Biotechnology Research and Innovation (2018) 2, 45–57

Biotechnology Research & Innovation

http://www.journals.elsevier.com/biotechnology-research-and-innovation/

RESEARCH PAPER

Optimized biodegradation of carcinogenic fungicide Carbendazim by *Bacillus licheniformis* JTC-3 from agro-effluent

Jigisha Panda*, **Tiyasha Kanjilal**, **Sumona Das**

Jadavpur University, Department of Chemical Engineering, Jadavpur, West Bengal, India

Received 21 June 2017; accepted 24 October 2017
Available online 16 November 2017

KEYWORDS
Carbendazim;
2-Hydroxybenzimidazole;
Second order kinetics;
Gompertz

Abstract The aim of this research work is to study biodegradation of Carbendazim, a globally used carcinogenic fungicide, by novel bacterial strain *Bacillus licheniformis* JTC-3, isolated from local agro-effluent, as it wrecks havoc on human immune, nervous and endocrine systems, upon consumption. The strain was characterized by various biochemical tests, antibiotic assay and identified by 16S rDNA analysis. High Performance Liquid Chromatographic studies showed its biodegradation capacity to be very high (73.2% of initial Carbendazim concentration), in Minimal Salt Media, within 24 h of incubation. Various growth parameters (temperature, agitation speed, pH, substrate concentration) were optimized by Central Composite Design to get the biodegradation rate of 0.305 mg/L of Carbendazim/h/mg biomass. Scanning Electron Micrograph, X-ray diffraction, Fourier Transform Infra-Red spectroscopic microanalysis and toxicity testing of metabolic end-product confirmed formation of non-toxic, crystalline 2-hydroxybenzimidazole. Accordingly, a plausible mechanism of biodegradation of Carbendazim has been proposed here. The isolate's growth curve and the rate kinetics mathematically fitted well with Gompertz model and second order reaction, respectively.

Introduction

Pesticides are biocidal substances used to destroy '*pests*' for protecting plants from weeds, fungal infection and insects (FAO-WHO Joint Report, 2012). Based on chemical composition these pesticides could be of various types: organophosphates, organochlorides, carbamates, pyrethroid, sulfenylurea, biopesticides etc. But depending on their inhibitory actions, these are mainly of three types: herbicides, insecticides and fungicides (Dam, 1974). Fungicides, like Carbendazim ($C_9H_9N_3O_2$; methyl 1H-benzimidazol-2-yl carbamate or MBC; relative molecular mass = 191.2), are widely used in agriculture and forestry to protect arable crops (cereals, oil, rice, maize, cotton, peas, grapes etc.), fruits, vegetables, ornamentals and medicinal herbs from fungal diseases, like sheath blight, brown spot, scab, leaf-spot etc., as they are designed to suppress the biochemical metabolism of the target phytopathogenic fungi (WHO Report, 1993). In fungi, it appears to binds to an unspecified site on tubulin and suppresses microtubule assembly dynamic. This results in cell cycle arrest at the G2/M phase and an induction of apoptosis (NPIRSD Report, 2016) Carbendazim, a well-known systemic benzimidazole fungicide, is an active

* Corresponding author.
E-mail: jigisha.panda@gmail.com (J. Panda).

https://doi.org/10.1016/j.biori.2017.10.004

ingredient in some commercial agents, like, Arrest, Bavistin, Cerezim, Desrosal, Funaben, Hinge, Kolfugo Super, Occidor, Sabendazim etc. It is also the major metabolic product of some other systemic fungicides, such as, benomyl and thiophanate-methyl (Montgomery, 1997). It is often commercially available as crystalline compounds (MacBean, 2010) or gray-to-white powder (O'Neil, 2013). Its melting point is between 302 and 307 °C and density is 1.45 at 20 °C (Haynes, 2015). World-wide, Carbendazim has extensive applications, with the global market worth over $200 million at user level, equivalent to over 12,000 tons at active ingredient level. About 1000 tons are produced every year in India, where the consumption has now reached over 700 tons per annum (Alexander, 1981). Carbendazim is a stable compound with long half-life: it takes about 3–6 months to be decomposed on turf soil, about 6–12 months on bare soil, and up to 25 months in water, under both aerobic and anaerobic conditions (Yu, Chu, Pang, Xiang, & Fang, 2009). Because of its extensive use and its relatively high stability, very high levels of this fungicide have been reported in air, water, soil as well as in foods and biological materials in India. The presence of high levels of Carbendazim in air, water and soil has a number of deleterious effects. The growth of microorganisms in lucerne planted soil is often inhibited by carbendazim. A significant inhibitory effect on soil ammonification, nitrification, and soil dehydrogenase activity has also been observed (Pattanasupong et al., 2004).

Carbendazim is particularly hazardous to living organisms, including humans. As per NPIRSD Reports (2016), 99.83% of Carbendazim may cause genetic defects (Germ cell mutagenicity – Category 1A, 1B), 99.65% of it can damage fertility (Reproductive toxicity – Category 1A, 1B) and 93.92–98.96% of it is very toxic to aquatic life with long lasting effects (acute hazard – Category 1). Its mutagenic, carcinogenic and teratogenic properties have been reported since Carbendazim may cause damage to the immune, nervous or endocrine systems (Fang et al., 2010; Singhal, Bagga, Kumar, & Chauhan, 2003; Xu et al., 2007). Therefore, for protecting our living organisms from the harmful effects of Carbendazim, this toxicant is required to be removed from the environment. Conventional physico-chemical methods, like chemical degradation in aqueous solution by UV-TiO_2 photocatalysis, evaporation, volatilization, hydrolysis, oxidation etc. are time consuming and expensive. On the other hand, climate-dependent process like phytoremediation has portability problems (Mazellier, Leroy, Laat, & Legube, 2003). The process of bioremediation/biodegradation has distinct advantages over conventional techniques: complete breakdown organic contaminants into other nontoxic chemicals; minimal requirement of equipment or chemicals and hence, low cost of treatment per unit volume of soil or groundwater compared to other remediation technologies; also, can be implemented as an in situ or ex situ method depending on conditions (Agnieszka, Jacek, & Wojeciech, 2010). Degradation rates of Carbendazim by physical and abiotic chemical processes are reported to be slow, with microbial metabolism thought to be the principal degradative process in natural soils. Only a limited number of Carbendazim-degrading bacterial strains have been previously reported. Highly efficient, ecologically competitive microbes are required to remediate a range of Carbendazim contaminated environments (Holtman & Kobayashi, 1997; Xinjian et al., 2013). Hence, bacterial biodegradation could prove to be an eco-friendly, efficient and cost-effective alternative. So degradation by microbes has evolved as a special area of interest to researchers world-wide. Some aerobic bacterial strains (*Nocardioides* sp., *Pseudomonas* sp., *Rhodococcus jialingiae* etc.) are reported to show the capability to degrade Cabendazim to 2-aminobenzimidazole, benzimidazole and 2-hydroxybenzimidazole, via specific mechanistic pathways, and use them as carbon and energy sources, but their degradation rates are very slow in synthetic media (up to 99% in 72 h). Furthermore, they have low activity/survival in real environment, since they are exposed to *abiotic* (pH, temperature inorganic nutrients etc.) and *biotic* (predation, competition etc.) stress there that they are never exposed to in laboratory environment (Pandey et al., 2010). Very few research works are reported, regarding biodegradation of Cabendazim in India (Sharma & Arya, 2014; Xiao et al., 2013). Hence, in this present study, a novel bacterial isolate *Bacillus licheniformis* JTC-3 has been reported, for fast and efficient biodegradation of Carbendazim, from agro-wastes.

Materials and methods

Reagents and chemicals

Analytical grade Carbendazim (99% pure MBC) from Sigma–Aldrich Inc., dipotassium hydrogen phosphate or K_2HPO_4 (E. Merck), potassium dihydrogen phosphate or KH_2PO_4 (E. Merck), magnesium sulfate or $MgSO_4$ (E. Merck), sodium chloride or NaCl (E. Merck), ammonium nitrate or NH_4NO_3 (E. Merck), commercially available Folin-Ciocalteau reagent (E. Merck), lysozyme (3× crystallized ext. egg white extrapure for biochemistry, supplied by SRL, India), methanol (E. Merck), HPLC grade water (E. Merck), acetic acid (E. Merck), Luria-Bertani Agar and Broth media (HiMedia, India), sodium carbonate or Na_2CO_3 (E. Merck), copper sulfate or $CuSO_4$ (E. Merck), disodium hydrogen phosphate or Na_2HPO_4 (E. Merck), sodium dihydrogen phosphate or NaH_2PO_4 (E. Merck), copper sulphate or $CuSO_4$ (E. Merck), sodium hydroxide or NaOH (E. Merck), potassium sodium tartarate or $KNaC_4H_4O_6{\cdot}4H_2O$ (E. Merck), bovine serum albumin standard protein (SRL, India), biochemical test kit for bacterial characterization (HiBacillus™ Identification Kit) from HiMedia, India, Agar (E. Merck), commercially available impregnated discs of various antibiotics: Ampicillin (A), Amicacin (AM), Streptomycin (S), Chloramphenicol (C), Azithromycin (AT), Penicillin (P), Gentamycin (G), Cephalosporin (CE), Tetracyclin (T) obtained from HiMedia, India, microtips (of 2–10 μL, 10–200 μL, up to 1000 μL capacities), gamma irradiation sterilized petriplates (Tarson, India), glasswares (Borosil, India).

