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ORIGINAL PAPER

Enhanced biodegradation of hexachlorocyclohexane (HCH) in contaminated soils via inoculation with *Sphingobium indicum* B90A

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Abstract Soil pollution with hexachlorocyclohexane (HCH) has caused serious environmental problems. Here we describe the targeted degradation of all HCH isomers by applying the aerobic bacterium *Sphingobium indicum* B90A. In particular, we examined possibilities for large-scale cultivation of strain B90A, tested immobilization, storage and inoculation procedures, and determined the survival and HCH-degradation activity of inoculated cells in soil. Optimal growth of strain B90A was achieved in glucose-containing mineral medium and up to 65% culturability could be maintained after 60 days storage at 30°C by mixing cells with sterile dry corncob powder. B90A biomass produced in water supplemented with sugarcane molasses and immobilized on corncob powder retained 15–20% culturability after 30 days storage at 30°C, whereas full culturability was maintained when cells were stored frozen at –20°C. On the contrary, cells stored on corncob degraded γ-HCH faster than those that had been stored frozen, with between 15 and 85% of γ-HCH disappearance in microcosms within 20 h at 30°C. Soil microcosm tests at 25°C confirmed complete mineralization of [^{14}C]-γ-HCH by corncob-immobilized strain B90A. Experiments conducted in small pits and at an HCH-contaminated agricultural site resulted in between 85 and 95% HCH degradation by strain B90A applied via corncob, depending on the type of HCH isomer and even at residual HCH concentrations. Up to 20% of the inoculated B90A cells survived under field conditions after 8 days and could be traced among other soil microorganisms by a combination of natural antibiotic resistance properties, unique pigmentation and PCR amplification of the *linA* genes. Neither the addition of corncob nor of corncob immobilized B90A did measurably change the microbial community structure as determined by T-RFLP analysis. Overall, these results indicate that on-site aerobic bioremediation of HCH exploiting the biodegradation activity of *S. indicum* B90A cells stored on corncob powder is a promising technology.

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Introduction

Hexachlorocyclohexane (HCH) has been extensively used in the recent past for pest control in agricultural production, food and seed storage, and forestry. HCH has mainly been applied in two formulations: i) pure grade γ-HCH (lindane) and ii) technical grade mixture of HCH isomers. Serious environmental problems have resulted from HCH usage, since all its isomers are toxic, highly persistent in the environment and tend to accumulate in biological tissues (Ballschmiter 1996; Ortiz et al. 2001; Zou and Matsumura 2003). Due to their toxicity and persistence, technical grade HCH and γ-HCH have gradually been banned and their use is now restricted in most countries. Unfortunately, HCH residues still remain present in many agricultural soils where they had been applied previously (Kannan et al. 1997; Kashyap et al. 2002; Mukherjee and Gopal 2002; Nawab et al. 2003; Simonich and Hites 1995), and a clear risk exists that HCH residues find their way from soil into vegetables, grains and fruits (Ahuja and Awasthi 1993; Sanghi and Tewari 2001).

Serious HCH contamination also occurred from HCH production itself. HCH is synthesized by photochemical chlorination of benzene (Windholz et al. 1976). During this process, several isomers are formed of which the most abundant ones are designated as α-, β-, γ- and δ-HCH. The isomers are formed in the approximate proportion of α-HCH (60–70%), β-HCH (5–12%), γ-HCH (10–15%) and δ-HCH (6–10%) (Walker et al. 1999). Of all the isomers only γ-HCH has the insecticidal activity. During γ-HCH purification, the other non-active isomers were buried or dumped without further treatment which resulted in highly contaminated areas, such as at former production sites in the Netherlands (Vijgen 2006; Willem and Wollent 2005), Spain (Oliveira et al. 2003), Brazil (Osterreicher-Cunha et al. 2003), Germany (Kalbitz and Popp 1999) and India (Prakash et al. 2004). Although HCH contamination from dump and production sites has mostly been removed to landfills or has been contained to avoid further distribution, the risk posed by the material remains as spontaneous HCH degradation proceeds too slowly (Sahu et al. 1990; Senoo and Wada 1989; Thomas et al. 1996; Tu 1976).

One of the options to mitigate HCH risks would be to mineralize it to CO_2, H_2O and chloride, which might be achievable by targeted bioremediation (Phillips et al. 2005). Bioremediation or bioaugmentation of contaminated soils through exploitation of microbial catalytic activities have been increasingly projected as an alternative to more costly physical and chemical methods such as incineration, and a few studies have convincingly demonstrated its usage (Pepper et al. 2002; Phillips et al. 2005; Swaminathan 2003; Vidali 2002). One important prerequisite for effective bioaugmentation is the availability of suitable bacterial catalysts, which can transform the compound(s) under consideration. In the case of HCH decontamination, several potentially suitable bacterial catalysts may exist among the various isolated HCH-degrading strains (Boltner et al. 2005; Ceremonie et al. 2006; Mohn et al. 2006; Sahu et al. 1990; Senoo and Wada 1989). The HCH-degradation pathways, in particular those of γ-HCH and to some extent of α-, δ- and β- HCH (Nagata et al. 1999; Nalin et al. 1998; Sahu et al. 1990; Senoo and Wada 1989; Sharma et al. 2006; Thomas et al. 1996) have been studied in particular detail for three of those strains, *Sphingomonas paucimobilis* strains B90A (Sahu et al. 1990), UT26 (Senoo and Wada 1989) and Sp+ (Ceremonie et al. 2006), now classified as *Sphingobium indicum* B90A, *Sphingobium japonicum* UT26 and *Sphingobium francense* Sp+, respectively (Pal et al. 2005).

Here we studied targeted HCH bioremediation in soil by applying *S. indicum* B90A, a bacterium degrading all HCH isomers (Dogra et al. 2004; Kumari et al. 2002; Suar et al. 2004; Sharma et al. 2006). We focused on the cultivation conditions necessary to maintain HCH degradation activity of the strain, we examined its survival during storage on corncob powder at ambient temperatures or whilst frozen, and analyzed its ability to degrade and mineralize γ-HCH in liquid culture and in soil microcosms, even at residual (μg per g) HCH concentrations.

