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Biodegradation of hexazinone by two isolated bacterial strains (WFX-1 and WFX-2)

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Abstract

Two hexazinone-degrading bacterial strains were isolated from soil by enrichment culture technique, and identified as *Pseudomonas* sp. and *Enterobacter cloacap*, respectively. The two purified isolates, designated as WFX-1 and WFX-2, could rapidly degrade hexazinone with half-lives of 3.08 and 2.95 days in mineral salts medium (hereafter referred to as MSM). In contrast, their mixed bacterial culture (herein abbreviated as MBC) was found to degrade hexazinone, at an initial concentration of 50 mg l^{-1}, by enhancing 2.3-fold over that when the isolates were used alone. The degradation of hexazinone by MBC in MSM clearly decreased concomitant with the increase of initial concentration, and the level of hexazinone that was toxic enough to totally inhibit degradation was in the range of 150–200 mg l^{-1}. The appropriately combined conditions for hexazinone degradation by MBC in MSM were studied, and found to be pH 5.5, 30 °C and at agitation of 120 rpm. The addition of MBC to soil had a greater impact on disappearance of hexazinone, which nearly increased fivefold over that of the control set. As a result, findings in the present investigation provide useful information for soil and water decontamination of hexazinone.

Introduction

The proliferation of soil microorganisms that use pesticides as C or N sources, or both, for growth forms the foundation of bioremediation technique (Racke & Coats 1990). At present biological decontamination of pesticidal wastes or spills has become an increasingly important area of research and it is preferable if a microbial or biological method of degradation is available for the various pesticidal compounds (Synal & Kulshrestha 2002). Hexazinone, whose chemical structure is shown in Figure 1, is a broad-spectrum herbicide used to control a wide variety of broad leaf weeds, grasses and woody plants. It is used for weed control in nurseries in forestry, in sugarcane and pineapple plantations, in highway or railway grasses and in industrial plant sites (James et al. 1997). As a contact and residual herbicide, hexazinone may be absorbed through either the roots or the leaves and acts as an inhibitor of photosynthesis. It is produced by Jiangsu Xinyi pesticide factory in China and marketed commercially as 5% Sentai GR (Granule) or 25% Sentai SL (Soluble concentrate).

Hexazinone is highly water soluble (33 g l^{-1}) and mobile in soil, which confers great potential for leaching, and is considered to be among the most likely pesticides to contaminate ground water (McRae 1991). The hydrolysis of hexazinone was reported by Zhonglin et al. (1998) who observed that hexazinone is stable in buffered solution at pH levels of 5–9.

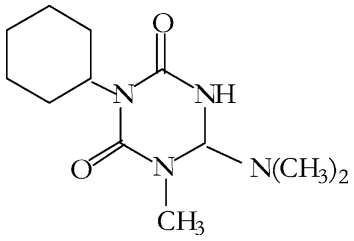

Figure 1. The chemical structure of hexazinone.

Hexazinone degradation in soil was mainly attributable to the indigenous microorganisms in spite of its slow rate and it was thought to be not susceptible to photolysis (Neary et al. 1983); thus, residual activity might be expected to last several months. Previous work on hexazinone dealt primarily with residual analyses of parent compound and its metabolites (Fischer & Michael 1995; Kubilius & Bushway 1998;); dissipation (Calderson et al. 2004); adsorption–desorption (Bouchard & Lavy 1985); leaching potential; mobility (Bottni et al. 1996; Zhu & Li 2002), and persistence (Jensen & Kimball 1987) in soils. Peterson et al. (1997) reported the toxicity of hexazinone to green algae, diatoms, cyanobacteria, and duckweed and found that all of them were sensitive to hexazinone. Metabolites of hexazinone were first identified by Reiser et al. (1983) who separated five degradates in plant seedlings. Chakravarty & Chatarpaul (1990) investigated the effect of hexazinone on microbial activities. However, little data are available on biodegradation of hexazinone. The objectives of this study aimed to (1) compare the degradative activities between two pure isolates and their mixed culture in MSM; (2) analyze the appropriately combined conditions for hexazinone degradation by MBC; and (3) evaluate the role of the mixed hexazinone-degrading bacterial strains in decontamination of hexazinone in soil.