Sample collection from agro-wastes

Carbendazim is known to be extensively used in the arable crops of the agricultural lands adjacent to Mathpukur, East Kolkata Wetlands, West Bengal, (Ramsar site No. 1208, 22° 25′40″ North, 88° 22′55″ East) India. Hence, 1 L of the effluent sample was aseptically collected, following the

conventional standard collection protocol (Panda & Sarkar, 2012a, 2012b), from an excess running water flow channel from the agricultural land, for the purposes of isolation of Carbendazim-degrading bacteria and also for lab-level study of effluent treatment. This agricultural area has a history of repeated Carbendazim application. The collected effluent was then analyzed to study its physico-chemical properties and BOD/COD values.

Isolation and characterization of the carbendazim-degrading bacterium from agro-wastes

The collected liquid agro-waste was serially diluted in sterile normal saline (0.89% NaCl) and plated on Minimal Salt Media plates (made selective by adding Carbendazim) having K_2HPO_4 1.5 g/L, KH_2PO_4 0.5 g/L, $MgSO_4$ 0.2 g/L, NaCl 1 g/L, NH_4NO_3 1 g/L, Carbendazim 10 mg/L, Agar 15 g/L, pH 7 (Fang et al., 2010). The plates were incubated for 24 h at 37 °C. One bacterial strain was isolated and its colony morphology was studied. This was further subjected to Gram staining for primary morphological study of the bacterial cells under Inverted Microscope (Axio Vert.A1, Zeiss, Germany). Biochemical characterizations were studied in HiBacillus™ Identification Kit (HiMedia, India) having mannitol salt agar media, urease production media, gelatine liquefaction media, lipase production media, carbohydrate utilization media (with, glucose, sucrose, maltose, fructose), amino acid utilization media (with glutamic acid, aspartic acid, phenylalanine, cysteine). Various tests such as Indole Methyl Red Voges-Proskauer Citrate utilization (IMViC), starch hydrolysis, motility, catalase production, and nitrate reduction were also performed with this strain. The results of biochemical tests were cross-checked with ''Bergey's Manual of Determinative Bacteriology'' and confirmed by 16S rDNA genome sequencing for the genus and species identification of the isolates, using forward and reverse primers, in DNA sequencer (MegaBACE 1000, GE Healthcare, USA). Antibiotic sensitivity assay was performed with lawn culture of this strain on Luria-Bertani agar media (pH 7) against commercially available impregnated discs of various antibiotics: Ampicillin (A), Amicacin (AM), Streptomycin (S), Chloramphenicol (C), Azithromycin (AT), Penicillin (P), Gentamycin (G), Cephalosporin (CE), Tetracyclin (T). Minimum Inhibitory Concentration (MIC) of Carbendazim at which no bacterial growth occurred, was detected by inoculating each culture in a series of LB broths having varying concentrations of Carbendazim – ranging from zero to 1000 mg/L. Then these were incubated for 24 h at 37 °C and turbidity of the broth culture observed as a measure of bacterial growth by UV-vis spectrophotometer (Helios γ UVG-094737, Thermospectronic, UK), at 600 nm wavelength (Panda & Sarkar, 2012a, 2012b).

Identification of the isolate

The genus and species of the said strain was identified by 16S rDNA gene sequencing. DNA was isolated from the culture and quality was evaluated on 1.2% Agarose Gel. Fragment of 16S rDNA gene was amplified by PCR using 8F and 1492R from the above isolated DNA. A single discrete PCR amplicon band of 1500 bp was observed. The PCR amplicon was purified and further used for the sequencing. Forward and Reverse DNA sequencing reaction of PCR amplicon was carried out with 704F and 907R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 1424 bp 16S rDNA gene was generated from forward and reverse sequence data using aligner software (Panda & Sarkar, 2012a, 2012b). The 16S rDNA gene sequence was used to carry out BLAST alignment search tool of NCBI genbank database. Based on maximum identity score first fifteen sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 5 (Panda & Sarkar, 2015).

Statistical designing of biodegradation of carbendazim by the isolate

Some critical environmental and physical parameters (Initial MBC concentration, Temperature, Agitation Speed, pH) for bacterial growth in MSM broth were used as the input in the Central Composite Design (CCD) in Design Expert 8.1 Software (Stat-Ease, Inc., Minneapolis, USA). Different combinations of the four parameters were obtained and then according to those batch cultures were inoculated and incubated at 37 °C for 24 h. After incubation, the cultures were removed via filtration (0.22 μm) the remaining MBC in the broth was detected and quantified by High Performance Liquid Chromatography or HPLC (Alliance® HPLC, Waters, USA) at 281 nm (Fang et al., 2010), against some standard MBC solution using Eq. (1):

$$\text{Conc. of unknown sample} = \frac{\text{Conc. of standard sample} \times \text{Peak of sample}}{\text{Peak of standard}} \quad (1)$$

The results were expressed as the degrading efficiency (% E) of MBC by the isolated bacterial strain or the % MBC consumption, which was quantified using Eq. (2):

$$\%E = \frac{C_0 - C_f}{C_0} \times 100 \quad (2)$$

where C_0 is the initial MBC concentration (mg/L), C_f is the concentration of MBC after degradation (mg/L). The percentage of MBC consumed by the said strain was given as the response for the different combinations. Response surface methodology was used to optimize the percentage degradation of MBC. To ascertain the reproducibility of the data, all experimental runs were conducted in triplicate.

Optimization of biodegradation potential of the isolate

Critical environmental (initial MBC concentration, pH) and physical parameters (temperature, agitation speed) for bacterial growth in MSM broth were varied to optimize the biodegradation potential of the strain. pH in the growth media was varied from 3 to 9 keeping Carbendazim concentration, temperature and agitation speed constant, respectively, at 10 mg/L, 37 °C and 100 rpm. Initial MBC

concentration was varied from 10 to 100 mg/L while temperature, agitation speed and pH were kept constant at 37 °C, 100 rpm and 7, respectively. Temperature for bacterial growth was varied from 10 to 45 °C when Carbendazim concentration was held constant at 10 mg/L, pH was maintained at 7 and agitation speed at100 rpm. Finally, agitation speed was varied from 40 to 100 rpm while keeping Carbendazim concentration, temperature and pH constant at 10 mg/L, 37 °C and7, respectively. To ascertain reproducibility of the data, all experimental runs were conducted in triplicate. After incubation, the cultures were removed via filtration (0.22 μm). The remaining MBC in the broth was detected and quantified by HPLC (Alliance® HPLC, Waters, USA), against some standard MBC solutions (Xiao et al., 2013).

The chromatographic separation was achieved on an XDB-C18 column (150 mm × 4.6 mm i.d., 5 μm) at room temperature. And 10 μL samples were analyzed by measuring the absorbance at 281 nm with an elution of methanol and water mixture (45:55, v/v) at a flow rate of 0.8 mL/min.

Studying the mechanism for biodegradation of carbendazim by the isolate

To study the mechanism for biodegradation of Carbendazim by the isolate and its interaction with the substrate, cells were subjected to Scanning Electron Microscopy (EVO LS 15, Zeiss, Germany), Fourier Transform Infra-Red spectroscopy (Nicolet S10, ThermoScientific, The Netherlands) and X-ray diffraction analysis (PW 3040/60, X'Pert PRO, PANanalytical, The Netherlands). For this purpose, 1% of broth-culture cells were grown in Minimal Salt Media broths having K_2HPO_4 1.5 g/L, KH_2PO_4 0.5 g/L, $MgSO_4$ 0.2 g/L, NaCl 1 g/L, NH_4NO_3 1 g/L, pH 7, both in absence and presence of Carbendazim (10 mg/L) as the sole hydrocarbon source. The broths were incubated for 24 h at 37 °C, and were finally examined under Scanning Electron Microscope for any change in morphological behavior (Panda & Sarkar, 2012a, 2012b).

