[Regular Paper]

Degradation of Carbazole by *Novosphingobium* sp. Strain NIY3

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Bacteria were screened to identify strains that could degrade carbazole, a model compound for the N-containing compounds present in fossil fuels which could be removed by biodenitrogenation instead of hydrotreatment. Enrichment cultures from 6 different habitats were examined with carbazole as a source of nitrogen. Bacterium strain NIY3 was isolated, which efficiently metabolized very low concentrations of carbazole. Strain NIY3 is a bright yellow Gram-negative immotile bacterium with a rod shape (0.7×0.7-1.0 μm). DNA base sequence analysis revealed that strain NIY3 belongs to genus *Novosphingobium* sp., most closely related to *Novosphingobium subarcticum* (98.3% similarity). *Novosphingobium* sp. strain NIY3 could degrade 95% of 100 ppm of carbazole in a culture within 3 days.

Keywords
Carbazole, Biodegradation, Denitrogenation, Fossil fuel

1. Introduction

The efficiency of hydrodesulfurization (HDS) processes is limited by the poisoning of the catalysts caused by nitrogen-containing organic compounds present in the feedstocks, typically at levels of a few hundred ppm. Therefore, such compounds must be removed prior to HDS, which is particularly difficult by conventional catalytic methods such as hydrodenitrogenation (HDN). In comparison, bio-processes have many advantages, including high substrate specificity; low energy consumption (processing at ambient temperature and atmospheric pressure); no hydrogen consumption; and no requirement for heavy and costly infrastructures. Bacteria that can degrade carbazole, which is representative of the refractory N-containing compounds present in the fossil fuels, have already been isolated[1)~12)]. As much as 46% of carbazole can be converted after 60 days using *Mycobacterium*[1)], but most of the active bacteria are members of *Pseudomonas*[2),4),5),8),11),12)] or *Sphingomonas*[3),6),9)10)]. *Sphingomonas* CDH-7 degraded 100% of carbazole within 50 h (original level of 2.99 mM, 1 M = 1 mol·dm^{-3}) and the degradation rate was 10 μmol·min^{-1}·g^{-1} [6)]. *Sphingomonas* GTIN11 degraded 82% of carbazole within 8 h (original level of 1.70 mM) and almost 100% of carbazole within 1 h (original level of 120 μM)[10)]. This bacteria degraded 95% of carbazole in petroleum within 16 h and the degradation rate was 8 μmol·min^{-1}·g^{-1}. *Pseudomonas* achieved somewhat lower degradation rates[11),12)].

The present study specifically searched for new microorganisms capable of degrading carbazole at low concentrations. A promising strain, *Novosphingobium* sp. strain NIY3 was isolated and subsequently characterized. Preliminary results were reported in the proceedings of meetings[13),14)].

2. Materials and Methods

2. 1. Media

Inorganic salts (IS) medium is a nitrogen-free synthetic medium containing 0.44 g of $MgCl_2 \cdot 6H_2O$, 0.50 m*l* of 10% solution of $CaCl_2 \cdot 2H_2O$, 0.20 g of Na_2SO_4, 23.60 g of KH_2PO_4, 11.46 g of $Na_2HPO_4 \cdot 12H_2O$, 0.20 g of yeast extract, and 1.00 m*l* of trace elements solution in 1 *l* of distilled water. The trace elements solution is prepared by dissolving 1.00 g of H_3BO_3, 0.24 g of $CuSO_4 \cdot 5H_2O$, 0.50 g of KI, 2.00 g of $FeCl_2 \cdot 6H_2O$, 1.0 g of $MnCl_2$, 0.40 g of $Na_2MoO_4 \cdot 2H_2O$, 0.475 g of $ZnCl_2$ and 0.20 g of $CoCl_2 \cdot 6H_2O$ in 1 *l* of distilled water. LB medium used for observation of

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the bacteria colony and for the maintenance of cultures consisted of 10.0 g of polypeptone, 5.0 g of yeast extract, 10.0 g of NaCl and 20.0 g of agar in 1 *l* of distilled water.