Materials and methods

Materials

Hexazinone (purity 99.7%) was purchased from Shenyang chemical engineering institute, China. Its stock solutions (500 mg l^{-1}) in methanol (HPLC grade) were prepared freshly every two weeks and stored in dark bottles at −20 °C until use. Analytical grade reagents and solvents locally procured were purified and distilled before use.

Loamy sand soil with pH 6.45, organic matter content (OMC) 22.31 mg kg^{-1}, cation exchange capacity (CEC) 15.26 cmol(+)/kg was collected at 5–10 cm depth from both sides of a railway line located in a suburb of Hangzhou city, Southeastern China. From 2000 to 2002, both sides of the railway line had been frequently applied with hexazinone (5% GR) to control weeds. Immediately following collection, fresh field soil samples were taken to the laboratory where large pieces of plant material, stones, and visible soil fauna were removed. Subsamples of the soil were air-dried, ground and passed through a 2-mm-diameter sieve.

Isolation and identification of hexazinone-degrading bacteria

Two hexazinone-degrading bacterial strains were isolated by enrichment culture technique (Xuedong et al. 2003) from the treated soil as described above. Soil enrichment was conducted by eight subsequent additions of 50 mg l^{-1} of hexazinone at seven day interval for each time. Soil suspensions were prepared up to 10^{-7}–dilution by sequential dilution with a sterilized 0.85% NaCl solution. An aliquot (1 ml) of the 10^{-7}-dilution was transferred to the sterilized nutrient agar medium (NAM), containing 3.0 g of beef extract, 5.0 g of peptone, 5.0 g of sodium chloride and 15.0 g of agar dissolved in 1 l of distilled water (pH 7.0–7.2) and incubated at 28 ± 1 °C for 48 h. After 48 h of incubation, a single colony was selected according to the different morphological and cultural characteristics, then purified by spread-plate technique and tested for the ability to use hexazinone (50 mg l^{-1}) as the sole source of carbon in mineral salts medium (MSM). MSM consisted of 1.0 g of sodium nitrate, 1.0 g of dipotassium hydrogen phosphate, 3.0 g of potassium dihydrogen phosphate and 0.5 g of magnesium sulfate in 1 l of distilled water (pH 7.0–7.2). After 10 days of incubation with shaking at 28 ± 1 °C, samples were analyzed by HPLC to determine the degradation rate of hexazinone. The abiotic degradation of hexazinone was checked in sterile MSM without inoculation. When the degradation of hexazinone was at least 20% greater than that in the media without inoculation, the

strain was regarded as a hexazinone-degrader (Eizuka et al. 2003; Gennari et al. 1995). Each isolated hexazinone-degrading bacterial strain was purified by spread-plate technique using NAM (Cappuccino & Sherman 1992). The results showed that two bacterial strains were capable of degrading hexazinone quickly. Their degradation rates after 10 days of incubation were 86.7 and 89.5%, respectively, and were significant at $P < 0.01$ compared with control. Based on morphological, cultural and biochemical characteristics (Gram stain, sporulative, motile, fluorescence, growth on McConkey agar, aerobe, acid produced from glucose, citrate utilization, gelatin liquefaction, starch hydrolysis, urea hydrolysis, casein hydrolysis, catalase, Oxidase, H_2S production, nitrate reduction and so on), two isolates, designated as WFX-1 and WFX-2, were identified as *Pseudomonas* sp. and *Enterobacter cloacap*, respectively (Xuedong et al. 2003).

Hexazinone degradation by two isolates and their MBC in MSM and bacterial growth

The degradation kinetics of hexazinone by the pure isolates was carried out using WFX-1 or WFX-2 and their MBC. Time-course hexazinone degradation and bacterial cell growth were examined concurrently in the same culture flasks. Cell cultures of WFX-1 or WFX-2 were grown in 100-ml sterilized enrichment medium containing beef extract 3 g, peptone 5 g and NaCl 5 g in 1 l distilled water at pH 7.0–7.2, incubated for 48 h and collected by centrifugation (4000 rpm). Supernatants were discarded and the cells were suspended in MSM. Bacterial cell densities were measured at 486 nm (A_{486nm}) and adjusted to A_{486nm} 0.4. The cell densities, which is at A_{486nm} 0.4, equivalent to 5.69×10^5 colony forming units (CFU)/ml for *Pseudomonas* sp.WFX-1, and 6.38×10^5 CFU/ml for *Enterobacter cloacap* WFX-2, respectively, were maintained at the beginning of the experiment unless otherwise mentioned as inoculum in degradation studies.