Scanning Electron Microscopic (SEM) images show difference in surface topography formed by the change in intensity of the secondary electrons emitted from the bacterial cell-surface, due to incident electron beam and hence are a good representation of the 3D structure of the cell. Fourier Transform Infra-Red (FTIR) spectroscopy of lyophilized powder form of bacterial cells (grown in presence of 10 mg/L Carbendazim and also in absence of any hydrocarbon substrate) was performed to acquire information regarding wave number changes of the functional groups. Spectra were obtained from 400 to 4000 cm^{-1}. Phase identification of samples was carried out with the help of X-ray diffraction analysis (XRD) at 40 kV voltage and 30 mA current and calibrated with 0.15406 nm radiation, λ; α a standard silicon sample, using Ni-filtered Cu K at a rate of 4° per minute, using 2θ value range of 0 to 90° (along the *x*-axis) versus intensity count (in a.u.) along the *y*-axis (Panda & Sarkar, 2014).

Biodegradation of carbendazim in real sample: kinetic study

100 mL of pre-collected agro-effluent (having 10 mg/L Carbendazim; pH 6.8) was treated with 2 mg/mL of lyophilized cells of log phase culture of the strain. Carbendazim degradation with the increase in biomass was monitored in this batch biodegradation study (Pattanasupong et al., 2004).

This study leads to the formation of biomass and the amount of biomass formed increases exponentially with time during the log phase. Furthermore, the increase in biomass concentration depends on the depletion of substrate concentration. As Carbendazim biodegradation is the result of bacterial activity, the kinetics of the degradation process is related to the specific growth rate of microorganisms. The specific growth rate of microorganism in turn is proportional to the number of microorganisms present at a given time in the log phase. In general, experimental values of the specific growth rates so obtained were used to test various growth kinetic models. In biodegradation processes, the rate of disappearance of substrate

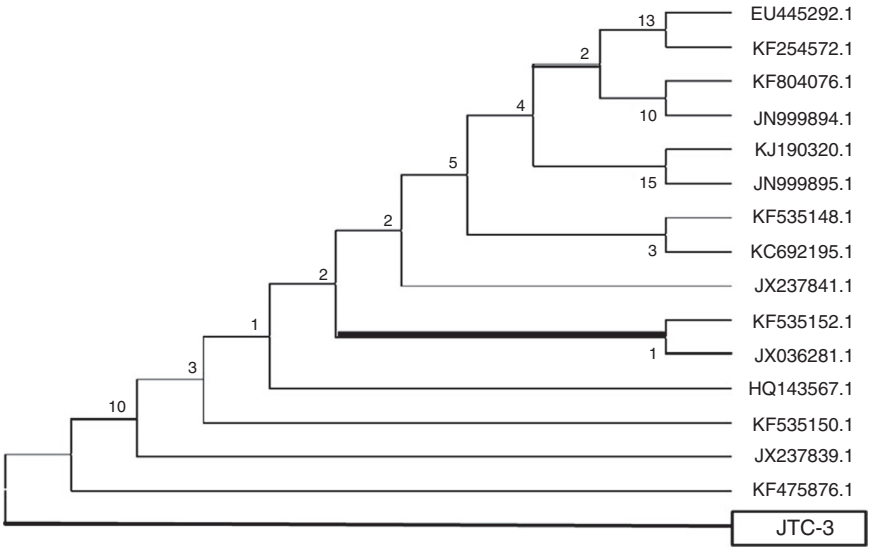

Figure 1 Phylogenetic tree of JTC-3 formed by neighbor-joining method.

(Carbendazim) is dependent on the substrate concentration. The change in substrate concentration with time was studied using zero-order, first-order and second-order rate kinetics. Likewise, biomass is produced simultaneously as substrate concentration decreases (Paszko, 2006). The optimized biodegradation rates of Carbendazim by *Bacillus licheniformis* JTC-3 was compared with the reported results (Table 5). Gui-Shan, Xiao-Ming, Tian-Fan, Xiao-Hang, and Yu-Hua (2005) reported collection of 7.5 g of contaminated soil, with Carbendazim concentrations between 0.2 and 0.8 g/L, for the purpose of bacterial isolation. Fang et al. (2010) reported collection of 5 g of 20 mg/L Carbendazim-contaminated soil sample. Both the authors isolated bacteria at 30 °C, pH7 and studied the complete biodegradation over 72 h duration. None of them reported the conventional standard processes of sample collection, though.

Toxicity testing of the bio-degraded end-products

The choice behind bio-degradation process over other conventional waste water treatment process lies on the ultimate fate of the substrate molecules. Safe discharge of wastewater requires meeting the associated regulatory standard. In this context, the degraded metabolite profile was investigated by HPLC. The antimicrobial susceptibility study of the degraded metabolites was carried out with *Pseudomonas aeruginosa* (*MTCC 434*), known to be one of the most prevalent microorganisms in environment. For this purpose, wells were aseptically bored (6 mm diameter) in LB agar media plated with lawn cultures of our isolate, and 10–100 mg/L concentrations of Carbendazim and its degraded metabolite product (identified and isolated by HPLC) were poured in those wells. Plates were incubated at 37 °C for 24 h (Das, Sarkar, Chakraborty, Choi, & Bhattacharjee, 2014) before results were observed and recorded (by measuring clear zone of inhibition of bacterial growth around the wells).

Results and discussion

Isolation and characterization of the carbendazim-degrading bacterium from agro-wastes

The collected effluent was found to have pungent odor at 30 °C and pH7. Its BOD and COD values were calculated, following standard protocols (APHA, 1989), to be 720 and 875 mg/L respectively. The conductivity and salinity values of the same were detected (with the help of standard equipments) to be 4 μS/cm and 1.7 respectively. The effluent was further used for isolation of Carbendazim-degrading bacterium and other degradation studies.

One Carbendazim degrading bacterium was isolated from MSM agar plates (with 10 mg/L Carbendazim) and was arbitrarily named as JTC-3. It showed discrete individual cells when grown without any stress (under 45× magnification of inverted compound microscope) and then cells formed extensive chain like structures (with bacterial cells being fused with each other) under stressful growth conditions. *Minimum Inhibitory Concentration* (*MIC*) of Carbendazim with JTC-3 showed that the isolate could withstand and grow in substrate concentrations as high as 1000 mg/L. JTC-3 gave positive results in mannitol salt utilization, urease production, gelatin liquefaction, citrate utilization, starch hydrolysis, various carbohydrates and amino acids utilization and also in nitrate reduction. In case of Indole production, Voges-Proskauer, lipase and catalase production tests it gave negative results. The results of these biochemical tests were cross-checked with ''Bergey's Manual of Determinative Bacteriology'' (Holt, Krieg, Sneath, Staley, & Williams, 1994) and confirmed by 16S rDNA genome sequencing for the genus and species identification of the isolates. In the antibiotic sensitivity assay, the strain was found to be resistant against Gentamycin, Azithromycin, Tetracyclin, Ampicillin, Chloramphenicol and Streptomycin and the same was sensitive to Amicacin, Penicillin and Cephalosporin.

Identification of the isolate

The strain was analyzed to be *B. licheniformis* (GenBank Accession Number: HQ143567.1) based on nucleotide homology and phylogenetic analysis (Fig. 1).

Statistical designing of biodegradation of carbendazim by the isolate

In order to examine the effects of individual parameters as well as their relative effects on the response variable, a general second order polynomial model, as shown below (Eq. (3)), was selected:

$$y = b_0 + \sum_{i=1}^{4} b_i X_i + \sum_{i=1}^{4} b_{ii} X_i^2 + \sum_{i=1}^{4} b_{ii} X_i^3 \sum_{j=1}^{4} b_{ij} X_I X_j \qquad (3)$$

where '*y*' is the response variable, b_o is the constant, b_i is the linear coefficient, b_{ii} the quadratic coefficient, b_{ij} the interaction coefficient and X_i is dimensionless coded variables (X_1 depicted for initial MBC concentration, X_2 for temperature, X_3 for agitation speed and X_4 for pH). The above equation was used to maximize '*y*' using a numerical optimization program. Response surface methodology was used to maximize the percentage degradation of MBC (Table 1).

Initial MBC concentration (*c*) was varied within the range of 10–100 mg/L, temperature (*t*) within the range of 10–45 °C, agitation speed (*a*) within the range of 20–100 rpm, pH (*p*) within the range of 3.0–7.0. A Central Composite Design (CCD) was created by entering values for '*c*', '*t*', '*a*', '*p*' in terms of ±1 level.

According to statistical model fit summary as reported in Table 2, a quadratic model was found to be the best fitted model with lower standard deviation (0.75), lowest PRESS value (49.08), higher adjusted and predicted R^2 value (0.99) as compared to other tested models. Analysis of variance (ANOVA) for quadratic model illustrated in Table 3 shows high R^2 value (0.96), and acceptable *p*-value (<0.00), which proves the adequacy of the quadratic model. According to ANOVA, model insignificant terms (*p*-value > 0.05), are

Table 1 Optimization of MBC consumed (*R*) as a response.