Materials and methods

Organism and culture conditions

S. indicum B90A was obtained from N. Sethunathan, Central Rice Research Institute, Cuttack, India in 1993. Strain B90A was usually grown aerobically at 30°C in mineral salt medium (SM, pH 7.0) containing per liter: 0.5 g $(NH_4)_2HPO_4$; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.1 g K_2HPO_4; 0.01 g $FeSO_4 \cdot 7H_2O$ and 0.01 g Ca $(NO_3)_2$, supplemented with 0.1% glucose (SMG). As complex medium we used Luria Broth with 3 g instead of 10 g NaCl per liter, or nutrient medium (HiMedia Laboratories Pvt. Ltd., Mumbai, India) solidified with 15 g agar per liter, if necessary.

For small-scale cultivation (up to 100 ml) precultures of *S. indicum* B90A in SMG medium were transferred in a 1/100 volumetric ratio to fresh SM medium or to tap water containing 1 or 2% (w/v) cane molasses (Deorala Sugar Factory, Meerut, India). For large-scale cultivation 100 ml precultures were grown to log phase on SMG (corresponding to a culture turbidity at 540 nm [OD_{540}] of 1.2 and to a cell number of approximately 10^9 per ml). The 100 ml culture was transferred to 1.5 l medium in 5 l flasks and shaken at 95 strokes per min (Edmund Bühler Labortechnik Materialtechnik, Germany). After about 36 h, the cell biomass equaled again approximately 10^9 cells per ml.

Storage of cultivated cells

Survival of B90A was tested on corncob powder as carrier material or as washed cell suspension at –20°C. Overnight grown cultures from different media and in different volumetric amounts (0.1, 0.2 up to 1.0 ml) were added to 0.1 g of corncob powder (Venkateshwara Bioproducts Pvt. Ltd, Bangalore, India) that was autoclaved twice consecutively for 30 min in 10 ml sterile plastic tubes. Three different temperatures (6, 30, and 37°C) were tested for storage. Culturability as number of colony forming units (CFU) at each time point during storage was then determined by sacrificing three tubes, resuspending the content of each tube in 2 ml of sterile saline solution (8.5 g NaCl per l H_2O), vigorously vortexing for 1 min and diluting immediately with a wide-bore tip (to avoid losing particulate matter). Dilutions were plated on SMG or complex medium agar plates, incubated at 30°C, and colonies were counted after 3 days.

B90A cells for freezing were recovered in 50 ml sterile plastic tubes from 20 ml cultures (containing 3×10^8 cells per ml) raised in SM plus 2% molasses. Cells were centrifuged at 4500 *g* for 10 min at 20°C, resuspended in 20 ml of SMG and stored at –20°C. At 10 day intervals three tubes were sacrificed, the content of which was resuspended in 2 ml of saline solution, serially diluted and plated on SMG or nutrient agar plates. Colonies were counted after 2 days of incubation at 30°C.

HCH degradation and mineralization in soil microcosms

The HCH-degradation activity of strain B90A after 1 month of storage (30°C on corncob powder or frozen) was first studied in soil microcosms, either by following degradation of γ-HCH by extraction and GC analysis, or via [^{14}C]-CO_2 evolution upon addition of [^{14}C]-γ-HCH. Soil (250 g) was contaminated with approximately 0.7 mg γ-HCH per kg as follows: five batches of 50 g of air-dried and sieved soil (from an agricultural field near Ghaziabad, U.P., India) were spread out to a thin layer ($\approx$1 cm) in a fume hood on a 20 × 20 cm piece of aluminum foil. 30 ml of a solution of 5 mg γ-HCH per liter acetone was homogenously sprayed on top of the soil. Acetone was let to evaporate for 1.5 h at 30°C in the fume hood. 20 g of the contaminated soil was transferred to 50 ml serum bottles making a total of 12 microcosms. Into six such microcosms, 0.3 g of corncob immobilized *S. indicum* B90A culture (raised in different growth media) were mixed; to two microcosms thawed batches of frozen B90A cells were added. The remaining four microcosms were kept as control, two of which received 0.3 g of sterile non-inoculated corncob powder. Water was added to the microcosms to maintain approximately 35% water holding capacity (WHC). It was determined by adding the measured volume of water to oven dried soil up to the level of saturation. Each microcosm was then incubated

at 30°C without shaking. After 20 h of incubation another 10 ml of water was added to each of the microcosms. Samples of approximately 3 g were withdrawn and extracted with 10 ml of a hexane: acetone mixture (4:1 v/v). 2.5 ml of sulfuric acid (18.7 M, facilitating the removal of soil bound HCH) was added to the hexane: acetone-soil mixtures. Soil mixtures were vortexed, and the hexane-acetone layer was recovered to a new tube and evaporated in a rotary evaporator to reduce the volume to one tenth of the original volume. Afterwards, the volumes of all samples were adjusted to 1.0 ml. A volume of 1 μl of the concentrated sample was injected into a gas chromatograph (GC), type Shimadzu (GC-17A, Japan) equipped with a ^{63}Ni electron capture detector and a BPX50 capillary column (internal diameter 0.32 mm; SGE Australia Pty. Ltd., Australia). The oven, injector and detector temperatures were maintained at 200, 220 and 250°C, respectively, and the nitrogen carrier gas flow rate equaled 27 ml per min. Extraction efficiencies of HCH residues by the protocol described varied between 74 and 98%.

