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(54) Title: MICROORGANISM CULTURE FOR REMEDIATION OF CHLORINATED HYDROCARBONS AND METHOD OF USE

(57) Abstract

The invention provides a method for removing a chlorinated hydrocarbon from soil or groundwater comprising the steps of adding to chlorinated hydrocarbon containing soil or groundwater a chlorinated hydrocarbon metabolizing microbial culture of the genus *Arthrobacter* and providing conditions supporting growth and reproduction of the microbial culture. The microbial culture removes chlorinated hydrocarbons including those selected from the group consisting of DDT, dieldrin, toxaphene, aldrin, 1,1,1–trichloroethane, 1,1–dichloroethane, 1,2–dichloroethene, trichloroethylene, methylene chloride, chloroform, or mixtures thereof. The soil or groundwater is treated in place, or removed, cleansed and returned. The invention provides a purified and isolated microbial culture of the genus *Arthrobacter* adapted to use chlorinated hydrocarbons as a sole carbon source.

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MICROORGANISM CULTURE FOR REMEDIATION OF CHLORINATED HYDROCARBONS and METHOD OF USE

TECHNICAL FIELD

This invention relates to the field of environmental remediation of chlorinated hydrocarbons. And particularly to the use of micro-organisms selected to degrade hydrocarbons, especially toxic chlorinated hydrocarbons.

BACKGROUND OF THE INVENTION.

Large quantities of wastes contain synthetic halogenated materials such as those found in dielectric fluids, flame retardants, refrigerants, heat transfer fluids, lubricants, protective coatings, pesticides, including herbicides, and insecticides. Many of these materials have been labeled as non-biodegradable. In many cases, these materials are byproducts from manufacture that have accumulated in landfills or disposal sites. current environmental regulations demand contaminated soil either be treated and disposed of and replaced with clean uncontaminated soil. Among the nonbiodegradable materials are chlorinated hydrocarbons such as toxaphene.

Use of micro-organisms to treat hydrocarbon wastes is known. For example U.S. Patent No. 3,871,957 discloses the use of freeze-dried cultures to treat beeches contaminated by hydrocarbons. Many aerobic organisms are listed as useful. including *Arthrobacter.* Patent 3,871,957 does not disclose the use of *Arthrobacter* for degrading chlorinated hydrocarbons. Three U.S. patents mention *Arthrobacter* among other genera present in composting plant materials, U.S. Patent Nos. 5,525,139; 5,100,455, and 4,511,657. While these patents indicate that chlorinated hydrocarbons are degraded by the composting plant material, none indicates any role of *Arthrobacter* species in the degradation. The references merely indicate that *Arthrobacter* species are present in the composting plant material used for remediation of contaminated soils.

Many *Arthrobacter* species are known to be useful in production of amino acids and coproporphyrins. Several patents disclose degradation of hydrocarbons and chlorinated hydrocarbons by micro-organisms but none disclose use of *Arthrobacter* adapted to use hydrocarbons or chlorinated hydrocarbons as a carbon source. Because indigenous micro-organisms may not be compatible with cultures isolated from other sites, new hydrocarbon degrading cultures are continually in demand for bio remediation applications.

SUMMARY OF THE INVENTION

The invention provides a method for removing a hydrocarbon from soil or groundwater comprising the steps of adding to hydrocarbon-containing soil or groundwater a hydrocarbon-metabolizing microbial culture of the genus *Arthrobacter* and providing conditions supporting growth and reproduction of the microbial culture. A preferred method uses a purified and isolated microbial culture of the genus *Arthrobacter* adapted to use chlorinated hydrocarbons as a sole carbon source. The invention also provides a purified and isolated chlorinated hydrocarbon metabolizing microbial culture of the genus *Arthrobacter*. In a preferred method the microbial culture removes a chlorinated hydrocarbon selected from the group consisting of DDT, dieldrin, toxaphene, aldrin, 1,1,1-trichloroethane, 1,1-dichloroethane, 1,2-dichloroethene, trichloroethylene, methylene chloride, chloroform, or mixtures thereof. The soil may be treated in place, or excavated, cleaned and replaced. Treatment in place is preferred.

DETAILED DESCRIPTION OF THE INVENTION

The microbes of the invention were first isolated and cultured from soil samples collected from a pesticide processing plant site in Texas. The site has been in operation since the late 1950s. The plant has manufactured a variety of insecticide-containing formulations. The site was contaminated with toxaphene, and dioxathione as well as non-pesticide products, such as xylenes, ethylbenzene,

naphthalene, and alkylnaphthalene. Low concentrations of chloropyrifos, malathion, methyl parathion, ronnel, aldrin, lindane, chlordane, DDT, and dieldrin, were also present. Site remediation has been underway since 1990. As part of the remediation plan groundwater is extracted, treated to drinking water standards, and about 75 percent is reinjected into the zone under remediation. Indigenous micro-organisms were assayed to determine whether there was sufficient bio remediation potential to discontinue groundwater treatment.