Run	Concentration (*c*) mg/L	Temperature (*t*) (°C)	Agitation speed (*a*) rpm	pH (*p*)	Response (*R*%)
1	80	35	70	5.5	55.87
2	70	40	80	6	79.21
3	80	45	70	6.5	59.33
4	80	45	90	6.5	35.21
5	80	45	90	5.5	72.03
6	60	45	90	5.5	65.78
7	70	40	80	6	79.21
8	50	40	80	6	62.79
9	60	45	90	6.5	36.85
10	60	45	70	6.5	75.5
11	90	40	80	6	58.34
12	60	35	70	5.5	55.6
13	80	35	90	5.5	76.75
14	70	40	100	6	59.06
15	80	35	90	6.5	53.38
16	70	40	60	6	62.77
17	70	50	80	6	50.97
18	70	40	80	5	65.41
19	70	30	80	6	57.52
20	60	35	90	5.5	75.64
21	60	35	90	6.5	40.14
22	70	40	80	6	79.21
23	80	35	70	6.5	53.81
24	70	40	80	6	79.21
25	70	40	80	7	44.21
26	70	40	80	6	79.21
27	80	45	70	5.5	62.16
28	60	45	70	5.5	70.53
29	60	35	70	6.5	65.67
30	70	40	80	6	79.21

Table 2 Statistical model fit summary.

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	11.15	0.27	0.15	0.00	4259.50	
2FI	9.07	0.63	0.44	0.39	2578.38	
Quadratic	*0.75*	*0.99*	*0.99*	*0.98*	*49.08*	*Suggested*
Cubic	0.54	0.99	0.99	0.93	290.21	Aliased

neglected and the final equation in coded form (Eq. (4)) expressed as:

$$R\% = 68.14 - 0.50X_1 - 3.42X_2 - 5.27X_3 + 1.32X_4 - 3.66X_1X_2 - 0.72X_1X_3 - 4.00X_1X_4 - 4.96X_2X_3 - 1.97X_2X_4 - 4.89X_3X_4 - 5.92X_1^2 - 1.55X_2^2 - 5.66X_3^2 - 2.32X_4^2 \quad (4)$$

The optimum process control conditions for MBC bioremediation efficiency by *Bacillus licheniformis* JTC-3 can be predicted from the three dimensional curves (Fig. 2a–c). The 3D curve in Fig. 2a is representing a definite number of combinations of two variables – initial MBC concentration and temperature with the initial MBC concentration maintained at the middle value. The maximum degradation of MBC was obtained at temperature 37 °C. The uptake of MBC by *Bacillus licheniformis* JTC-3 reduced as the temperature decreased from 37 °C, whereas; the concentration became higher as the temperature increased from 30 °C till it reached the optimum condition of 37 °C.

The 3D curve, in Fig. 2b is representing a definite number of combinations of initial MBC concentration and agitation speed with the initial MBC concentration maintained at the middle value. It's evident from here that the maximum MBC biodegradation was obtained at 80 rpm. The biodegradation rate increased as the agitation speed increased from 60 rpm till it reached an optimum value of 80 rpm.

Likewise, in Fig. 2c, the 3D curve is representing a definite number of combinations of pH and temperature. Hence, it is clear that the maximum efficient biodegradation of MBC was obtained at pH 5.0 and at temperature 37 °C. Below pH 5.0 and temperature 40 °C, the biodegradation ability of *Bacillus licheniformis* JTC-3 is low.

Table 3 ANOVA analysis for response surface quadratic model.

Source	Sum of squares	*F* value	*p* value	Significant
Model	4936.69	33.03	<0.00	
A = initial MBC concentration	27.68	2.59	0.12	
B = temperature	6.92	0.65	0.43	
C = agitation speed	105.78	9.91	0.00	
D = pH	1029.25	96.41	<0.00	
AB	31.33	2.94	0.10	
AC	191.69	17.96	0.00	
AD	14.81	1.39	0.25	
BC	332.40	31.14	<0.00	
BD	10.66	1	0.33	
CD	1140.19	106.80	<0.00	
A^2	514.26	48.17	<0.00	
B^2	958.12	89.75	<0.00	
C^2	493.63	46.24	<0.00	
D^2	912.47	85.47	<0.00	
R^2				0.96
C.V%				5.19

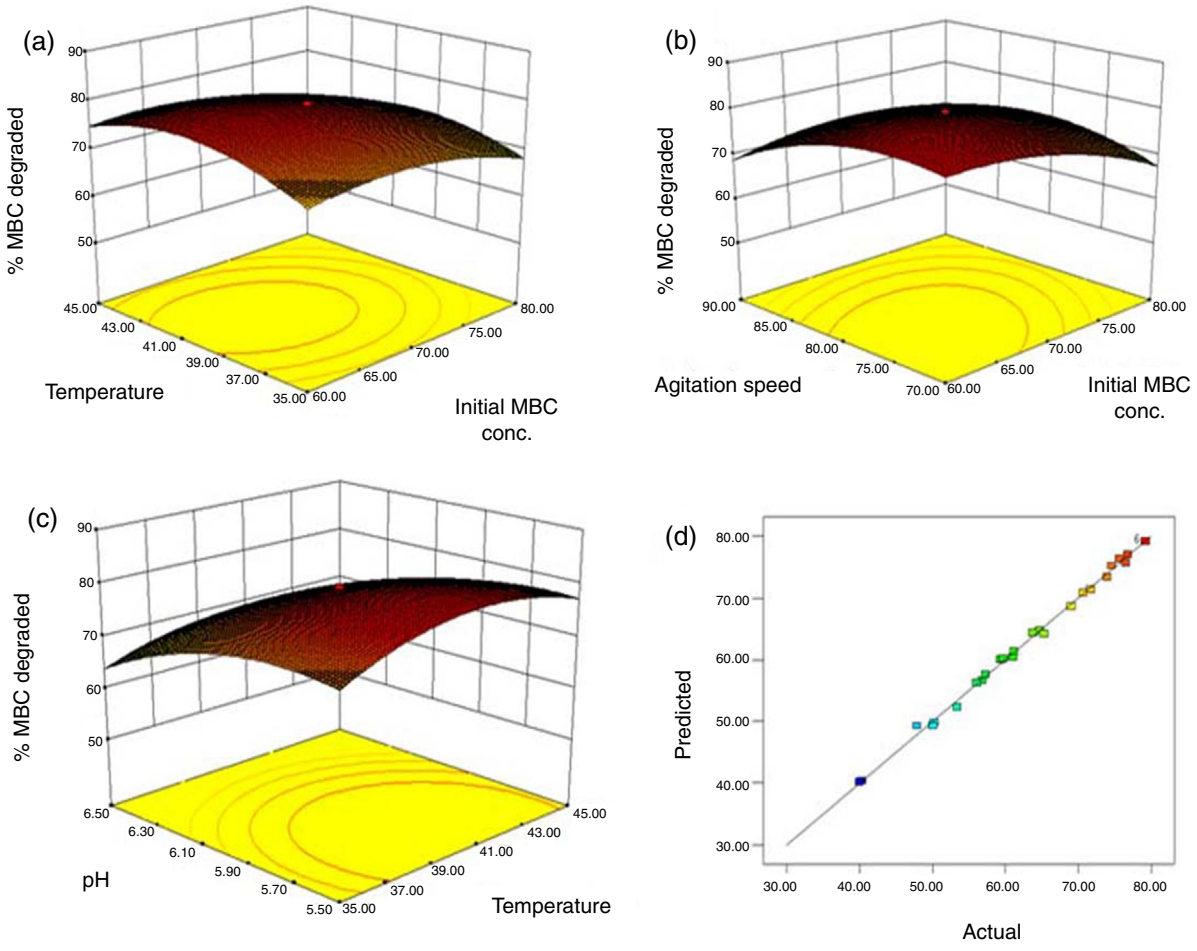

Figure 2 Optimization of biodegradation potential of the isolate by studying (a) initial MBC concentration v/s temperature, (b) initial MBC concentration v/s agitation speed, (c) pH v/s temperature and (d) predicted response value versus the actual degradation value.

The optimization of the degradation parameters of MBC using CCD revealed that all the lab scale parameters had significant influence in controlling the percent consumption of the pesticide, which illustrated positive linear correlation with all the four variables. All the variables showed positive effect in the consumption efficiency of MBC. Fig. 2d showed the plot of predicted response value over the actual degradation value which demonstrates the significance of the model for maximization of the MBC biodegradation.