An aliquot (50 ml) of the bacterial suspension was added aseptically to the flask amended with 50 mg l^{-1} hexazinone. The flasks in four replications were swirled for even distribution of the organisms in the medium and incubated in the dark at 28 ± 1 °C under aerobic conditions. For each experiment, four control flasks without inoculation were run in parallel to test possible chemical degradation. For hexazinone degradation by MBC, WFX-1 and WFX-2 were mixed in equal volume proportions of their original suspensions at A_{486nm} 0.4, which was regarded as their MBC. The degradation capacity of MBC was tested under the same conditions as that for the single ones. The appropriately combined conditions for hexazinone degradation in MSM by MBC were carried out to compare the degradation rate under the respectively intersecting conditions: pH 3.5, 5.5, 7.0, and 8.0; temperature 20, 30, 35, and 45 °C; incubation agitation speed at 80, 120, 160, and 200 rpm, respectively.

At seven intervals (0, 1, 2, 3, 5, 7, and 10 days) from initial incubation, aliquots of MSM for degradation study were taken for analyses of hexazinone residues and bacterial growth assay. For analyses of hexazinone residues, the samples were acidified to 5 with 0.1 mol l^{-1} HCl, and subjected to HPLC analysis as described in a following section. For bacterial growth assay, an aliquot (100 μl) of the culture was plated onto NAM agar plates and the colonies that appeared in two days of incubation were counted as CFU/ml. In the present investigation, we laid emphasis on MBC capacity for degrading hexazinone. Therefore, the bacterial cell numbers of MBC composed of WFX-1 and WFX-2 were monitored, while monitoring of the single ones was not conducted.

Hexazinone degradation in soil by MBC

The tested soil with pH 5.58, OMC 17.9 mg kg^{-1}, CEC 16.45 cmol(+)/kg, was collected at 5–10 cm depth from the experimental farm of Huazhong agricultural university located in a suburb of Wuhan city, Central China and treated as described earlier. As far as our information goes, the soil had not been received any application of hexazinone formulation. A rate kinetics study by MBC was carried out in non-sterilized soil fortified at 20 mg kg^{-1} hexazinone concentration. An aliquot (2 ml) of MBC was added to 10 g of the pretreated soil for each treatment. The soil was mixed well with occasional stirring for even distribution of the cells, and allowed to air-dry for 2 h. The calculated amount of sterile distilled water was then added to the soil to maintain 60% of maximum water-holding capacity. The flasks were weighed, incubated at

28 ± 1 °C for 40 days in the dark and the weight loss due to evaporation of soil moisture was maintained by periodical addition of water at 5-day intervals during the whole incubation period. MSM (2 ml) without inoculation of MBC was added to the soil (10 g), which served as control set. Each set in four replicates was taken at specific time intervals of 0 (2 h after application), 1, 5, 10, 20, 30, and 40 days after treatment, and processed as described below for analysis.

Extraction and clean-up of samples in soil and MSM

Each soil sample was spiked with 50 ml extraction solution (methanol : water, 70 : 30 by volume), and then acidified to pH 5 with 0.1 mol l^{-1} HCl. The solution was shaken vigorously for 1 h on a mechanical shaker and filtered through a Buchner funnel using Whatman No 1 filter paper under vacuum with repeated washing using methanol. The methanol was evaporated from the filtrate using a vacuum rotary evaporator. The remaining aqueous portion was then extracted three times with dichloromethane (15 + 10 + 10 ml). The organic layer was dehydrated over anhydrous sodium sulfate and its volume reduced to 1∼2 ml with a vacuum rotary evaporator. The concentrated dichloromethane extracts were transferred to a glass column (1.0 cm i.d., 20 cm length) packed with a mixture of Florisil (80–120 mesh) and acidic aluminum oxide (1 : 1 by weight), and followed by a rinse with a mixing solvent (25 ml) of methanol and ethyl acetate (20 : 80 by volume). The eluate was evaporated to dryness on a rotary evaporator, and the residue was redissolved in methanol (5 ml) for estimation by HPLC.

