BACTERIUM CAPABLE OF BIODEGRADATION OF DIOXIN-LIKE SUBSTANCES

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Introduction

Isolation of novel strains of Bacillus midousuji, a species of thermophilic bacteria which degrades various toxic and non-toxic waste materials including industrial wastes. These particular wastes present various problems under current disposal methods. This thermophilic microorganism has optimal growth at 62 degrees Celcius or higher (up to the boiling point of water) enabling a novel accelerated method for degrading waste in contrast to any other microorganism process in current use. This microorganism degrades fish waste into protein and DHA (a food supplement), plastic into CO2 and water and hazardous compounds into inert byproducts. Several of the compounds that this microorganism safely degrades now present a difficult and hazardous degradation
problem in many superfund and contaminated sights around the world.

Materials

Bacterium strain, Bacillus midousuji SH2A was deposited on January 21, 1997 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A., under the provisions of the Budapest Treaty For The International Recognition Of The Deposit Of Microorganisms For The Purpose Of Patent Procedure.
Bacterium strain SH2A has been accorded ATCC Accession Number 55926.
Bacterium strain, Bacillus midousuji SH2B was deposited on October 24, 1997 with the ATCC under the provision of the Budapest Treaty. Bacterium strain SH2B has been accorded ATCC Accession Number 202050.
Strains SH2A and SH2B were isolated from a sample of compost collected in Osaka, Japan.

Results and Methods

1 The heat –resistance of the cultured cells was determined in trypticase soy broth. After overnight incubation at 62C, and more higher temperature up to 95C, means both SH2A and SH2B strains are thermophilic extremophiles and require at least 62C to grow.
Strain SH2A is sensitive to both kanamycin at concentrations of 50ug/ml and to ampicillin at concentration s of 50ug/ml. Strain SH2B is resistant to kanamycin at concentrations of 50ug/ml, but sensitive to ampicillin at concentrations of 50 ug/ml.

2 16S rRNA sequence studies

Purification of genomic DNA, and amplification and purification of the 16S rRNA gene segment from isolate SH2A and SH2B were performed using commercially available kits (Gene Amp kit, Geneclean spin). The purified PCR product was directly sequenced. Sequencing was performed on an ABI automated DNA sequencer using a Prism dideoxy
Terminating cycle sequence kit as recommended by the manufacturer (Applied Biosystem, LTD).

The primers used for amplification and sequencing are 16SRR I: cag cag ccg cgg taa tac and 16SRR8: gat tag ata ccc tgg ta. The resulting DNA sequence for the 16S rRNA gene segment was aligned with 16S rRNA sequences obtained from the Ribosomal Database Project and Genebank, and compared. The resulting DNA sequence for the 16S rRNA gene sequences of these two strains were unique and identical compared to known bacteria.

3 Biodegradation of Dioxin-like by B. midousuji SH2B

One mL of Tipticase Soy Broth (BBL) containig 1ppm of nonchloro dibenzo furan (Wako Pure Chemiccal Inc.) and 2,3-dichloro dibenzo-p-dioxin, 2,8-dichloro dibenzo-p-dioxin, 2,3,7-trichloro dibenzo-p-dioxin, and 2,3,7,8-tetrachloro dibenzo-p-dioxin (AccuStandard Inc.) were incubated respectively with 10 to the 6th bacterial cell suspension of SH2B strain in sealed glass tube and shaken 25 strokes/ minute at 65C for 22hours.

Assay was done by GC-MSD HP6890 with SIM system in Towa Kagaku Co., Ltd.

4 Method for analyzing Dioxin-like substances

Analysis Method using Quadrupole

Analysis using quadrupole was applied to measurement analysis where specific isomers like dioxin, dibenzofuran, mono-tetra chlorodioxin in high concentration, were added, and has undergone degradation tests.

Substrates after the tests were shipped test tubes sealed with screw caps. Volume of all substrates were around 1ml. In order to determine volumes of all substances to be analyzed that are contained the substrates, volume of substrates shipped were not measured.

To achieve levels of 20m mo-H+ or greater, chloric acid was added to the substrates, and was left for one hour, shaken occasionally. The purpose of chloric acid treatment was to separate substances to be analyzed from bacteria that are either adhering or surrounding the substances as much as possible.

Distilled water was added to substrates treated with chloric acid, and was prepared to volume of

around 4ml. To this solution, 37Cl4-2,3,7,8-TCDD was added as clean-up spike. Total added volume was 10ng. From 37Cl4-2,3,7,8-TCDD solution that was prepared to 1ng/ul, 10ul was extracted using a micro syringe, and was added to all test solutions.

Five hundreds ul of Toluene was added to test solutions with clean-up spike added. After they were sealed, test solutions were shaken for 10 minutes, then extracted. After extraction, toluene layers were separated by pipettes, and were placed in 3ml mini-vials. Volume was adjusted by either concentration (in case to low concentration) or dilution (in case of high concentration) according to the concentration of the substances to be analyzed.

2ul of extracted solution was injected into the quadrupole, then measured, with 37Cl4-2,3,7,8-TCDD as internal standard.

The results of biodegradation rate of these compounds by SH2B strain were as follows:

nonchloro dibenzo furan: 1213.3pg/uL to 576.9pg/uL (52.5% degradation)

2,3-dichloro dibenzo-p-dioxin: 494.8 to 22.5 (95.4%)

2,8-dichloro dibenzo-p-dioxin: 105.8 to 13.2 (87.5%)

2,3,7-trichloro dibenzo-p-dioxin: 216.0 to 191.0 (11.6%)

2,3,7,8-tetrachloro dibenzo-p-dioxin: 4013.4 to 3990.8 (0.6%)

5 Searching for DNA sequence of key enzyme in the degradation pathway of Dioxin-like substances

Purification of plasmid pSA101 in SH2A strain(HA1002) and pSB301 in SH2B strain(HB1030) was performed by Qiagen plasmid purification protocol (Qiagen, Velencia CA), and the pulse field gel electrophoresis was applied to these plasmid identification.

Three plasmids were obtained from both strains and indicated the DNA size of 18k bases, 45k bases, and 400k bases respectively. Restriction fragment length polymorphism of these plasmids suggested these plasmids had different sequences in each other.

The partial sequencing of this 18k bases plasmid indicated 80% homology to the plasmd pTB19 of

Bacillus stearothermophilus strain.

Discussions

Important recent developments in genetic engineering have extended the scope and potential of industrial fermentation technology. By using genetic engineering, one may construct new combination of inheritable material by inserting the foreign genes into bacteria and other suitable organisms. One of the workhorses of the fermentation industry, Bacillus, may be a suitable bacterial host for such genetic engineering experiments.
Thermophilic Bacillus producing high-temperature-stable enzymes, which have the ability to degrade substrates up to 100C, have not only streamlined substrate-hydrolysis processes but have also created an impetus to isolate or construct other enzymes with highly stable properties. Bacteria can express proteins at high levels. Gram-negative bacteria are general not good extracellular protein secretors; gram-positive bacteria are significantly better at secreting extracellular proteins. Therefore, thermophilic Bacillus, such as SH2A and SH2B, may be a suitable producer of industrial enzymes.

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