Optimization of biodegradation potential of the isolate

Initial MBC concentration was varied from 10 to 100 mg/L when *Bacillus licheniformis* JTC-3 was grown at 37 °C, with 100 rpm agitation speed, at pH7. It was observed that the strain was capable of degrading a very high concentration of substrate (100 mg/L). This is in congruence with the results of MIC studies. Temperature for bacterial growth was varied from 10 to 45 °C when Carbendazim concentration, pH and agitation speed were kept constant respectively at 10 mg/L, 7 and 100 rpm. It was observed that the optimum biodegradation rate was achieved at 37 °C. Keeping Carbendazim concentration, temperature and agitation constant respectively at 10 mg/L, 37 °C and 100 rpm, the pH was varied from 3 to 9. The optimum biodegradation was achieved at around pH 5. Finally, Agitation Speed was varied from 40 to 100 rpm when Carbendazim concentration was kept constant at 10 mg/L, temperature at 37 °C and pH at 7. The optimum biodegradation rate was achieved at around 80 rpm. These results (Table 4) are in congruence with the experimental design as mentioned in the previous section. The rates were calculated by comparing the MBC concentration (via HPLC) before and after inoculation with JTC-3.

Biodegradation of 10 mg/L Carbendazim at zero hour of incubation in MSM media was studied at 37 °C, pH 5 with 80 rpm agitation speed and the results were analyzed using information from the HPLC chromatogram (Fig. 3a) The HPLC chromatogram showed a peak of Carbendazim at retention time 2.120 min, with peak height of 27,594 μA and area of 1,247,580 μV s. When the same was studied (Fig. 3b) under similar conditions at 12 h of incubation, the degraded peak (in HPLC chromatogram) of Carbendazim was found at retention time 2.070 min, with peak height of 15,306 μA and area of 675,608 μV s. The second peak in the chromatogram is of the degraded product 2-hydroxybenzimidazole (2-HB) at retention time 9.942 min, with peak height of 18,789 μA and area of 1,588,193 μV s.

Plausible mechanism for biodegradation of carbendazim by the isolate

Scanning Electron Micrographs of *Bacillus licheniformis* JTC-3, when grown in absence of Carbendazim, showed discrete individual cells (Fig. 4a) while those grown in presence of 10 mg/L of Carbendazim, fused with each other forming extensive chain like structures, under stress (Fig. 4b). Furthermore, in Fig. 4b, some crystalline structures are observed along with cell-chains, which could be of 2-hydroxybenzimidazole (2-HB), the metabolic end-product of Carbendazim biodegradation, as suggested by the HPLC chromatograms.

Table 4 Biodegradation rates of *B. licheniformis* JTC-3 by varying growth parameters.

Parameters	Biodegradation rates (mg/L of Carbendazim/h/mg biomass)
1. pH[a]	
pH 3	0.14
pH 5	0.30
pH 7	0.15
pH 9	0.12
2. Temperature[b]	
10 °C	0.12
25 °C	0.14
37 °C	0.30
45 °C	0.19
3. Agitation[c]	
40 rpm	0.11
60 rpm	0.12
80 rpm	0.30
100 rpm	0.12
4. Carbendazim[d]	
10 mg/L	0.30
30 mg/L	0.91
50 mg/L	1.01
70 mg/L	1.32
100 mg/L	2.15

[a] When Carbendazim concentration was 10 mg/L, at 37 °C, 100 rpm agitation.
[b] When Carbendazim concentration was 10 mg/L, at pH 7, 100 rpm agitation.
[c] When Carbendazim concentration was 10 mg/L, at 37 °C, pH 7.
[d] At 37 °C, 100 rpm agitation, pH 7.

Fourier Transform Infra-Red spectroscopy of lyophilized powder form of bacterial cells grown in presence of 10 mg/L Carbendazim and also in absence of any hydrocarbon substrate, was performed (Fig. 4c) to acquire information regarding wave number changes of the functional groups. Spectra were obtained from 400 to 4000 cm^{-1}. Peaks were observed in case of bacterial cells grown in presence of substrate at 1355.7, 1399.1 and 1529.2 cm^{-1}. 1355.7 and 1399.1 cm^{-1} corresponds to trans-CH bonds and 1529.2 cm^{-1} corresponds to C=C bonds. Due to the presence of Carbendazim as the sole hydrocarbon source, bacterial cells interacted and utilized this for its growth. In case of bacterial cells grown in absence of any hydrocarbon source, no such peaks in that region are observed.

In Fig. 4d, the X-ray diffraction report shows two major peaks at 21.09° and 33.46° with respective intensity counts of 1276.39 a.u. and 773.45 a.u. in case of bacterial cells grown in absence of Carbendazim (shown by blue color in the graph). Cells when grown in presence of Carbendazim (shown by red color in the graph) showed diminished peaks

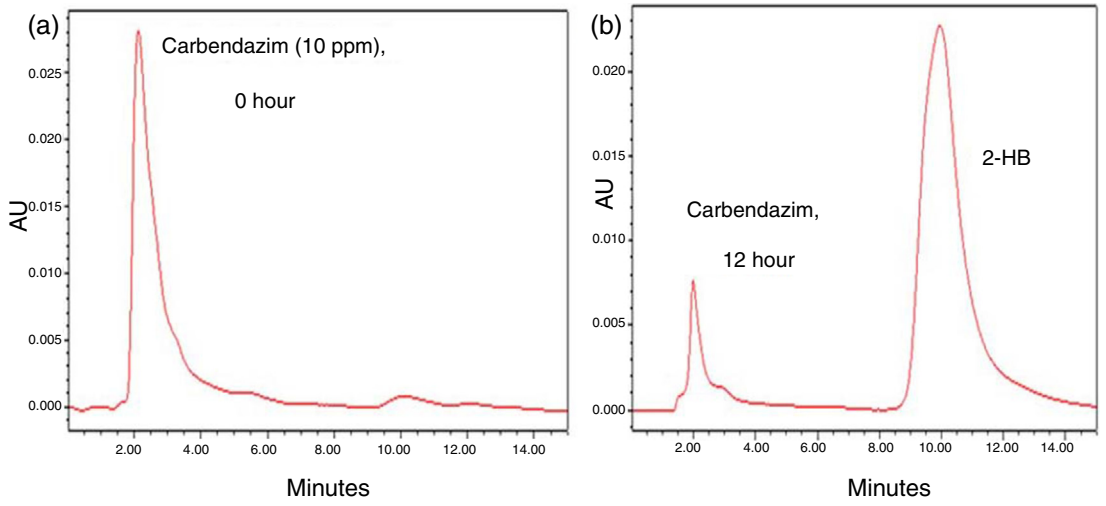

Figure 3 Biodegradation of 10 mg/L Carbendazim, by *Bacillus licheniformis* JTC-3, at (a) zero hour of incubation and (b) 12 hour of incubation in MSM media, at 37 °C.

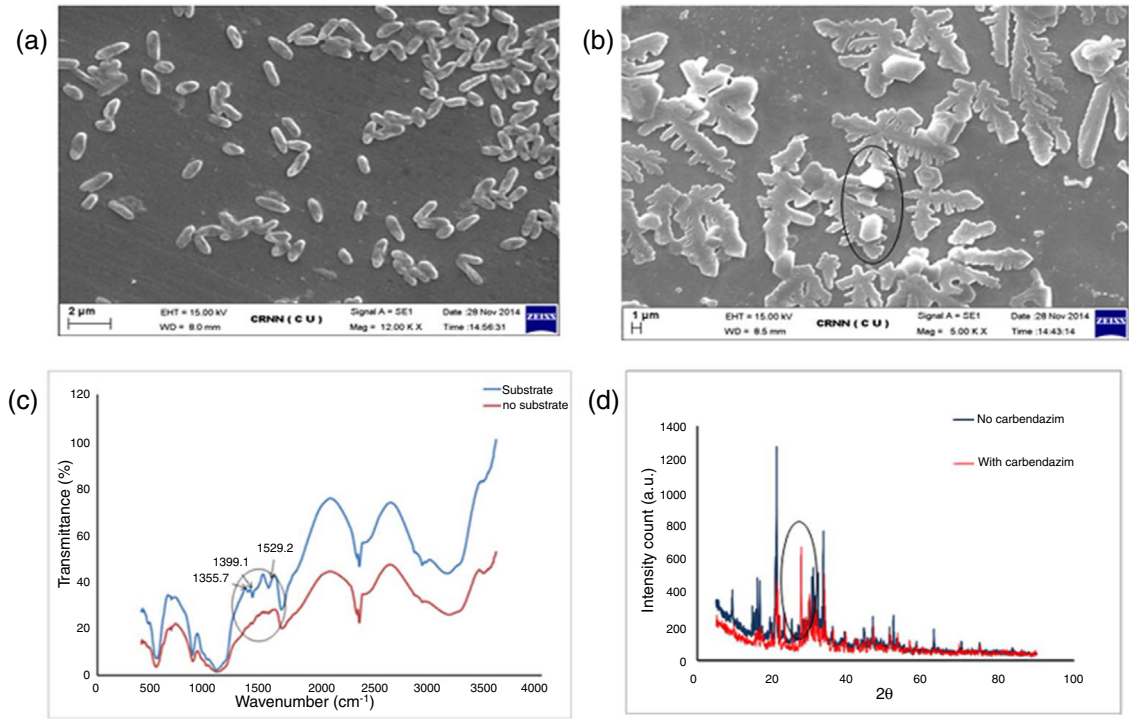

Figure 4 Bacteria-substrate interaction study by (a) Scanning Electron Micrographs of *Bacillus licheniformis* JTC-3 grown in absence of substrate, (b) SEM of the same grown under the stress of 10 mg/L Carbendazim, (c) Fourier Transform Infra-Red spectroscopic microanalysis and (d) X-ray diffraction graphs of *Bacillus licheniformis* JTC-3, grown in presence and absence of Carbendazim.