An aliquot (20 ml) of the MSM sample was partitioned three times with 10 ml of dichloromethane for each time. The extracts were pooled, dehydrated over anhydrous sodium sulfate, concentrated in a rotary evaporator (water-bath at 40 °C) to dryness and brought to final volume with methanol (5 ml). The final samples were subjected to HPLC analyses as described in a following section.

Recovery study

To estimate the recovery of hexazinone residue, a recovery study was carried out by spiking the soils with hexazinone standard solution in methanol to obtain a series of concentrations of 0.05, 0.1, 1, 10 mg kg^{-1} in soil. The results showed the average recoveries were in the range of 82.3–97.4% and the relative standard deviations ranged from 1.66 to 3.71% (data not shown). For hexazinone residues in MSM, the average recoveries at a series of the spiked concentrations (0.1, 1, 5, and 10 mg l^{-1}) varied between 86.0 and 96.7% and the coefficient of variance varied between 2.05% and 5.13% (data not shown). Accordingly, the methods adopted for analysis of hexazinone residue in soil and MSM were satisfactory.

HPLC analysis

HPLC analysis was performed using an Agilent 1100 model instrument with diodearray detector under the following conditions: cartridge column, Nova-Pak C_{18} (150×4.6 mm i.d., 5 μm particle size); mobile phase, methanol-water (70:30 by volume); flow rate, 1 ml min^{-1}; detection wavelength, 247 nm and injection volume, 20 μl. An external standard method was used for calibration.

Results and discussion

Degradation of hexazinone by WFX-1 and WFX-2 pure isolates in MSM

The hydrolytic rate of hexazinone in MSM at termination of the incubation period (10 days) was found to be only 2.84%, which showed that hexazinone was degraded very slowly in aqueous solution. Zhonglin et al. (1998) reached the comparable conclusion that hexazinone was stable in buffered solution at pH 7. The preceding results demonstrated that the contribution of hydrolysis to hexazinone disappearance in aqueous solution was minute.

However, when inoculated by WFX-1 in MSM, the degradation rate was 3.5% for the first day, followed by 25.9% and 39.6% in the second and third days (Figure 2). There was an increase in the rate of degradation as much as 28.6% from 5 days to 10 days of incubation. After 10 days of incubation, 86.7% of hexazinone was degraded with a half-life of 3.08 days based on the first-order kinetics equation. Similarly, only 10.5% of the

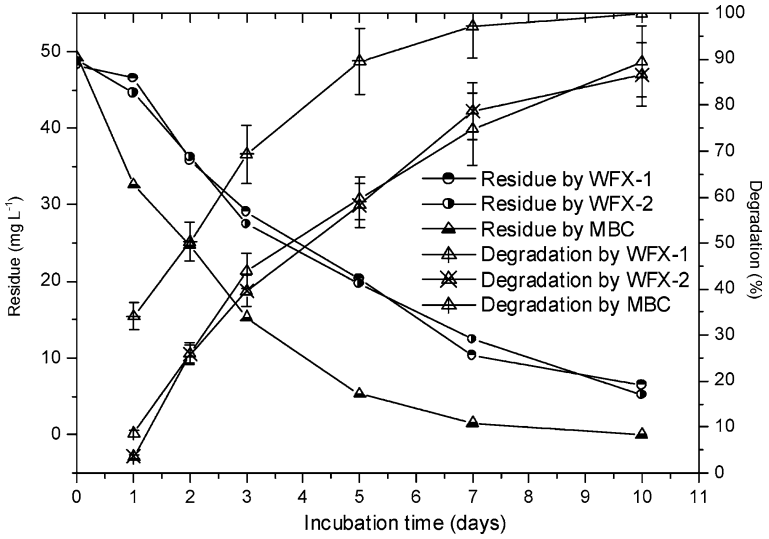

Figure 2. Degradation of hexazinone by WFX-1, WFX-2 pure isolates and their mixed cultures.

fortified hexazinone in MSM was recovered from WFX-2 at the end of the experimental period (10 days), and the half-life was found to be 2.95 days which was comparable to that of WFX-1.