2. 2. Isolation of Carbazole Degrading Bacteria

Several strains of bacteria with the potential to use carbazole as a source of nitrogen were isolated from 6 habitats (soil samples) by enrichment culture in L-shaped test tubes (external diameter: 18 mm; length of the basal part: 120 mm; height: 100 mm) containing a 10 m*l* solution of IS medium supplemented with 300 ppm of carbazole and 16.7 vol% of succinic acid by shaking at 30℃ and 120 rpm. After sufficient bacteria growth, 1/10 of the liquid culture was transferred to the same fresh medium. This operation was repeated three times. Single colony isolation was then repeated on IS medium containing 1.5 wt% of agar and 2 wt% of glucose. Isolates were maintained on slants of IS medium supplemented with 300 ppm of carbazole and 2 wt% of glucose or LB medium.

2. 3. Growth Measurement and Taxonomy

Growth of bacteria in the L-tubes was observed by measuring the OD_{610} using a colorimeter (ICM-21100, Asahi Kagaku). Bacteria motility was checked after methylene blue staining by direct observation under a microscope with a magnification of 400-1000. Gram staining was confirmed by comparison with Gram-positive *Rhodococcus* BIM (obtained from Tamagawa University).

Full analysis of the 16S rDNA gene sequence (1500 bp) was performed by NCIMB Japan Co., Ltd. Genomic DNA, which was extracted using an InstaGene Matrix kit (BIO RAD, CA, USA), was used as a template and PCR-amplified using Ready-To-Go PCR Beads (Amersham Pharmacia Biotech, NJ, USA) and primers 9F and 1510R. An ABI Prism BigDye Terminator v3.1 Kit (Applied Biosystems, CA, USA) was used for cycle sequencing with 8 sequencing primers with a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems, CA, USA). The DNA sequencer was an ABI PRISM 3100 DNA Sequencer (Applied Biosystems, CA, USA). The DNA fragments were then assembled with AutoAssembler 2.1 software (Applied Biosystems, CA, USA). Bacteria were then identified by homology search (from the decoded base sequence of 16S rDNA) using the Basic Local Alignment Search Tool (BLAST)[15] from the National Center for Biotechnology Information (NCBI) and other international nucleic acid databases (GenBank, DDBJ, EMBL).

2. 4. Carbazole Degradation

Culture media contained 50, 100, 200 or 300 ppm of carbazole in 100 m*l* of IS medium with 0.6 vol% of acetone and 0.4 vol% of ethanol to help complete dissolution of the carbazole crystals. Aliquots (10 m*l*) of culture medium were loaded into L-tubes, which were then plugged with silicon foam rubber stoppers and autoclaved. During this step, the acetone and ethanol evaporated. After cooling, 0.2 m*l* of cell suspension was inoculated into each L-tube. Cultures in these L-tubes were incubated at 30℃ and 120 rpm for 1 to 7 days. The cell suspensions used for inoculations were prepared by addition of 5 m*l* of NaCl solution at 0.85% to the cells grown on the respective LB medium slant cultures.

2. 5. Chemicals

Carbazole (special grade, purity higher than 99%) was obtained from Tokyo Chemical Industry, Co., Ltd., Japan and was used without further purification. The other chemicals (Kishida Chemical Co., Ltd.; Wako Pure Chemical Industries, Ltd.; MERCK; Kanto Kagaku Chemicals Inc.; SIGMA; Kokusan Chemical Co., Ltd.; Nihon Pharmaceutical Co., Ltd.) were first or special grade compounds.

2. 6. Extraction of Carbazole and Metabolites

Carbazole and metabolites were recovered by ether extraction of the culture. After incubation, bacteria growth was stopped by adding 5 drops of 6N HCl to adjust the pH to about 1 in the L-tube, which was then stored in a refrigerator until extraction. The culture liquid was centrifuged at 3000 rpm for 15 min to separate the cells from the culture liquid. Ether extractions of the pellet (cells) and the aqueous phase (culture liquid) were performed with 3 m*l* of ether. The interfacial phase formed between the aqueous phase and the ether phase was transferred into a separate vessel and then extracted with 3 m*l* of ether. Ether extraction was performed 3 times on each phase. Ether was evaporated and all the extracts from one L-tube were combined and redissolved in 1 m*l* of ethanol.