at 21.09° and 33.46° with respective intensity counts of 454.30 a.u. and 517.52 a.u. A new peak was observed in this case at 27.56° with intensity count of 678.34 a.u. It is evident that the peaks of dried mass of bacterial growth in the absence of Carbendazim (presumably due to the presence of some polysaccharides and fatty acids in the cell wall) have diminished in appearance in the XRD patterns of bacterial cells associated with precipitate of the crystalline end-product 2-hydroxibenzimidazole (Cemil, Selma, Halil, Taner, & Seda, 2003).

Plausible mechanism of biodegradation of Carbendazim could be illustrated (Fig. 5).

Carbendazim was degraded by *Bacillus licheniformis* JTC-3, first into 2-aminobenzimidazole with the exclusion of methyl formate, and finally into 2-hydrozybenzimidazole with the release of ammonia (Cemil et al., 2003).

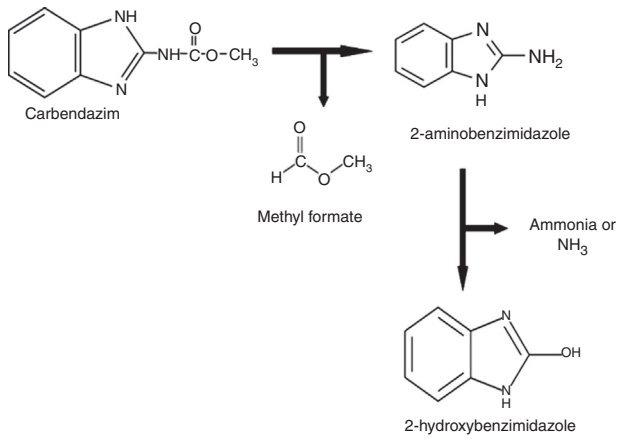

Figure 5 Plausible mechanism of bacterial biodegradation of Carbendazim to 2-hydroxybenzimidazole.

Biodegradation of carbendazim in real sample for kinetic studies

Biodegradation of real agro-effluent was carried out by *Bacillus licheniformis* JTC-3 and the degradation was checked by HPLC as in Fig. 6a (Agnieszka et al., 2010). HPLC chromatogram of *real agro-waste* having Carbendazim, at 12 h of incubation in at 37 °C, with 100 rpm agitation speed, showed the degraded peak of Carbendazim at retention time 2.107 min, with peak height of 45,532 μA and area of 1,893,622 μV s; second peak is of the degraded product 2-hydroxybenzimidazole (2-HB) at retention time 9.889 min, with peak height of 5247 μA and area of 383,508 μV s.

Rate equations with zero, first and second order kinetics were tested using the HPLC data. As we know, in case or zero order reaction the biodegradation rate is independent of any parameters involved in bacterial growth, as evident in Eq. (5):

$$[A]_t = -kt + [A]_0 \quad (5)$$

where $[A]_0$ is the initial substrate concentration and $[A]_t$ is the substrate concentration after time 't'. A straight line graph was plotted, here, with '$[A]_t$' vs 't' and the R^2 value was found to be 0.69 (Fig. 6b).

In case of first order reaction, the rate is dependent on only one critical parameter, as per Eq. (6):

$$\ln [A]_t = -kt + \ln [A]_0 \quad (6)$$

Here also a graph was plotted with '$\ln[A]_t$' vs 't' and the R^2 value was found to be 0.84 (Fig. 6c).

Actually, the rate of biodegradation of Carbendazim by *Bacillus licheniformis* JTC-3 was dependent on more than one parameters like substrate concentration, pH, temperature, agitation speed etc. Hence, it fitted best with the second order reaction rate (Fig. 6d), following Eq. (7):

$$1/[A]_t = 1/[A]_0 + kt \quad (7)$$

A graph was plotted here with '$1/[A]_t$' vs 't' and the R^2 value was found to be 0.90.

The growth kinetics of the bacterium in the real sample was also studied and it was found to fit in Gompertz model (depending on the R^2 value of the curve). The modeling was performed using Origin Pro 8.5.0 SR1 software (Origin Labs). The Gompertz function is based on an exponential relationship between specific growth rate and population density.

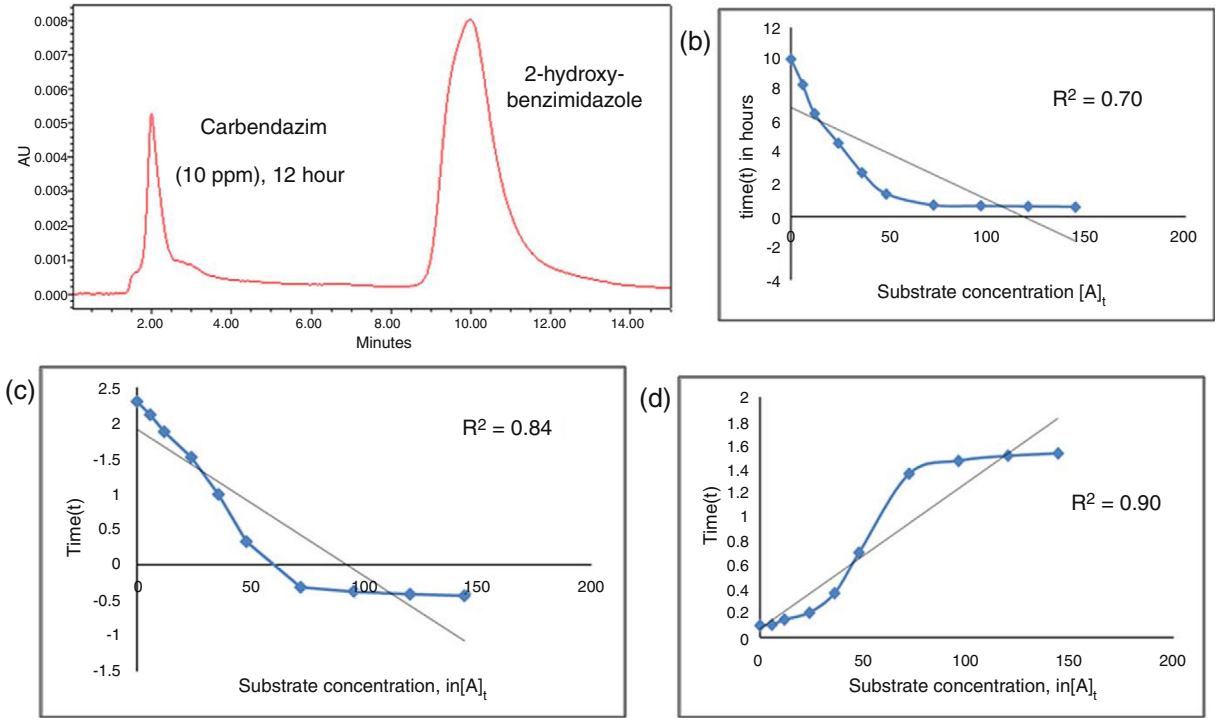

Figure 6 Kinetic study of biodegradation of Carbendazim in real sample in (a) HPLC chromatogram of *real agro-waste* having Carbendazim, (b) zero order degradation curve, (c) first order degradation curve and (d) second order degradation curve of Carbendazim.