Samples were collected in mid-1993, consisting of two liters of soil and groundwater. From these materials, two petrophillic strains and one heterotrophic strain were isolated that can utilize the constituents found at the site as an energy and growth source. A slightly modified version of the National Environmental Technology Applications Center method was utilized for all bacterial counts.

The samples were first screened for petrophillic character by growth on Bushnell-Hass media in order to determine if the microbes could use gasoline as a sole carbon and energy source. The Bushnell-Hass media contains only gasoline as a carbon source, therefore growth in this medium indicates petrophillic organisms.

If the microbial samples demonstrate that the organisms are petrophillic, then other constituents are added to be Bushnell-Hass medium. In this case toxaphene, naphthalene and benzene were added to the Bushnell-Hass medium in order to determine if the microbes could utilize the added constituents as an energy and carbon source.

The samples than demonstrated growth in the presence of toxaphene naphthalene or benzene were streaked onto nutrient agar plates and incubated at room temperature for 24-48 hours. The resulting colonies were transferred to agar slants to prepare stock cultures for further characterization and identification. The cultures obtained by this method were further characterized by their ability to grow on chlorinated hydrocarbon materials. One strain showed excellent initial growth with benzene and naphthalene. With extended growth and many transfers, the

strain began to grow with toxaphene as a sole source of carbon. After full adaptation the strain's growth rate on toxaphene approached its growth rate on naphthalene. On further examination the strain was identified as a member of the genus *Arthrobacter.*

The new *Arthrobacter* strain was able to use either benzene, ethylbenzene, naphthalene, or toxaphene, as a sole carbon source. The result was surprising because no reference has been located wherein an *Arthrobacter* species has been reported to use chlorinated hydrocarbons as a sole carbon source.

Example 1

The combined culture the two novel *Arthrobacter* strains was compared to the indigenous micro-organisms from the original site as well as microbes from two other contaminated sites referred to as Site 1 and Site 2. All cultures also contained heterotrophic organisims isolated from the same site. Viability studies were conducted with a slightly modified version of the National Environmental Technology Applications Center method. Cultures were analyzed at time zero for microbial counts (petrophillic and heterotrophic). All cultures were incubated at room temperature in individual closed containers out of direct sunlight. Aliquots were removed from each container for viability counts at the following time: Day 0, Day 3, Day 5, Day 10, Day 14, Day 30. The results are set out in Table 1 below. Altogether six solutions were examined. They were an indigenous culture from the contaminated site (Control), Control plus nutrient, the combined culture, combined culture plus nutrient, site 1 culture and site 2 culture. The samples were made up of soil and water slurries from the original isolation site and the preceding ingredients added except in the case of the control sample to which nothing was added.

TABLE 1

Day/ Type	Control	Control Plus Nutrients	Combined Culture	Combined Culture Plus Nutrients	Site 1 Culture	Site 2 Culture
0-Het	10^6	10^6	10^6	10^6	10^6	10^6
0-Pet	10^6	10^6	10^6	10^6	10^6	10^6
3-Het	10^8	10^7	10^8	10^8	10^7	10^8
3-Pet	10^7	10^6	10^6	10^5	10^6	10^5
5-Het	10^8	10^8	10^8	10^8	10^7	10^8
5-Pet	10^6	10^6	10^6	10^5	10^6	10^6
10-Het	10^{10}	10^{10}	10^{10}	10^{10}	10^{10}	10^{10}
10-Pet	10^4	10^4	10^4	10^6	10^5	10^4
14-Het	10^8	10^8	10^8	10^7	10^8	10^8
14-Pet	10^4	10^4	10^4	10^5	10^4	10^4
30-Het	10^6	10^6	10^7	10^6	10^6	10^6
30-Pet	10^3	10^3	10^4	10^4	10^3	10^4

NOTES: All measurements are CFU./ml

Het = heterotrophic

Pet = petrophillic

After treatment for 18 months with areation of the site to increase activity of the indigenous petrophillic organisms the site was again sampled and indigenous organisms compared to the combined culture. The test results are set out in Table 2 using the same conventions.