Mineralization of γ-HCH in soil was tested with *S. indicum* B90A cells stored on corncob, as described above (0.6 ml of a culture of OD_{600} 0.5 in SMG to 0.1 g sterile corncob powder in 10 ml sterile glass tubes). Two replicates were used immediately for counting viable cells (4.0×10^7 cells per corncob tube), the others (10 replicates) were stored at 30°C. After 14 days storage the cells were used for the mineralization experiments. Simultaneously stored corncob powder immobilized cells were diluted and plated for viability. Contaminated soils were prepared as above, except that 42 g of air-dried soil was contaminated with 30 ml of a 10 mg per l γ-HCH solution in acetone plus approximately 10^6 dpm of [^{14}C]-γ-HCH (UL γ-HCH -14C, L2034, 95% purity, specific activity 20 mCi per mmol, Sigma). After acetone evaporation the soil was mixed in a 500 ml Schott flask for 16 h on a tube-roller at room temperature. To one half of the soil-HCH mixture (20 g), three batches of the corncob stored cultures were added and mixed with a spatula, then placed back on the tube roller for 1.5 h (total volume approximately 25 ml). To the other half, 0.3 g dry sterile corncob was added, plus another 1.8 ml sterile SM medium, after which the mixture was rotated for 1.5 h as well. Each of the mixtures was then divided in three portions of approximately 8 ml and transferred to 100 ml gas tight flasks, after which another 0.9 ml of sterile demineralized water was added. The flasks were closed with grey butyl rubber septa (No. 27232, Supelco, Fluka Chemie, Buchs, Switzerland), sealed with crimped aluminum rings and incubated at ambient temperature (20–25°C). Each of the flasks was connected through Teflon tubings, low pressure fittings (Bio-Rad), and steel needles with a series of three 25 ml gas tight vials, containing 6 ml of 0.5 N NaOH solution and closed with septa and aluminum crimp seals. In each of the vials, the incoming needle was placed into the NaOH-solution, whereas the outgoing needle was not in contact with the solution. Tubing from the last vial was connected to a vacuum pump (operating at –100 mbar). [^{14}C]-CO_2 evolution (and ^{14}C resulting from any other volatile HCH-metabolite dissolving in the NaOH solution) was measured on day 1, 3 and 20 by pumping for 1 h and sampling 0.5 ml of the NaOH solution in each of the vial 'traps' (after sampling, 0.5 ml fresh NaOH solution was replenished in the traps). To each of the samples, 3.5 ml of Lumagel (Lumac LSC B.V., Groningen, The Netherlands) was added, mixed by vortexing and measured on a liquid scintillation counter (Tri-Carb 2500 TR, Packard Instrument Company, Meriden, CT 06456, USA). After the final measurement on day 20, 15 ml of a hexane:acetone solution (4:1 v/v) was added to each of the incubations, shaken for 20 s by hand, and 0.5 ml of this solution was sampled for [^{14}C]-content. The total [^{14}C]-activity recovered in the experiments (= total amount of [^{14}C]-CO_2 plus [^{14}C] in the soil divided by the amount [^{14}C]-γ-HCH added) was about 66%.

Field trial with HCH contaminated soils in pits

A small-scale field trial experiment was conducted in pits of 1 square meter and 20 cm depth during March–April 2006. Experimental soil (300 kg) was brought from a contaminated field close to a γ-HCH production factory (India Pesticide Ltd. [IPL] in Lucknow, U. P. India),

sieved through a 4 mm mesh, dried and kept at 28°C for 72 h before its transfer to the pits. This soil contained 17.6% sand, 24% silt, 58% clay and 0.92% organic carbon, with a pH_{H2O} of 8.4. Ambient temperature during the experiment in Delhi fluctuated between 25 and 30°C. In total around 100 kg of air-dried and sieved soil was transferred to each of the three plots (plot A, B and C), which were lined at the bottom with an impermeable membrane ('geomembrane', purchased from a local market in Delhi, India) in order to maintain the proper containment for bacteria as well as pollutants. Only corncob immobilized B90A formulation was used in this experiment (as the use of frozen cells was found not to be practical). Plot A served as control. Soil in plot B was mixed with 1 kg of corncob to which 3 l of SM plus 2% molasses but no cell culture was added. Plot C was mixed with 1 kg corncob powder to which 3 l of *S. indicum* B90A culture (at 10^9 cells per ml raised in SM plus 2% molasses) had been added. Depending upon the availability of the corncob immobilized cells formulations were either used immediately or after storage at 30°C until a maximum period of 1 month. An approximate final number of 4.0×10^8 cells of strain B90A per gram of dry soil was obtained. Moisture content of the soil in all the plots was raised up to 35% WHC by addition of water which was estimated periodically by weighting the soil after homogenous mixing and loss in weight (moisture) after every week was adjusted by adding the equal amount of water from outside and soil was aerated by manual tilling after every 8th day. After day 8 and 16 a new portion of corncob-immobilized B90A was mixed into the soil plot C at $\approx 1.0–3.0 \times 10^8$ cells per g soil, whereas plots A and B received another 3 l of SM plus 2% molasses or 1 kg corncob plus molasses, respectively. Five soil samples from different locations within a pit and up to a depth of 20 cm were withdrawn at 0, 8, 16 and 24 day and mixed to form a composite sample. From this composite sample, three replicates of ten grams each were taken, from which HCH was extracted and analyzed on GC-ECD as described above. From the same composite sample one g of soil was taken for determining the culturability of strain B90A.

Field trial at the agricultural site

This experiment was carried out during March–April 2006 directly at an agricultural field with a history of HCH application. The soil in this field was composed of 67.6% sand, 20.0% slit, 12.4% clay, and 0.45% organic carbon, with a pH_{H2O} of 7.6. Levels of HCH contamination at the agricultural site were ~4 μg per g soil. An area of 6×3 m was selected and divided into two plots of 3×3 m^2 each. The plots were ploughed and the soil was mixed thoroughly before the start of the experiment. Soil moisture was measured and adjusted to 35% of WHC by spraying the water from outside as before. Corncob immobilized *S. indicum* B90A (9 l of B90A culture immobilized on 3 kg of corncob) was applied on one of the plots whereas the second served as control receiving only an equal amount of corncob plus SM and 2% molasses. Sampling for HCH residues was done immediately after the application of corncob and also on every 8th day as described above. In order to maintain a high population of B90A, a fresh inoculum of B90A in corncob was added on every 8th day as described above. Ambient temperature during the experiment was between 25 and 32°C.