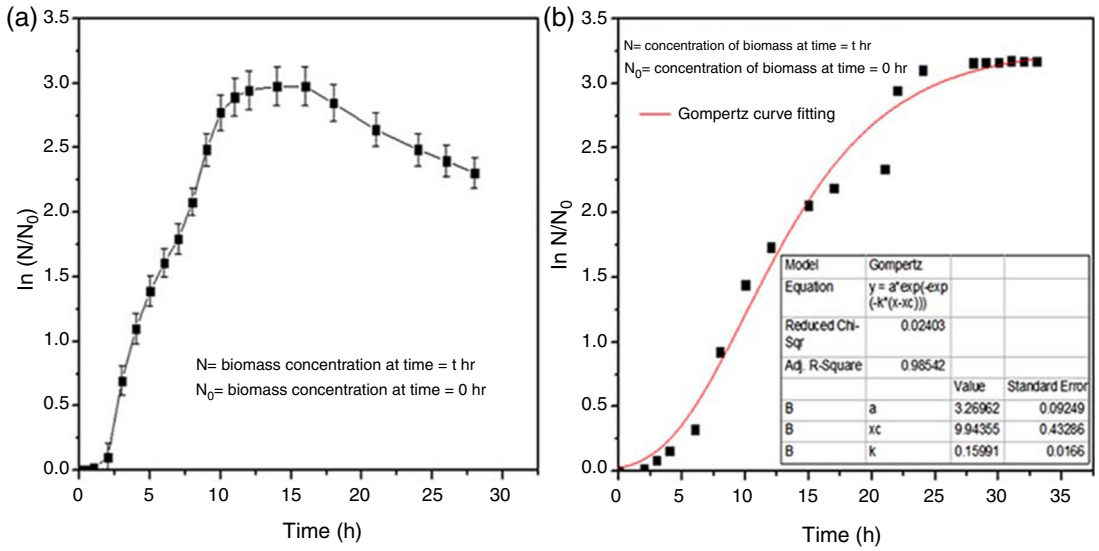

Figure 7 Studying growth kinetics *Bacillus licheniformis* JTC-3 in real sample by (a) plotting bacterial growth curve in real sample plotted with log value of initial ($\ln N_0$) and final ($\ln N$) biomass concentration against time and (b) fitting the growth curve in Gompertz kinetic model.

Table 5 Comparison of biodegradation rates of Carbendazim by various strains.

Serial no.	Name of strains	Biodegradation rates (in 24 h)
1.	*Ralstonia* sp. (Guin-Shan et al., 2005)	19.16% of initial Carbendazim concentration, without Yeast Extract and 95.96% of the same with Yeast Extract, in Minimal Salt Media
2.	*Pseudomonas* ap. CBW (Fang et al., 2010)	33.03% of initial Carbendazim concentration in Minimal Salt Media
3.	*Bacillus licheniformis* JTC-3 (our reported strain)	73.2% of initial Carbendazim concentration in Minimal Salt Media, with Carbendazim as the sole source of carbon

A curve was plotted (Fig. 7a and b) with log value of initial ($\ln N_0$) and final ($\ln N$) biomass concentration against time (h) and the R^2 value was found to be 0.98.

The biodegradation rate of Carbendazim by *Bacillus licheniformis* JTC-3 (our proposed strain) was compared (Table 5) with that of other reported strains and it was calculated that, within 24 h, *Bacillus licheniformis* JTC-3 could degrade 73.2% of Carbendazim in MSM (with Carbendazim as the sole source of Carbon), whereas *Ralstonia* sp. (Gui-Shan et al., 2005) and *Pseudomonas* sp. CBW (Fang et al., 2010) could degrade only 19.16% and 33.03% of Carbendazim in MSM, respectively.

Toxicity testing of the bio-degraded end-products

The zone of inhibition of bacterial growth around wells with 10–100 mg/L of Carbendazim, on LB agar of lawn culture of *Pseudomonas aeruginosa* (*MTCC 434*), varied from 13 to 22 mm in diameter (Fig. 8a), whereas the same in case of 2-hydroxybenzimidazole (metabolic end-product of Carbendazim) showed no such zone (Fig. 8b). Hence, the product was considered to be non-toxic.

Novelty of the research work: This highly efficient Carbendazim-degrading bacterium *Bacillus licheniformis* JTC-3 was isolated from liquid agro-effluent, unlike from contaminated soil sample as reported in previous literatures. This effluent was collected from agricultural areas adjacent to East Kolkata Wetlands, West Bengal, India, where the contaminated effluent is mixing with the adjacent water bodies which is again used in agriculture. This, in turn, is contaminating crops, which is consumed by local people who are facing the subsequent health hazards. Very few original research works have been reported regarding biodegradation of Carbendazim in India. Of these, in most of the biodegradation processes Carbendazim was incompletely converted to 2-aminobenzimidazole, which is again an acute oral toxicant and skin-irritant (Gui-Shan et al., 2005). In contrast, our isolate *Bacillus licheniformis* JTC-3 was capable of completely degrading Carbendazim into its non-toxic metabolic end product 2-hydroxybenzimidazole.

Conclusion

The difference in retention time, peak height and area in the HPLC reports, taken at zero and 12 h of bacterial incubation, showed remarkable degradation rate of 73.2% of Carbendazim, by *Bacillus licheniformis* JTC-3, into its metabolic end-product 2-hydroxybenzimidazole. SEM and XRD characterizations confirmed the formation of the crystalline 2-HB.

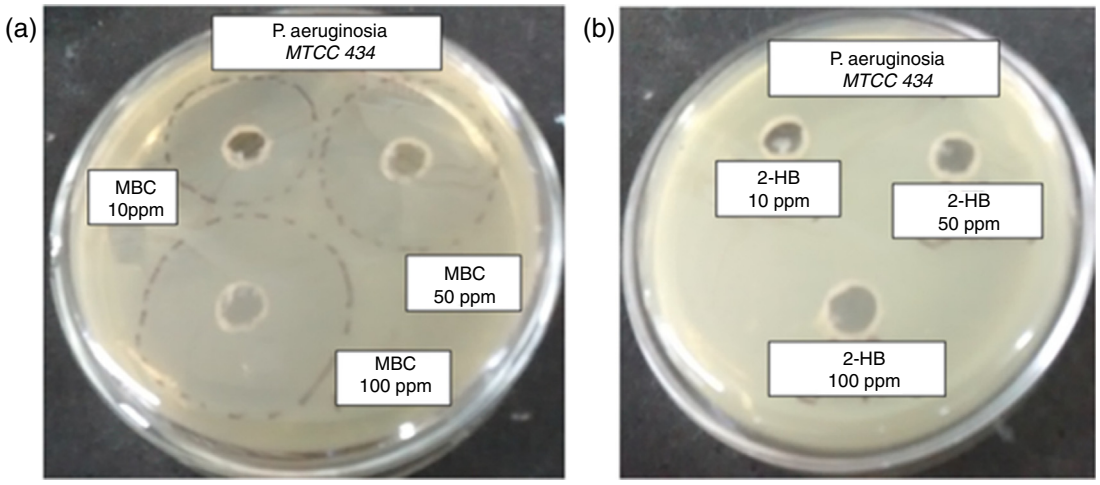

Figure 8 Toxicity testing of (a) Carbendazim or MBC (10–100 mg/L) and (b) 2-HB (10–100 mg/L), on *Pseudomonas aeruginosa* (MTCC 434).

Toxicity testing proved this 2-HB to be non-toxic. Process optimization by Central Composite Design (CCD) showed that all the parameters involved (temperature, agitation speed, initial MBC concentration and pH) were significant for biodegradation.

Though the followings have not been covered under the current domain of research, these could be considered by future researchers as subsequent steps in congruence with the present research. It has been advocated that, regarding pesticide wastewater treatment, biological processes can provide an environmentally sustainable and less expensive alternative to physicochemical methods. In many cases, however, the pure bacterial monoculture introduced to contaminated sites fails to degrade the pollutants due to their poor survival or low activity in the environment (Alvarado-Gutiérrez et al., 2017). Moreover, microbial bioremediation works slowly, taking weeks to months to achieve substantive remediation, and it is not suitable for cleaning up contaminated water because of the low aeration and low-nutrient state of the water. On the other hand, the introduction of a microbial consortium has a higher possibility of success in biodegradation than that of a single strain because such a consortium has a higher ability to adapt to these stresses. Moreover, a microbial consortium contains both the degraders of target compounds and strains that can utilize the metabolic intermediates of the target compounds. Toxic intermediates sometimes remain when using a single degrader strain (Arya, Kumar, Mishra, & Sharma, 2017). Also, there has been increasing interest in the concept of using formulated enzymes rather than live microbes as bioremediation agents, a process known as enzymatic bioremediation (Bai et al., 2017). This is particularly suitable for situations like water treatment, where rapid remediation is needed and/or microbial growth and survival are not desirable. Accordingly, the bacterial gene product responsible for such biodegradation is cloned and expressed in suitable vector for rapid remediation; for example, the cognate gene *mhel* for Carbendazim-hydrolyzing esterase, isolated from *Nocardioides* sp. strain SG-4G and heterologously expressed in *E. coli*, has been reported for rapid bioremediation (Pandey et al., 2010). Furthermore, the efficiency of the developed protocols could be evaluated in membrane/column/stirred-tank bioreactors with biologically enhanced pure bacterial monocultures or with non-enhanced bacterial consortium for rapid biodegradation of Carbendazim from both synthetic solution and actual environmentally contaminated agricultural wastes (Singh et al., 2016).