Degradation of hexazinone by MBC in MSM and bacterial growth

Considering in natural aqueous system a bacterial community is present and not only a single strain, study on degradation behavior of the two isolated strains in their mixed culture is important and interesting. Utilization of hexazinone by MBC was examined on the basis of the bacterial growth, as determined by CFU/ml counting. The result in Figure 3 demonstrated that the growth of MBC including WFX-1 and WFX-2 was accompanied by the disappearance of hexazinone, giving almost complete degradation of the added hexazinone after 8 days of incubation. The bacterial cell numbers of MBC reached a maximum in 48 h of the degradation study and afterward decreased slightly, which was in general agreement with Kim et al. report (2004), who found the maximum

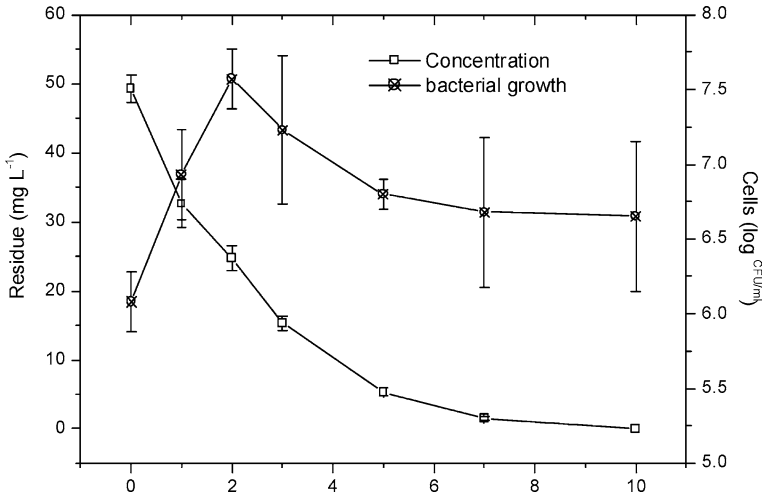

Figure 3. Degradation of hexazinone by MBC and bacterial growth based on CFU counting. The data given are the means of four measurements.

growth of *Sphingomonas* sp. Strain SB5 occurred in 24 h of incubation and then showed a declined trend in time-course degradation of carbofuran by SB5. The recovered hexazinone by MBC in MSM with an initial concentration at 50 mg l^{-1} at different intervals of time is shown in Figures 2 and 3. The highest degradation rate was monitored in the first day and followed by an increase of 16.0%, 19.1%, 20.3% at an interval from 1 to 2, 2 to 3, and 3 to 5 days of incubation, respectively. At the end of the incubation, no residue of the added hexazinone was detected in MSM. The half-life was calculated to be 1.30 days according to the first-order kinetics equation, which proved that MBC exhibited a higher activity to hexazinone degradability than that of individual strains. The synergistic effect of the two isolates in their mixed culture on degradation was present, and Synal & Kulshrestha (2002) also observed the same phenomenon in the metabolism of metolachlor by fungal cultures.

Appropriately combined degradation conditions by MBC in MSM

Study on the appropriate conditions for hexazinone degradation by MBC was carried out considering the variables pH, temperatures, and agitation speeds. In fixing agitation speed at 120 rpm and constant temperature at 30 °C, as can be seen from Table 1, the degraded hexazinone was dependent on variable pH. The highest degradation (63.4%) was found at pH 3.5, while the lowest (21.8%) was observed at pH 8.0, possessing the highest pH value in this study. It was evident from the preceding results that the lower pH was concomitant with higher degradation rate of hexazinone. However, although at pH 3.5 the degradation rate was slightly increased, no considerable difference was observed between hexazinone degradation at pH 3.5 and pH 5.5. As a result, the appropriate pH value was considered to be 5.5, which was comparable with the result obtained by Reventos et al. (2004) who investigated on degradation of thiobencarb by the newly isolated *Aspergillus niger*.