Tracking of introduced B90A bacteria in the soil

The number of culturable *S. indicum* B90A cells in soil was determined via plating. Hereto 1 gram of soil was mixed with 9 ml of sterile saline solution, vortexed for 2 min, serially diluted and plated onto LB plus 200 μg per ml streptomycin and 30 μg per ml nystatin. *S. indicum* has a natural resistance to streptomycin (Vanbroekhoven et al. 2004) and nystatin. B90A colonies were identified among a variety of other soil-borne morphologies after 4–5 days of incubation at 30°C, mostly on the basis of its yellow color and the production of a brown pigment that diffused into LB agar plates. A total of 25 brown pigmented and 25 yellow pigmented colonies were picked at random and were further identified via PCR amplification of *linA1* and *linA2* DNA (Dogra et al. 2004; Kumari et al. 2002). Due to the high sequence identity of *linA1* and *linA2* a common

sense primer was developed (5′-GCGGATCCGC ATG AGT GAT CTA TAG ACA GAC TT, located at the start of *linA1/linA2*), whereas antisense primers were designed individually (5′-ACGAATTC ATC AGG CGG CTG CT-3′ end of *tnpA* of IS6100 flanking *linA1*, and 5′-ACGAATTC ATG ACG GAT TTC AAG T-3′ end of *tnpA* of IS6100 flanking *linA2*). Because of the different location and orientation of IS*6100* nearby *linA1* and *linA2* this amplification would give products of 1.2 and 2.8 kb, respectively, with *S. indicum* B90A DNA. PCR cycling was performed according to standard procedures.

T-RFLP analysis

For T-RFLP analysis a total of 12 samples (10 g each) were collected from each pit immediately after treatment start, and at 8, 16 and 24 days afterwards. Soil samples from each pit were mixed homogeneously and stored at -70°C until use. Total DNA from each sample mix was extracted using the Power Soil DNA isolation kit (Mo Bio, Carlsbad, CA, USA). 16S rRNA gene fragments were amplified in duplicate reactions in the PCR using the 6-carboxyfluorescein labeled primer 8F, 5′-AGAGTTTGATCCTGG-CTCAG-3′ and unlabeled primer 534R, 5′-TTA-CCGCGGCTGCTGG-3′ (Relman, 1991; Muyzer et al. 1993). Amplicon size was verified by agarose gel electrophoresis. Replicate reactions were pooled together and DNA was further purified (Montage PCR, Millipore Corporation). Quality and purity of the amplified DNA was verified on a NanoDrop ND-1000 spectrophotometer (Witec-AG, Switzerland). Equal DNA amounts (100 ng) were then digested at 37°C for 4 h with *Hae*III, *Msp*I or *Rsa*I. A volume of 1.0 µl of the digestion mixture was mixed with 0.5 µl ROX-500 dye (Applied Biosystem Incorporated, Foster City, Calif.) and 8.5 µl of deionized formamide. This mixture was incubated at 95°C for 3.0 min and immediately transferred on ice for 5 min. The entire sample (10 µl) was transferred to a 96 well sequencer plate and loaded on a capillary sequencer (Type 3100 Genetic Analyzer, ABI, Foster City, Calif.) equipped with the GeneScan software.

Data analysis

Data generated by the 3100 Genetic Analyzer were transferred to Microsoft Excel. Fluorescently labeled terminal restriction fragments (T-RF) were identified on the basis of their length (in bp) whereas their respective peak area was taken for further analysis, the peak area being considered more appropriate than peak height for such kind of analysis (Grant and Ogilvie 2003). T-RFs below 50 fluorescent units, below 50 bp and above 500 bp in length were considered as instrument base line noise and/or PCR artifacts and were not taken into account for further analysis. Data files were created in the T-align software format, a freely available web based tool (http://inismor.ucd.ie/~talign), in order to normalize data matrices for all samples. Data matrices were further manually corrected and clustered using the Bray-Curtis similarity, distance measure and UPGMA (Unweighted Pair Group Method with Arithmetic Mean). All other statistical analysis was done using the Multivariate Statistical Analysis Package (MVSP) of Kovach Computing Services (KCS, Anglesey, Wales).

Results

Mass cultivation of *S. indicum* B90A

In order to find the most optimal medium for growing *S. indicum* B90A, while retaining economic considerations of possible mass cultivation, we compared growth on minimal media with glucose as sole carbon and energy source with growth on tap water with molasses only (as C and N source). As expected, fastest growth of B90A was observed on SM medium with 0.3 and 1%, and slightly slower with 0.1% glucose (Fig. 1). In comparison, growth on SM medium with 1 or 2% molasses as C source was considerably slower. Growth on tap water with 1 or 2% molasses was slowest, but still the cells reached a culture turbidity of about 0.8 after 30 h of growth (Fig. 1) and interestingly, survived even beyond 72 h, whereas cell culturability rapidly declined after 24 h in cultures grown in SM plus glucose. For mass scale cultivation the strain was

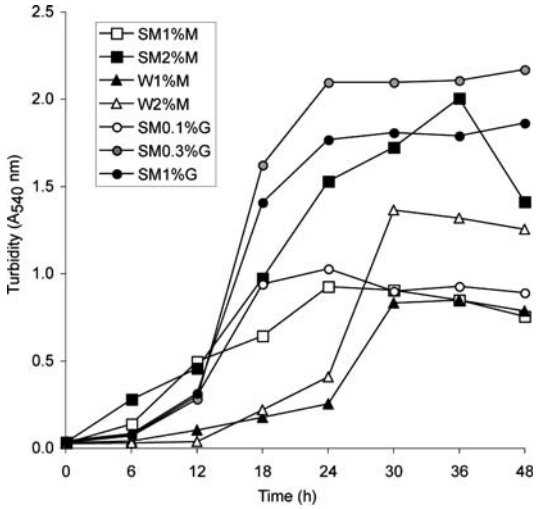

Fig. 1 Growth of *Sphingobium indicum* B90A in SM containing 0.1, 0.3 or 1% glucose (SM 0.1%G, etc.), 1 or 2% molasses (SM 1 and 2% M) and in tap water containing 1 or 2% molasses (W1 and W2% M). Growth was measured as turbidity (at 540 nm) of the inoculated media at different time points as against a blank of non-inoculated medium. Cultures in SM containing glucose stopped growing after 24 h; therefore, their turbidity is not shown afterwards

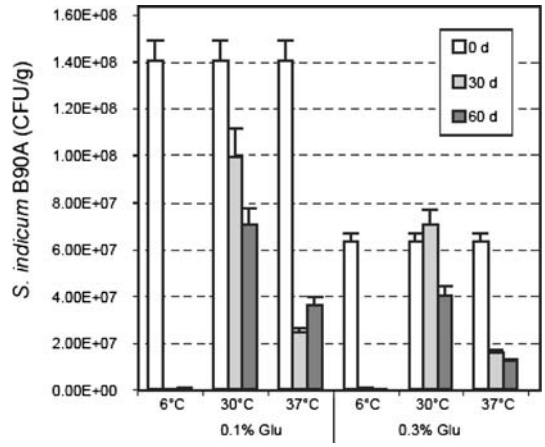

Fig. 2 Survival of *Sphingobium indicum* B90A in corncob powder incubated at 6, 30 and 37°C after cultivation on SM plus 0.1 or 0.3% glucose

incubated in 5 l flasks with 1.5 l SM plus 2% molasses for about 36 h, resulting in a cell number of $\approx 10^{10}$ cells per ml.