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors are thankful to Bio-Process Engineering Laboratory (Jadavpur University) for providing all necessary infra-structural support for conducting this work. The authors wish to convey their sincere thanks to the University Grants Commission (UGC) UPE Phase II, GoI, for their financial support to carry out the present work.

References

Agnieszka, K., Jacek, K., & Wojeciech, D. (2010). Biodegradation of Carbendazim by epiphytic and neustonic bacteria of eutrophic Chełmżyńskie Lake. *Polish Journal of Microbiology*, *57*, 221–230.

Alexander, M. (1981). Biodegradation of chemicals of environmental concern. *Science*, *9*, 132–138.

Alvarado-Gutiérrez, M., Ruiz-Ordaz, N., Galíndez-Mayer, J., Santoyo-Tepole, F., Curiel-Quesada, E., García-Mena, J., et al. (2017). Kinetics of carbendazim degradation in a horizontal tubular biofilm reactor. *Bioprocess and Biosystems Engineering*, *40*, 519–528.

American Public Health Association. (1989). *Standard methods for the examination of water and wastewater* (18th ed.). Washington, DC, USA

Arya, R., Kumar, R., Mishra, N., & Sharma, A. (2017). Microbial flora and biodegradation of pesticides: Trends, scope, and relevance. *Advances in Environmental Biotechnology*, http://dx.doi.org/10.1007/978-981-10-4041-2_15

Bai, N., Wang, S., Abuduaini, R., Zhang, M., Zhu, X., & Zhao, Y. (2017). Rhamnolipid-aided biodegradation of carbendazim by

Rhodococcus sp. D-1: Characteristics, products, and phytotoxicity. *Science of the Total Environment*, *590*, 343–351.

Cemil, O., Selma, Y., Halil, B., Taner, A., & Seda, T. (2003). A theoretical study of substituent effects on tautomerism of 2-hydroxybenzimidazoles. *Journal of Molecular Modeling*, *9*, 390–394.

Dam, W. (1974). Photolysis of methyl benzimidazol-2-yl carbamate. *Chemosphere*, *5*, 239–240.

Das, R., Sarkar, S., Chakraborty, S., Choi, H., & Bhattacharjee, C. (2014). Remediation of antiseptic components in wastewater by photocatalysis using TiO_2 nanoparticles. *Industrial and Engineering Chemistry Research*, *53*, 3012–3020.

Fang, H., Wang, Y., Gao, C., Yan, H., Dong, B., & Yu, Y. (2010). Isolation and characterization of *Pseudomonas* sp. CBW capable of degrading carbendazim. *Biodegradation*, *21*, 939–946.

Food and Agriculture Organization USA. (2012). *Pesticide residues in food: REPORT*. Joint Food and Agriculture Organization/World Health Organization Meeting on Pesticide Residues. http://www.fao.org/agriculture/crops/thematic-sitemap/theme/pests/jmpr/en/ (accessed 15.11.16)

Gui-Shan, Z., Xiao-Ming, J., Tian-Fan, C., Xiao-Hang, M., & Yu-Hua, Z. (2005). Isolation and characterization of a new carbendazim-degrading *Ralstonia* sp. *World Journal of Microbiology and Biotechnology*, *21*, 265–269.

Haynes, W. M. (2015). *CRC handbook of chemistry and physics* (95th ed.). FL, USA: CRC Press.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., & Williams, S. T. (1994). *Bergey's manual of determinative bacteriology* (2nd ed.). Baltimore: Williams and Wilkins.

Holtman, M. A., & Kobayashi, D. Y. (1997). Identification of *Rhodococcus erythropolis* isolates capable of degrading the fungicide carbendazim. *Applied Microbiology and Biotechnology*, *47*, 578–582.

MacBean, C. (2010). *British Crop Protection Council Report on Carbendazim* (15th ed.). UK: Alton.

Mazellier, P. E., Leroy, J., Laat, D., & Legube, B. (2003). Degradation of carbendazim by UV/H_2O_2 investigated by kinetic modeling. *Environmental Chemistry Letters*, *1*, 68–72.

Montgomery, J. H. (1997). *Agrochemicals desk reference* (2nd ed.). Florida: CRC Press Inc.

National Pesticide Information Retrieval System's Database on Carbendazim (2016). http://npirspublic.ceris.purdue.edu/ppis/ (accessed 11.08.17).

O'Neil, M. J. (2013). *An encyclopedia of chemicals, drugs, and biologicals*. Cambridge, UK: Royal Society of Chemistry.

Panda, J., & Sarkar, P. (2012a). Bioremediation of chromium by novel strains *Enterobacter aerogenes* T2 and *Acinetobacter* sp. PD12 S2. *Environmental Science and Pollution Research*, *19*, 1809–1817.

Panda, J., & Sarkar, P. (2012b). Isolation, identification, characterization and field application of Cr(VI) resistant bacteria. *Journal of Environmental Science and Health Part A*, *47*, 237–244.

Panda, J., & Sarkar, P. (2014). Biosensing and bioremediation of Cr(VI) by cell free extract of *Enterobacter aerogenes* T2. *Journal of Environmental Science and Health Part A*, *49*, 600–608.

Panda, J., & Sarkar, P. (2015). Biosorption of Cr(VI) by calcium alginate-encapsulated *Enterobacter aerogenes* T2, in a semi-batch plug-flow process. *Water Air Soil Pollution*, *226*, 2157–2166.

Pandey, G., Dorrian, S. J., Russell, R. J., Brearley, C., Kotsonis, S., & Oakeshott, J. G. (2010). Cloning and biochemical characterization of a novel carbendazim (methyl-1H-benzimidazol-2-ylcarbamate)-hydrolyzing esterase from the newly isolated *Nocardioides* sp. strain SG-4G and its potential for use in enzymatic bioremediation. *Applied and Environmental Microbiology*, *76*, 2940–2945.

Paszko, T. (2006). Sorptive behavior and kinetics of carbendazim in mineral soils. *Polish Journal of Environmental Studies*, *15*, 449–456.

Pattanasupong, A., Nagase, H., Sugimoto, E., Hori, Y., Hirata, K., Tani, K., et al. (2004). Degradation of carbendazim and 2,4-dichlorophenoxyacetic acid by immobilized consortium on loofa sponge. *Journal of Bioscience and Bioengineering*, *8*, 28–33.

Sharma, A., & Arya, R. (2014). Screening, isolation and characterization of *Brevibacillus borstelensis* for the bioremediation of carbendazim. *Journal of Environmental Science and Sustainability*, *2*, 12–14.

Singh, S., Singh, N., Kumar, V., Datta, S., Wani, A., Singh, D., et al. (2016). Toxicity, monitoring and biodegradation of the fungicide carbendazim. *Environmental Chemistry Letters*, *14*, 317–329.

Singhal, L. K., Bagga, S., Kumar, R., & Chauhan, R. S. (2003). Down regulation of humoral immunity in chickens due to carbendazim. *Toxicology in Vitro*, *17*, 687–692.

World Health Organization. (1993). *International programme on chemical safety: Geneva. Environmental Health Criteria, Carbendazim.*. http://www.who.int/ipcs/publications/ehc/ehc_numerical/en/ (accessed 15.05.17)

Xiao, W., Wang, H., Li, H., Zhu, Z., Zhang, J., He, Z., et al. (2013). Bioremediation of Cd and carbendazim co-contaminated soil by Cd-hyperaccumulator *Sedum alfredii* associated with carbendazim-degrading bacterial strains. *Environmental Science and Pollution Research International*, *20*, 380–389.

Xinjian, Z., Yujie, H., Harvey, P. R., Hongmei, L., Yan, R., Jishun, L., et al. (2013). Isolation and characterization of Carbendazim-degrading *Rhodococcus erythropolis* djl-11. *PLOS ONE*, *8*(10), e74810. http://dx.doi.org/10.1371/journal.pone.0074810

Xu, J. L., He, J., Wang, Z. C., Wang, K., Li, W. J., Tang, S. K., et al. (2007). *Rhodococcus qingshengii*, a carbendazim-degrading bacterium. *International Journal of Systematic and Evolutionary Microbiology*, *57*, 2754–2757.

Yu, Y., Chu, X., Pang, G., Xiang, Y., & Fang, H. (2009). Effects of repeated applications of fungicide carbendazim on its persistence and microbial community in soil. *Journal of Environmental Science (China)*, *21*, 179–185.