Hexazinone degradation was monitored in MSM at different temperatures (20, 30, 35, and 45 °C) under conditions of fixed agitation at 120 rpm and at pH 7.0. The higher (45 °C) and lower (20 °C) temperature in this investigation could result in the lower degradation rate (21.4% and 27.8 %), whereas the highest degradation (50.2%) was observed at 30 °C. Moreover, the significant difference at $P < 0.05$ between the degradation rate at 30 °C and 35 °C was also observed, which revealed that the appropriate temperature for hexazinone degradation by MBC was 30 °C under conditions of fixed agitation at 120 rpm and at pH 7.0.

In fixing temperature at 30 °C and at pH 7.0, the degradation at different agitation conditions (80, 120, 160, and 200 rpm) was monitored after 48 h of incubation. As can be seen from Table 1, the different agitation speeds could lead to different degradation rates. The highest degradation was observed in the highest agitation treatment of 200 rpm, and the lowest degradation was found in that of the lowest treatment (80 rpm). Nevertheless, no considerable difference was found among the four different agitation treatments, which demonstrated that the effect of incubation agitation speed on degradation was not as much as in the case of pH values. As a whole, the appropriately combined conditions for degradation of hexazinone were as follows: temperature 30 °C, pH 5.5, and 120 rpm of agitation.

Table 1. Effect of pH values, temperatures and agitation speeds on hexazinone degradation

pH (30 °C, 120 rpm)	Degradation (%) (±SD)	Temperature (pH 7.0, 120 rpm)	Degradation (%) (±SD)	Agitation (rpm) (30 °C, pH 7.0)	Degradation (%) (±SD)
3.5	63.4 ± 5.2%a	30 °C	50.2 ± 4.2%a	200	52.5 ± 3.2%a
5.5	61.2 ± 4.7%a	35 °C	42.3 ± 3.3%b	120	50.2 ± 4.2%a
7.0	50.2 ± 4.2%b	20 °C	27.8 ± 2.3%c	160	48.8 ± 5.3%a
8.0	21.8 ± 1.9%c	45 °C	21.4 ± 1.7%c	80	47.5 ± 4.6%a

Degradations (%) are the average of four replications determined at 48 h from incubation.
Different lower case letters represent treatments that are significantly different at $p < 0.05$.

Effect of the initial concentration on hexazinone degradation

The toxic concentrations of hexazinone employing the step-up levels of 50, 100, 150, 200 μg ml^{-1} was carried out by inoculating MBC in MSM and incubating under the following conditions: pH 7.0, temperature 30 °C, and 120 rpm of agitation. Figure 4 demonstrated that 50 mg l^{-1} was completely degraded (below detectable limit, <0.001 mg l^{-1}) within 10 days of incubation, whereas 100 mg l^{-1} degraded after 20 days of incubation. When fortified at 150 mg l^{-1}, the initiation of hexazinone degradation was delayed until 24 h and only 2.7 mg l^{-1} was recovered from MSM at the end of the experimental period of 20 days. The residues at different intervals of time were fitted to the first-order model and the half-lives were estimated to be 1.30, 2.31, and 3.21 days, respectively, at 50, 100 and 150 mg l^{-1} concentration of hexazinone which revealed that the degradation clearly decreased with the increase of initial concentration. However, with regard to the 200 mg l^{-1} treatment, the degradation curve did not accord with first-order kinetics equation because no occurrence of degradation was observed (Figure 4). As a result, the toxic level of hexazinone that was toxic enough to totally inhibit degradation was approximately in the range of 150–200 mg l^{-1}.

Effect of incorporation of MBC into soil on hexazinone degradation

The difference of rate kinetics was investigated between treatments by incorporation of MBC into soil, as described above, and control sets. The amount of hexazinone recovered from soil with the herbicide (20 mg kg^{-1}) at different time intervals is presented in Figure 5. For the control set, the recovered hexazinone from soil after 40 days was close to 50%, and the calculated half-life was 43.9 days on the basis of the first-order kinetics equation ($R^2=0.9691$). The half-life obtained in this study was in general agreement with the result acquired by Zhonglin et al. (1998), who found that the half-life of hexazinone was 40.3 days in black soil collected in Helongjiang province, northeastern China. However, the reported field dissipation half-life was 79 days for hexazinone (USDA-ARS, 2002), which showed that a considerable difference occurred for hexazinone degradation in soils with different physicochemical properties (Zhu & Li 2002).