Survival of B90A under different storage conditions

Next, we addressed the effect of prolonged storage in different media and conditions on culturability of strain B90A. Cells simply stored at 30 or 37°C in the medium they had been growing on, rapidly lost viability within a few days (not shown). To improve cellular viability we tried freezing cells at -20°C, or mixing the cultures with autoclaved corncob powder, which is an agricultural waste material with excellent wetting properties. Interestingly, cells pregrown on SM plus 0.1% glucose, washed and frozen at a cell density of 3.0×10^8 cells per ml in SMG remained completely viable during the 40 days period of our tests (data not shown). Cells mixed with corncob powder at a cell density of approximately 10^8 cells per gram dry corncob powder lost about 50% culturability after 60 days storage at 30°C, when precultivated on SM plus 0.1% glucose (Fig. 2). Survival was slightly better for cells that had been precultivated on SM plus 0.3% glucose (35% culturability loss after 60 days storage). The culturability of cultures stored at 37°C diminished to about 20% of the initial amount of colony forming units. Storage at 6°C resulted in about 0.1% survival after 30 d. Optimal survival was found for a volumetric-solid ratio of 0.7 ml cell suspension per 0.1 g dry corncob. Cells pregrown on SM plus molasses survived much poorer than on glucose. In these cultures, only about 15–20% of the cells remained viable after 30 d storage at 30°C (not shown).

γ-HCH degradation activity of stored B90A cells

Corncob-immobilized cells after 30 days storage were mixed with an aqueous solution of 0.6 mg γ-HCH per liter to test their degradation activity. All cultures had not only survived but also degraded γ-HCH directly (Fig. 3A). As expected from the survival data, cells stored at 30°C performed the best, although even cells that had been stored at 6°C still degraded half of the added γ-HCH. Subsequently, we tested whether corncob-stored B90A cell batches could degrade γ-HCH in artificially contaminated soil microcosms. Analyses after 20 h incubation showed that cultures that had been grown on SM plus glucose had degraded up to about 15% of the original γ-HCH concentration (Fig. 3B). No

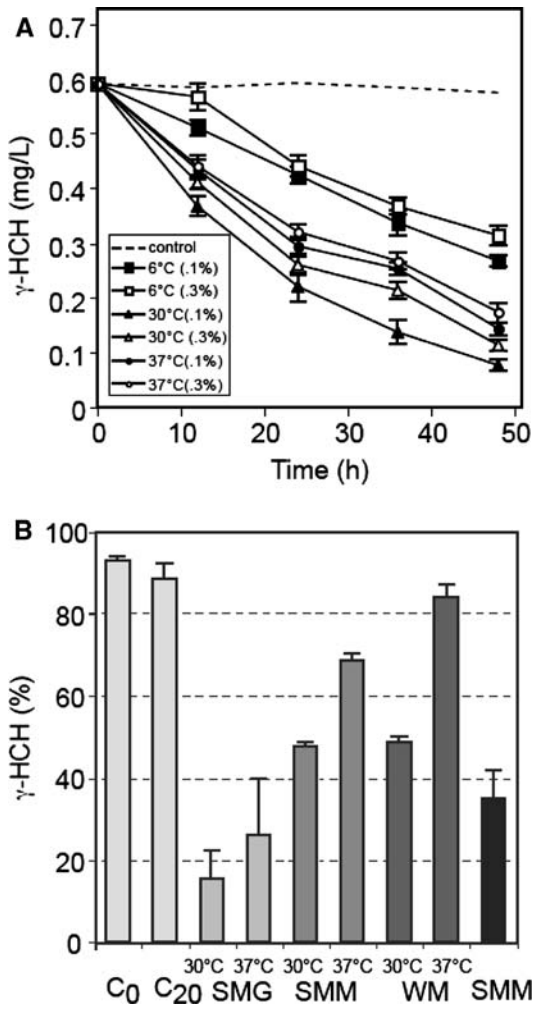

Fig. 3 Degradation of γ-HCH by corncob immobilized B90A cells or cell slurries from frozen pellets in (**a**) aqueous slurries and (**b**) in soil microcosms. C0, C20, non-inoculated control at 0 and 20 h, respectively; SMG 30°C, SMG 37°C, cells cultivated in SM glucose before immobilization and storage at 30 or 37°C; SMM 30°C, SMM 37°C, B90A cells cultivated in SM molasses; WM 30°C, WM 37°C, B90A cells cultivated in water plus molasses; SMM, frozen and thawed cells as inoculum, raised in SM + 2% molasses. Data points are averages from three independent incubations with calculated standard deviations

statistical difference was found for batches that had been stored at 30 or 37°C when they had been pregrown on SM plus glucose. Cultures that had been stored from cells pregrown on SM or tap water plus molasses were slower in degrading γ-HCH (around 50% γ-HCH disappearance after 20 h), but no statistical difference was found among them, except for cells that had been pregrown with molasses and stored at 37°C (20–30% γ-HCH disappearance after 20 h incubation time). Frozen cells of *S. indicum* B90A that had been pregrown on SM plus molasses degraded γ-HCH to about 35% of the original amount after 20 h of incubation, slightly better than those pregrown on molasses and mixed with corncob powder. The difference in amount of γ-HCH degraded after 20 h in all cases more or less reflected the amount of viable cells determined after 30 days of storage (Fig. 2). Therefore, we conclude that the cultivation medium does not directly affect the activity of the surviving cells after 30 days of storage, but only the proportion of culturable cells.