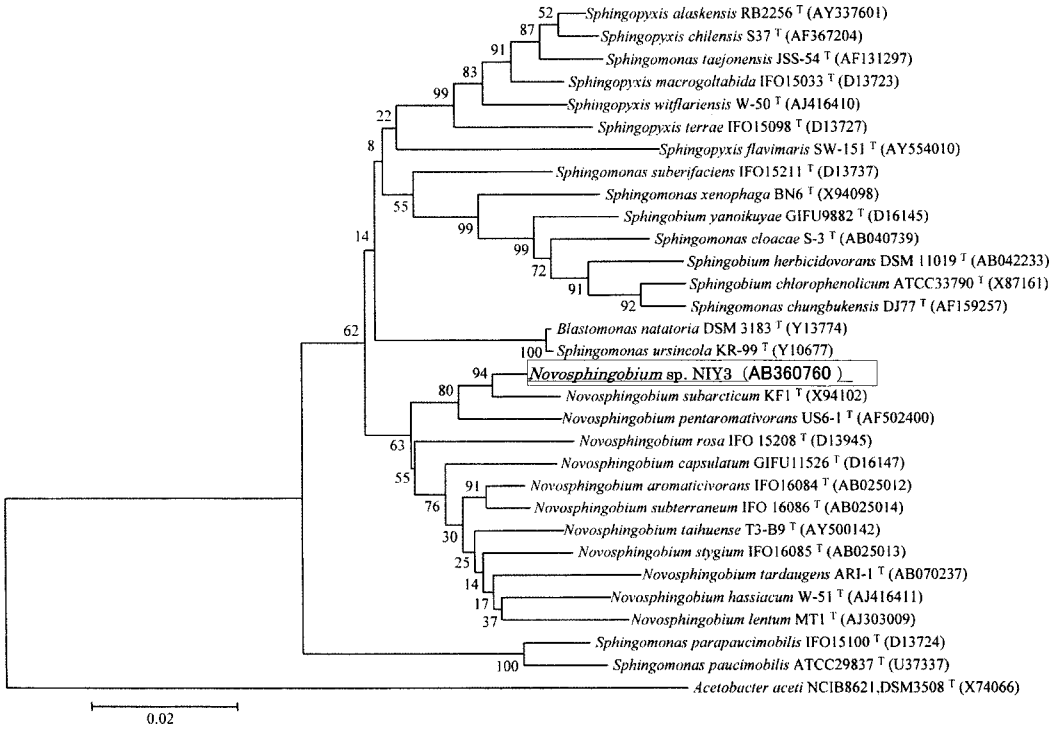
2. 7. Analytical Methods

Concentrations of carbazole and metabolites in the samples were determined by a TN-100 (Mitsubishi Chemical Corp.) to measure the total nitrogen content in the samples; a gas chromatograph equipped with a flame thermoionic detector (GC-FTD; GC-2010, Shimadzu Science) to detect and quantify the N-containing products; and a gas chromatograph in line with a mass spectrometer (GC-MS; QP5050, Shimadzu Science) to identify the metabolites.

3. Results and Discussion

3. 1. Taxonomic Characteristics of Isolate NIY3

Several bacteria that can degrade carbazole were isolated by enrichment culture using IS medium supplemented with 300 ppm carbazole and 16.7 vol% of succinic acid. Subsequently, their activity for carbazole degradation was tested without succinic acid. Among the carbazole utilizing isolates, isolate strain NIY3 exhibited the highest carbazole degradation activity, and so was selected and used throughout this study. Strain NIY3



Bootstrap values are indicated near nodes; the scale bar corresponds to 0.02 change per nucleotide; a 'T' after the name of a strain indicates a type strain.

Fig. 1 Phylogenetic Tree of the 16S rDNA Sequence from Strain NIY3 and Reference Strains (accession numbers in parenthesis)

is a Gram-negative, immotile aerobic bacterium, which forms rod cells with dimensions of 0.7×0.7-1.0 μm. Colonies of strain NIY3 grown on LB nutrient agar medium are round, regular, entire, smooth, slightly mucoid, bright yellow, opaque and convex. 16S rDNA[16] analysis indicated that strain NIY3 exhibits high homology of the 16S rDNA sequence with *Novosphingobium* sp. and especially with *Novosphingobium subarcticum* (98.3%). **Figure 1** shows the phylogenetic tree of the 16S rDNA sequences from *Novosphingobium* sp. strain NIY3 and reference strains, and *Novosphingobium* sp. strain NIY3 is considered to be closely related to *Novosphingobium subarcticum*. Note that the genus *Sphingonomas* first proposed in 1990[17] was divided into four different genera in 2001, *Novosphingobium*, *Sphingobium*, *Sphingomonas* and *Sphingopyxis*[18], but again reassigned to the genus *Sphingomonas* in 2002[19]. Therefore, the name '*Novosphingobium* sp. strain NIY3' might further change to '*Sphingomonas* sp. strain NIY3.'

