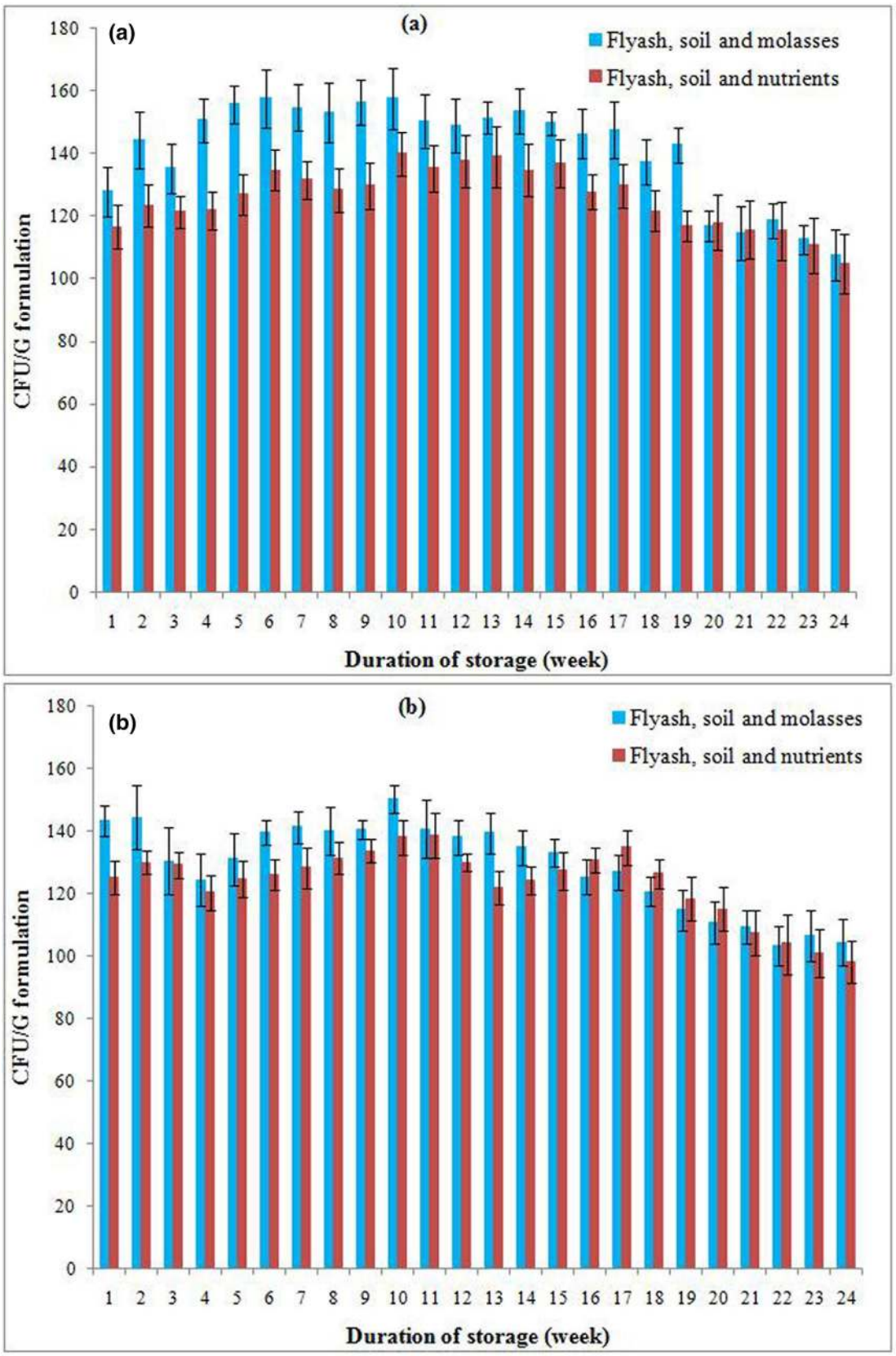
In the present study, hydrogen cyanide and ammonia production by the *A. caviae* JAS15 remained unaffected under fungicide stress. Similarly, hydrogen cyanide and ammonia production by rhizobacterial strain has been reported earlier (Abraham and Silambarasan 2016; Ahmed and Khan 2012). Rhizobacteria protect the growing plants from phytopathogen attack by directly killing parasites by producing hydrogen cyanide (Kang et al. 2010). The ammonia released by the rhizobacterial strain plays a signaling role in the interaction between rhizobacteria and plants and also increases the glutamine synthetase activity (Chitra et al. 2002). The present study suggested that the intrinsic ability of this multifunctional bacterial isolate producing plant growth-promoting substances in both the presence and absence of fungicide could be used to increase the growth of plants under a MBC stressed environment.

Formulation of strains *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15

The molasses is a good source of carbon needed by the bacteria and fungi (Abraham and Silambarasan 2016). The sawdust with 5% molasses provided large, porous and light substrate for proper colonization and multiplication of the bacteria and fungi (Khan et al. 2011). Wood is a preferred substrate for the colonization of a large number of microorganisms (Morsy et al. 2009). Therefore, in the present study, saw dust, soil and molasses was used for mass production of the microorganisms which provides sustained nutrition for the multiplication and survival of the propagules. The formulated bacterial products were viable and without any significant microbial contamination during the storage, they multiplied and maintained a higher colony forming units (Fig. 4). Microorganism other than the formulated bacterial culture was rarely observed and contamination when detected was 1 or 2 colonies in all Petri plates of treatment.

Conclusions

In this study, *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15 were isolated and characterized as an efficient MBC degrading bacterium. The *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15 were able to utilize MBC as the sole carbon source for growth and degrade MBC. Results of the MBC degradation kinetics indicated that the zero-order model fit well. A possible degradation pathway of MBC by *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15 has been proposed based on GC–MS analysis. Phytotoxicity and cytogenotoxicity studies proved that the less/non-toxic metabolites were generated. The developed powder based formulation with fly ash, soil and molasses maintained higher cell viability of bacterial strains up to 24 weeks. *A. caviae* JAS15 showed positive results for the phosphate solubilization, Zn solubilization, and IAA, hydrogen cyanide and ammonia productions. This work highlights that *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15 promise the great potential to remove MBC and provide a practical approach towards the rapid degradation of MBC-contaminated soils. However, since this study was conducted under in vitro conditions, further research in MBC affected field soil is necessary to utilize *Chryseobacterium* sp. JAS14 and *A. caviae* JAS15 as efficient bioinoculants for degrading MBC in natural ecosystems.


(a)
Flyash, soil and molasses
Flyash, soil and nutrients
CFU/G formulation
Duration of storage (week)
(b)
Flyash, soil and molasses
Flyash, soil and nutrients
CFU/G formulation
Duration of storage (week)

◂**Fig. 4** **a** Shelf life test of *Chryseobacterium* sp. JAS14 and **b** *A. caviae* JAS15. The inoculant was formulated in fly ash, soil and molasses/nutrients, and stored for 24 weeks at room temperature. Cell viability in terms of CFU g^{-1} was enumerated at 1 week intervals. Data presented as means of three replicates with standard deviation

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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