To establish whether the *S. indicum* B90A cells stored in corncob powder were not only degrading but also mineralizing γ-HCH, we incubated batches of corncob-stored cells with soil in microcosms that had been artificially contaminated with γ-HCH plus [^{14}C]-labeled γ-HCH, and measured the formation of [^{14}C]-CO_2 over time. Samples measured after 1, 3 and 20 days of incubation at 25°C showed a steady evolution of [^{14}C]-CO_2 from the microcosms inoculated with corncob-stored cells, but not from microcosms inoculated with sterile corncob only (Fig. 4). After 20 days, a total of 53% of the initially added [^{14}C]-γ-HCH was found back as [^{14}C]-CO_2. The total overall recovery ([^{14}C]-CO_2 plus [^{14}C]-extractable material from soil) across all incubations was 66%, indicating that part of the label or [^{14}C]-CO_2 had either volatilized and had not been trapped in the NaOH containing vials or remained as unextractable fraction in the soil. In the uninoculated controls, 13% of the radioactive label was recovered after 21 days of incubation in the NaOH-containing vials and 63% in the hexane extract of the soil. This clearly showed that γ-HCH mineralization had occurred and had been mediated by the corncob-immobilized *S. indicum* B90A cells.

Bioaugmentation experiments under field conditions

Residual concentrations of α-, β-, γ- and δ-HCH in the soil used to fill the pits were 46.7, 7.7, 1.4, and 4.9 μg per g soil, respectively (total amount of

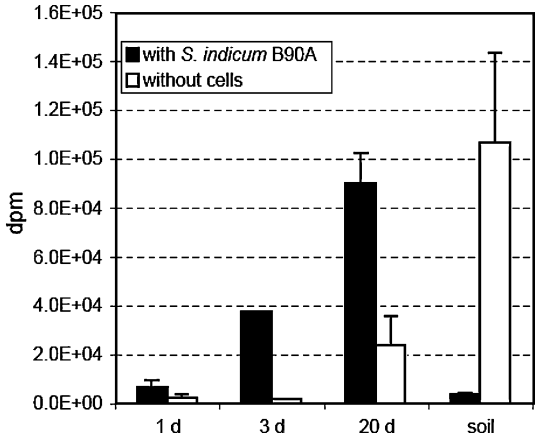

Fig. 4 Mineralization of radioactive γ-HCH by *S. indicum* B90A in soil. The bar diagram represents the cumulative formation of [^{14}C]-CO_2 from γ-HCH by corncob immobilized B90A cells after 1, 3 and 20 d of incubation time. Cells were stored for 14 days at 30°C in corncob powder before inoculation. Control vials (open bars) received corncob powder without cells. Radioactivity recovered in a hexane extract from the soil after 20 d is depicted in the final two bars ('soil'). Values represent averages from three independent replicate incubations with their average deviations

63.3–69.7 μg HCH per g soil in the different pits) (Fig. 5). The cell number of strain B90A introduced via corncob at the start of the experiment and determined via the selective plating and identification procedure was 2.0×10^8 cells per g of soil (Table 1). Pit C inoculated with B90A in corncob showed a 76% reduction in HCH concentration after 8 days of incubation, compared to 7% in pit B (receiving only corncob and SM plus molasses) and no significant decrease in the control pit A (Fig. 5). The number of CFU of strain B90A obtained from the soil after 8 days had decreased by nearly 75% (5×10^7 CFU per g soil) compared to the time of inoculation. Colonies of strain B90A could be differentiated from other streptomycin resistant yellow colonies appearing on plates with soil bacteria extractions from the pits via their brown pigment (96% of all yellow and streptomycin-resistant colonies on plates derived from pit C; none from pit A and pit B). PCR with *linA1*- and *linA2*- specific primers on 25 colonies with brown pigment and on 25 only yellow colonies randomly selected consistently showed amplification products of 1.2 and 2.8 kb only in brown-pigmented ones, which is indicative for strain B90A.

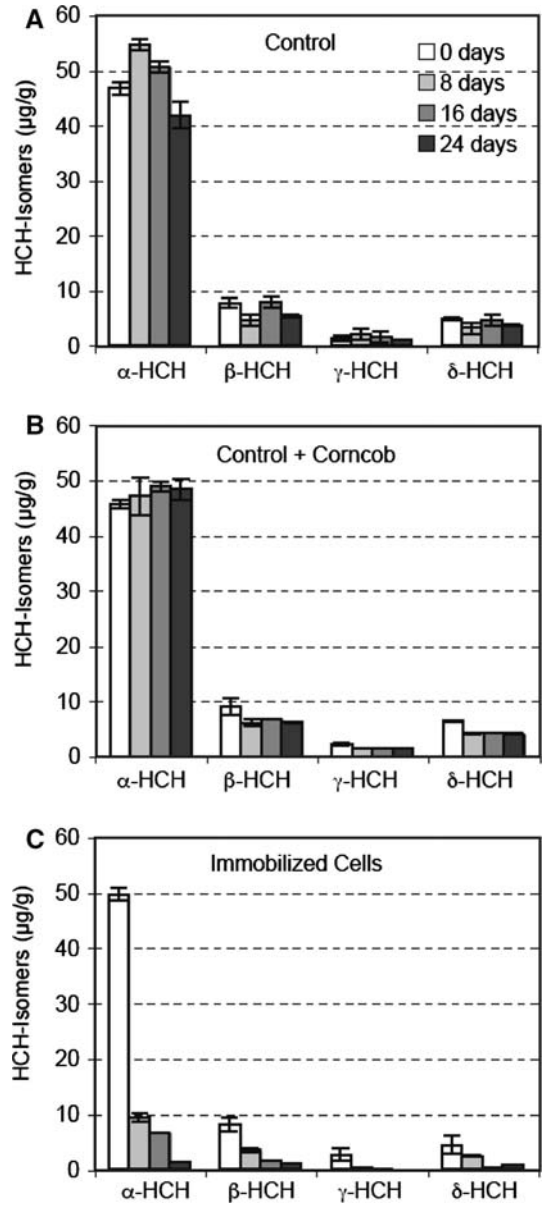

Fig. 5 Degradation of HCH isomers by *Sphingobium indicum* B90A in $1 \times 1 \times 0.2$ m^3 scale soil plots. (**A**) Soil with medium only, (**B**) soil incubated with corncob alone, and (**C**) soil to which corncob powder immobilized cells were added (≈10^8 cells per g of soil). Values represent averages from three replicates from a composite sample taken at five different positions in the plot