3. 2. Carbazole Utilization by *Novosphingobium* sp. Strain NIY3

To confirm that *Novosphingobium* sp. strain NIY3 can effectively metabolize carbazole, a degradation experiment with 1 wt% of carbazole powder was performed without using ethanol–acetone for carbazole dissolution, or yeast extract. Microscopic observation revealed that large numbers of *Novosphingobium* sp. strain NIY3 cells assembled on the surfaces of carbazole particles.

Figure 2 shows the growth of *Novosphingobium* sp. strain NIY3. In the culture without carbazole (0 ppm), OD_{610} did not significantly vary throughout incubation period. In the cultures with 100 ppm and 300 ppm of carbazole, OD_{610} increased on the first incubation day and then decreased for longer incubation periods. In the culture with 50 ppm of carbazole, OD_{610} did not change on the first day of incubation and then decreased for longer incubation periods.

The OD variation during the early stage of the reaction was attributed to the combination of two main factors. First, increase in the number of cells was obviously responsible for the increase in OD. Second, insoluble carbazole particles also contributed to the value of OD_{610}. We observed undissolved whitish particles of carbazole surrounded with cells, which were suspended with bacteria in the culture after shaking the solutions prior to OD measurements. For this reason, OD_{610} at initial time ($t=0$) increased as the carbazole concentration increased. Therefore, apparent OD_{610} was the combination of the OD_{610} due to cells and that due to carbazole particles. On the first day of incubation, OD_{610} increased proportionally to the initial carbazole concentration and then decreased. The OD_{610} decrease observed for any carbazole concentration does

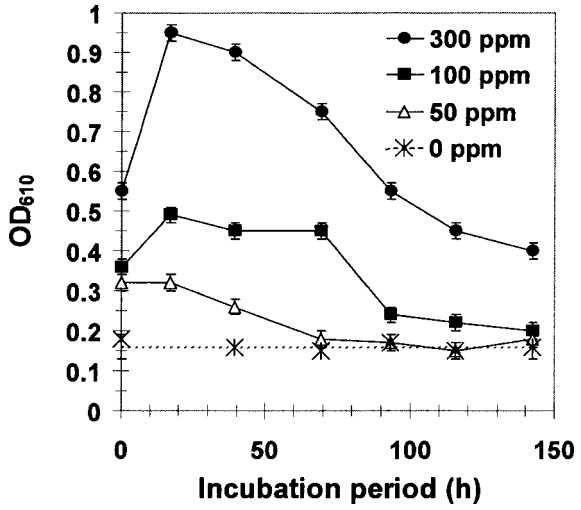


Fig. 2 Growth of *Novosphingobium* sp. Strain NIY3 in Cultures Containing Different Concentrations of Carbazole

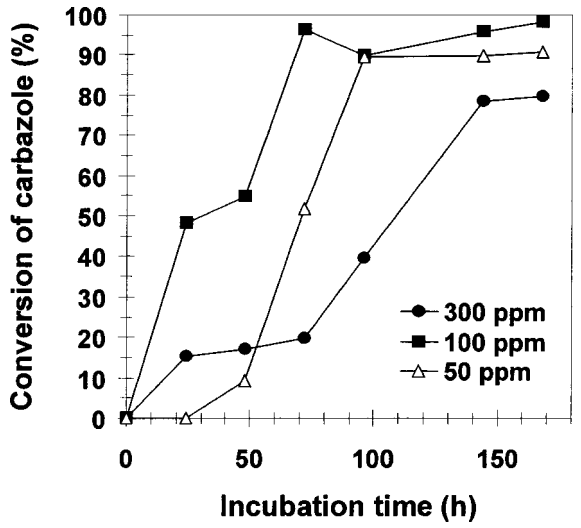


Fig. 3 Carbazole Denitrogenation by *Novosphingobium* sp. Strain NIY3 Determined from TN-100 Results for Three Carbazole Concentrations in IS Medium

not mean standstill of bacteria growth, because the number of cells was still observed microscopically to increase. The apparent OD_{610} possibly decreased due to lowering of the cell refractive index induced by production of some mucoid substance in the culture liquid.