TABLE 2

Day/ Type	Control	Control plus Nutrients	Combined Culture ATCC No. _____
0-Het	2.05×10^6	*	*
0-Pet	1.29×10^9	*	*
18-Het	1.96×10^6	2.15×10^6	2.04×10^6
18-Pet	2.31×10^9	3.59×10^9	4.22×10^9
25-Het	1.38×10^6	6.50×10^5	4.50×10^6
25-Pet	2.22×10^9	2.29×10^9	5.69×10^9
32-Het	1.52×10^6	5.10×10^6	6.00×10^6
32-Pet	2.96×10^9	2.96×10^9	4.72×10^9
39-Het	1.32×10^6	1.00×10^6	4.52×10^6
39-Pet	5.06×10^9	3.12×10^9	3.23×10^9
45-Het	1.05×10^6	1.10×10^6	5.15×10^6
45-Pet	2.51×10^9	2.55×10^9	2.33×10^9

It is notable that after 18 months of treatment with supplemental oxygenation the population ratio of petrophillic organisms to heterotrophic organisms indigenous to the site changed from 1:100 to 1,000:1, a change of five orders of magnitude. It is of further note that the combined culture continued to perform as well as the now highly adapted petrophillic indigenous strains without inhibition.

Example 2:

Use of Cultures in Site Remediation:

The ability of the *Arthrobacter* culture to reduce chlorinated hydrocarbon contamination at a site was also demonstrated in a test conducted as inTable 2 of

Example 1 above. The samples as in Table 2 above were spiked with 5,500 parts per billion ("ppb") toxaphene. After 18 days the combined culture reduced the concentration of toxaphene to 2,600 ppb as measured by ERA Sw846 Method. At day 32 toxaphene concentration was below the detection limit of the analytical system. Similiar results were obtained with the highly adapted indigenous strains from the treated site.

The activity of the *Arthrobacter* culture observed is surprising in that it metabolizes toxaphene even in the presence of other carbon sources. Normally one observes that the "easier" hydrocarbons are depleted from the site before chlorinated hydrocarbons are utilized. In the present case, the *Arthrobacter* strains degrade toxaphene in the presence of other carbon sources.

Example 3:

Arthrobacter cultures isolated as described bove are also useful for bioremediation of sites contaminated with chlorinated hydrocarbons other than toxaphene. For treatment of a site a culture is provided with at least 10^8 CFU/ml metered into a groundwater injection system. Preferably the soil of the site is also provided with supplemental oxygen and nutrients to favor rapid expansion of the petrophillic micro-organism population. In this manner chlorinated hydrocarbons such as 1, 2-dichloroethane or 1,1,1-trichloroethane are efficiently removed from the soil with no residual contamination. Treatment times vary with the initial level of contamination to be removed and the age of the contaimination.

CLAIMS:

I claim:

1. A method for removing a hydrocarbon from soil or ground water comprising innoculating chlorinated hydrocarbon containing soil with a cholornated hydrocarbon metabolizing microbial culture of the genus *Arthrobacter* and providing or maintaining conditions supporting growth and reproduction of the microbial culture.

2. A method according to claim 1 wherein the *Arthrobacter* culture is adapted to use toxaphene as a sole carbon source.

3. A method according to claim 2 wherein the microbial culture metabolises a chlorinated hydrocarbon in addition to toxaphene.

4. A method according to claim 1 wherein the microbial culture metabolises a chlorinated hydrocarbon selected from the group consisting of DDT, dieldrin, aldrin, 1,1,1-trichloroethane, 1,1-dichloroethane, 1,2-dichloroethene, 1,1,2-trichloroethylene, methylene chloride, chloroform, or mixtures thereof.

5. A method according to claim 1 wherein the soil or groundwater is treated in place.

6. A purified and isolated microbial culture of the genus *Arthrobacter* adapted to use chlorinated hydrocarbons as a sole carbon source.

7. A microbial culture according to claim 6 adapted to grow on toxaphene as its sole cabon source.

8. A microbial culture according to claim 6 wherein the microbial culture metabolises a chlorinated hydrocarbon in addition to toxaphene.

9. A microbial culture according to claim 6 wherein the microbial culture metabolises a chlorinated hydrocarbon selected from the group consisting of DDT, dieldrin, aldrin, 1,1,1-trichloroethane, 1,1-dichloroethane, 1,2-dichloroethene, 1,1,2-trichloroethylene, methylene chloride, chloroform, or mixtures thereof.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/09851

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :B09B 3/00; C07C 4/00, 6/00; C12N 1/00, 1/20

US CL :435/252.1, 262, 262,5, 264, 830

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/252.1, 262, 262.5, 264, 830

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, EPO, JPO

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,525,139 A (GILL) 11 June 1996, col. 6, line 51 - col. 8, line 24.	1-5 ---- 6-9
X --- Y	US 4,535,061 A (CHAKRABARTY et al) 13 August 1985, abstract, col. 2, lines 26-51, col. 6, lines 51-58, col. 11, line 56 - col. 12, line 44, and claim 2.	1, 3-5 ------ 2, 6-9

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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