At this point (day 8) another fresh inoculum was added so as to increase the number of B90A CFU to 1.4×10^8 per g soil. After 16 days, the HCH residual levels in pit C were further reduced from 15.9 to 8.8 μg per g soil (total reduction of

Table 1 Viability of *S. indicum* B90A inoculated at the HCH contaminated field site

Sampling time (days)	Control soil with medium(CFU)[a]		Control soil with corncob(CFU)		Soil with cells in corncob(CFU)	
	Before[b]	After[c]	Before	After	Before $\times 10^7$	After $\times 10^8$
0	–	–	–	–	–	3.0
8	–	–	–	–	7.5	1.5
16	–	–	–	–	3.5	1.0
24	–	–	–	–	4.0	1.8

[a] Number of colony forming units with yellow and brown pigment per gram of soil

[b] Before inoculation or renewed inoculation of strain B90A in soil

[c] After inoculation (primary or renewed) of strain B90A in soil

86% compared to time zero), with no further reduction in pits A and B. After 24 days (i.e., the end of the experiment) the extractable levels of α-, β-, γ- and δ-HCH were 1.4, 1.1, 0.0 and 0.8 μg per g of soil, respectively, and nearly more than 95% of total HCH had disappeared. On the contrary, there was no further HCH disappearance after 8 days (initial decline of 7.2%) in pits A and B.

In a final experiment, we tested whether *S. indicum* B90A cells were capable of degrading HCH residues directly on site in a contaminated agricultural soil under conditions prevailing in India. Hereto, one inoculated and one uninoculated plot were prepared on site and followed for 24 days. The α-, β-, γ- and δ-HCH residues at the time of inoculation amounted to 1.8, 0.06, 3.1 and 0.04 μg per g dry soil, respectively. Compared to the uninoculated control plot (Fig. 6), the plot that received B90A cells via corncob powder had degraded 95, 52, 81 and 50% of the initial α-, β-, γ- and δ-HCH amounts, respectively, by day 8. At that time point another batch of corncob-stored B90A cells was mixed with the soil. After 24 days the amounts of HCH had dropped to 0.1, 0.59 and 0.02 μg per g soil of α-, γ- and δ-HCH, respectively, whereas the β-HCH concentration remained at 29 ng per g soil. In none of the samples were any known HCH-degradation products, such as pentachlorocyclohexene, detected via GC-ECD. Estimations of the B90A population size showed that cells survived under field conditions but that the number of CFU had dropped by 89% after 8 days. A similar trend in reduction of cell culturability was observed after 16 and 24 days (after renewed B90A application).

T-RFLP community analysis

The highest numbers of reproducible T-RFs was obtained when 16S rRNA gene amplicons from

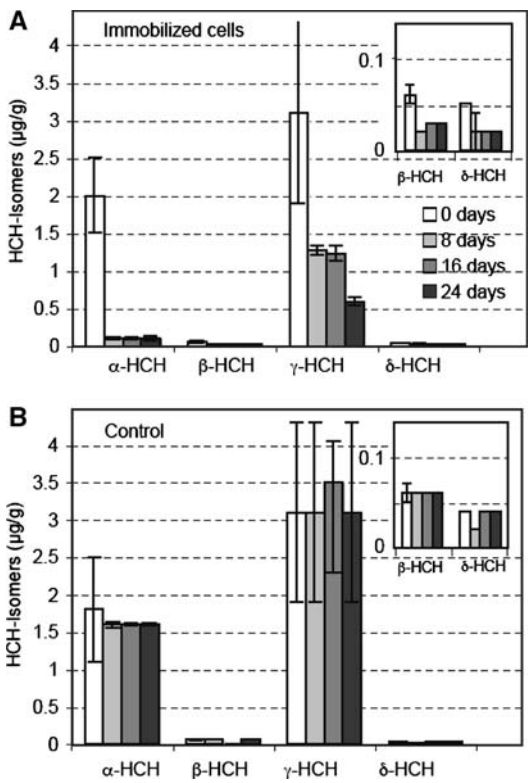

Fig. 6 Degradation of HCH isomers by *Sphingobium indicum* B90A under field conditions in $3 \times 3 \times 0.2$ m^3 plots. (**A**) plot inoculated with corncob powder immobilized cells ($\approx 10^8$ cells per g of soil); (**B**) plot mixed with corncob without cells. Values represent averages from three samples taken at different positions plus or minus calculated average deviation. Insets show an enlargement of the data sets for β- and δ-HCH

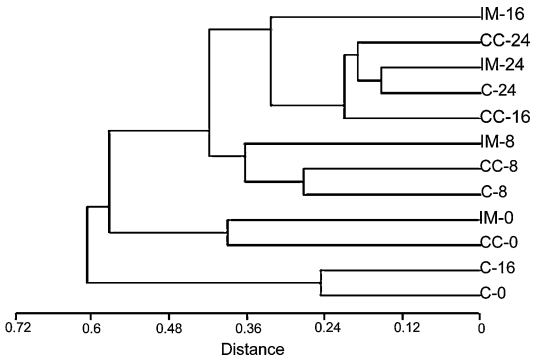

Fig. 7 Sample clustering generated from T-RFLP *Hae*III data, using Bray-Curtis distance measure and UPGMA clustering. Symbols: C, control pit with HCH contaminated soil, CC, contaminated soil amended with corncob powder only, IM, soil treated with corncob-immobilized *S. indicum* B90A. Numbers behind the symbols indicate sampling time

soil were digested with *Msp*I (96), followed by *Rsa*I (86) and *Hae*III (63). The Shannon's diversity index was highest based on the number of *Msp*I-generated T-RFs, whereas the sample's evenness was found to be 1.0 for all the three enzymes and in all 12 samples. This indicated that the overall diversity of the microbial community was the same in all the pits. Cluster analysis via the Bray-Curtis similarity and distance measures, and by UPGMA clustering showed a grouping for samples taken at the same time point, but no clustering as a function of the plot treatment (Fig. 7). This suggests that addition of *S. indicum* cells did not influence the microbial community composition as far as T-RFLP could reveal.