However, when MBC was incorporated into soil, 14.6% of the added hexazinone was metabolized in the first day, which was followed by 46.9% and 60.4% in 5 and 10 days. After 40 days of incubation, only 0.8 mg kg^{-1} of the spiked hexazinone was recovered from soil, that is to say, more than 95% degradation of hexazinone

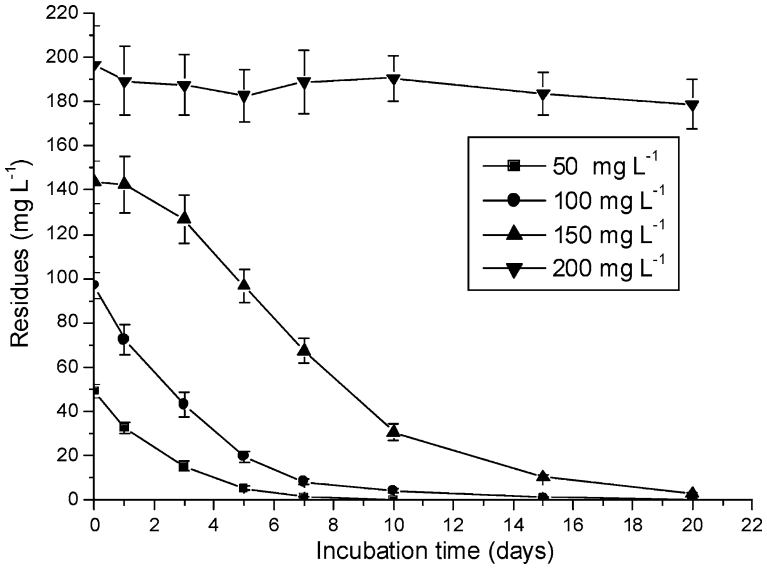

Figure 4. Time-course of hexazinone degradation by MBC in MSM with different intial concentrations.

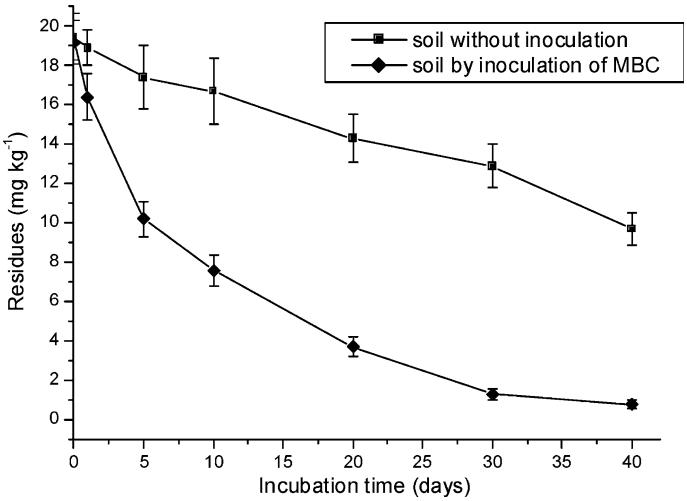

Figure 5. The effect of incorporation of MBC into soil on hexazinone degradation.

occurred with a half-life of 8.9 days. As a result, the degradation rate for MBC-added treatment almost enhanced fivefold faster than that for control set. Accordingly, although the intrinsic microorganisms in soil could degrade hexazinone, the degradation was rather slow, whereas the addition of the effectively hexazinone-degrading bacteria could lead to its quick disappearance. The results in this study showed an important application value in decontamination of hexazinone.

Conclusions

Two hexazinone-degrading bacterial strains were isolated from soil, both of them had high degrading capabilities for hexazinone in MSM, and moreover, their MBC showed higher degradation activity and resistance to hexazinone. The appropriately combined conditions for MBC to degrade hexazinone were pH 5.5, 30 °C and agitation of 120 rpm. The incorporation of MBC into soil resulted in nearly increasing fivefold over the control set for hexazinone degradation. From the stand point of environmental protection, the present observation of using MBC for remediation of hexazinone may be exploited further in environmental biotechnology for the effective detoxicification of the hexazinone-contaminated areas. However, many case studies show that results from laboratory studies can differ greatly from results in field studies due to many more variables with the latter. Therefore, more considerations should be given in application of MBC in fields.

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