Discussion

Currently HCH decontamination is mostly needed in (i) sites near HCH production facilities and (ii) production dumps, and (iii) agricultural fields with low residual HCH levels resulting from γ-HCH application. Since incineration is presently not a realistic alternative for detoxification of HCH residues (Vijgen 2006), bioremediation or bioaugmentation with HCH-degrading bacteria (Boltner et al. 2005; Ceremonie et al. 2006; Lal et al. 2006; Mohn et al. 2006; Sahu et al. 1990; Senoo and Wada 1989) could be a potentially useful way for restoring sites with residual contamination. Here we showed that enhanced HCH-mineralization in soils with residual HCH contamination can be achieved by using the strain *S. indicum* B90A, but that the degradation rates depend on the media used for strain cultivation and storage. The strain achieves highest growth rates and yields in defined medium with glucose compared to tap water with cane molasses. Not only did strain B90A grow more slowly and to lower yield on cane molasses but its viability upon storage (determined as plate culturability) was reduced 2- to 3 fold when compared to cells grown in medium containing glucose. Still, high biomass quantities of strain B90A ($\approx 10^{10}$ cells per ml) could be produced within 30 h in large batch cultures on cane molasses in water, and HCH-degradation activity of the surviving cells was sufficient for HCH detoxification in the field. Since cane molasses is an inexpensive carbon and nitrogen source, which is essentially a waste product from the sugar industry, and easily and freely available in sugar-producing countries like India, the cost of biomass production can thus be substantially reduced when compared to glucose as growth substrate (which costs around 20 times more). Therefore, for larger scale applications the lower cost of biomass production may be the more decisive parameter than the lower biodegradation rates.

Strain B90A survived quite well in sterile corncob powder, although this depended on the solid to liquid ratio. For example, 50–65% culturability was retained after 60 days storage at 30°C and with 6–7 ml culture per g material. Because of the ease of this immobilization and storage procedure (30°C), the simplicity of transporting the material to remote agricultural places and its mixing with the soils, we found that the use of corncob powder was superior to cell washing and freezing, despite the higher maintenance of culturability in that procedure. In fact, aqueous cell suspensions mixed very poorly with soils, unless complete slurry was made, which would have been unfavorable for aerobic HCH degradation. *S. indicum* B90A cells stored on corncob powder remained capable of degrading HCH in soil microcosms and in the field. In microcosms, between 15 and 65% of γ-HCH had disappeared after 20 h of incubation at 30°C. Up to 95% of the

residual HCH content disappeared in field plots inoculated twice in weekly distance with strain B90A immobilized on corncob powder. Our data provided strong evidence that inoculation of B90A was responsible for HCH disappearance in the field. γ-HCH was not only transformed but completely mineralized, which became apparent from incubating radioactively labeled γ-HCH with corncob immobilized B90A cells (around 53% of the initially added [^{14}C]-γ-HCH was found back as [^{14}C]-CO_2 at a total recovery of 66%). This further suggested that the complete pathway for γ-HCH degradation was functional and not only the constitutively expressed part, which could have resulted in formation of chlorinated metabolites (Suar et al. 2004). It was previously established that the *linA, linB* and *linC* genes are constitutively expressed as compared to the lower pathway genes (*linD*, and *linE*), which are induced during exposure to α- and γ-HCH but not measurably to β- and δ-HCH (Suar et al. 2004). It should be noted, however, that *lin* gene induction in that study could not be detected below 0.7 mg HCH per l, which is still 10 fold higher than the residual HCH-concentrations in the soils used here. Since we did not detect obvious HCH metabolites in hexane: acetone soil extracts via GC-ECD, we assume that the cells even at HCH concentrations in the range of 0.04–3 μg per gram soil still degrade HCH to completion, but this might be subject to further verification.

Corncob had been reported before as a potential good carrier material (Labana et al. 2005). We hypothesize that the good influence on B90A survival was caused by a number of factors. First, corncob has excellent swelling capacities, which may have prevented drying out of the cells on the matrix. Secondly, corncob may have provided certain nutrients or carbon sources to the cells while mixed with the soil, ensuring a long-term maintenance. Thirdly, the corncob provided a surface for attachment and perhaps may have protected the cells from predation in the soil. It has been realized before that encapsulated or immobilized bacterial cells survive significantly better in soil compared to free cells (Trevors et al 1990; van Elsas et al. 1992), probably via protection from phage and protozoan predators (Cassidy et al. 1996; Smit et al. 1996). Predators have earlier been proposed to be an important factor for reducing the size of the introduced bacterial populations in inoculation experiments (van Veen et al. 1997; Vidali 2002). On the other hand, we did not study the location of the cells on corncob particles specifically and it is not clear if close association to the corncob surface can reduce transport of HCH toward the cells. In any case, we can assume that the cells obtained sufficient HCH through diffusion to allow an effective overall biodegradation.

T-RFLP data suggested that the composition of the soil microbial community changed as a function of time but not as a function of treatment type. Perhaps soil tilting and watering during the bioaugmentation period contributed to the time effect seen in all pits. Since no effects on the community composition could be measured of addition of *S. indicum* B90A and/or corncob, we conclude that neither corncob nor the augmented *Sphingobium* cells disturb the diversity of the microbial community and thus could be potentially safe from point of view of microbial community changes.

We have thus for the first time been able to show effective bioaugmentation of HCH residues with sphingomonads, from the small lab-scale to the field, and under local environmental conditions in India. Our data imply that even residual HCH concentrations in soil can be significantly further reduced by bacteria application. Our results reinforce those of previous reports showing enhanced effectiveness of immobilization/encapsulation of microbial cultures instead of addition of free cells in environmental applications (Briglia et al. 1994; Cassidy et al. 1997; Errampalli et al. 1997). Even though many aspects of strain application need to be studied further (e.g., HCH intermediate formation, fate of introduced bacteria, overall toxicity reduction via bacteria inoculation), the work presented here forms a good basis for optimism to reduce the impact of HCH isomers and to clean-up environmental sites from HCH contamination. Perhaps the use of corncob powder for strain immobilization might also turn out to be favorable for other potential strains for bioremediation, which until now were too difficult to maintain.

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