3. 3. Carbazole Degradation and Metabolites

Figure 3 shows the denitrogenation conversion of carbazole by *Novosphingobium* sp. strain NIY3 for three initial carbazole concentrations (50, 100 and 300 ppm). The conversion of carbazole was determined using TN-100, so conversion (%) corresponds to denitrogenation rate (%). Carbazole conversion increased with incubation time for any initial carbazole concentration. Conversion for 300 ppm of carbazole was lower than that for 100 ppm of carbazole, due to

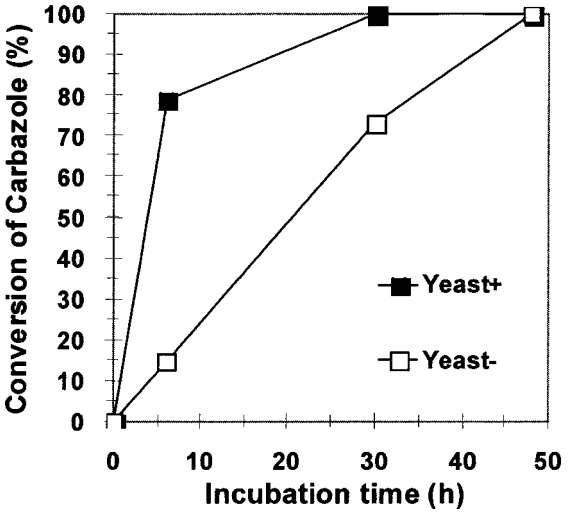


Fig. 4 Comparison between Experiments with and without Yeast Extract (carbazole concentration 100 ppm)

the higher quantity of substrate to be metabolized with an equivalent initial cell concentration. In contrast, conversion for 50 ppm of carbazole was significantly lower than that for 100 ppm or 300 ppm of carbazole for incubation times shorter than *ca.* 2 days. However, for longer incubation times, conversion for 50 ppm of carbazole became higher than that for 300 ppm of carbazole, and was close to that for 100 ppm of carbazole after *ca.* 4 days. An initial concentration of carbazole of 50 ppm was presumably too low to provide adequate nutrient availability (longer lag phase).

For the carbazole degradation experiments, 50-300 ppm of carbazole was added as a nitrogen source to IS medium. However, IS medium includes 200 mg/*l* of yeast extract, which might act as a nitrogen source. Therefore, we performed the experiments with and without yeast extract as shown in **Fig. 4**. The initial concentration of carbazole was 100 ppm (0.60 mM) and growing cells were used. The cell concentration was higher than that of preceding experiments using resting cells (**Fig. 3**). The initial OD_{610} was about 1.2 and the cell concentration was about 0.77 g dry cell/*l*. In these experiments, 75-80% of carbazole was degraded within 6 h with yeast extract and within 30 h without yeast extract. Large amounts of growing cells were used in these experiments, so that the degradation of carbazole proceeded rapidly even without yeast extract although no increase in OD was observed. This finding indicates that carbazole was degraded by enzymes in the growing cells added initially. The initial rate of carbazole degradation corresponds to 1.7 $\mu mol \cdot min^{-1}$ (g dry cell)$^{-1}$. This value is a comparable rate to the degradation by *Sphingomonas* CDH-7 of 100% of carbazole (2.99 mM) within 50 h with degradation rate of 10 $\mu mol \cdot min^{-1} \cdot g^{-1}$ [6].

To confirm whether NIY3 can utilize carbazole as a

sole source of nitrogen, carbon or energy, separate experiments using agar plate culture were performed in the presence and absence of yeast extract and glucose as carbon and nitrogen sources other than carbazole. Growth of NIY3 was rapid in the presence of yeast extract, indicating that NIY3 can grow better in the presence of carbon and nitrogen sources such as yeast extract.

GC-FTD chromatograms of samples from degradation experiments incubated with *Novosphingobium* sp. strain NIY3 showed new peaks attributable to metabolites after incubation, which could not be reliably identified by GC-MS analysis due to the low concentrations. Ion chromatography was used to investigate the final ionic products, but ions such as NH_4^+ and NO_3^- could not be detected, possibly due to the low concentrations.

One proposed mechanism for the metabolization pathway of carbazole with *Pseudomonas* (note that *Pseudomonas* and *Novosphingobium* are proposed to be synonymic[19]) involves the oxidation of carbazole *via* the formation of some intermediates possessing NH_2 groups that are further denitrogenated[2]. Compounds such as indole-3-acetic acid and 2-(2,3-dihydro-4-oxo-1H-quinoline-2-yl) acetic acid methyl ester (AAME) may be formed by *Pseudomonas* LD2[4]. More recently, investigation of the bacterium strain CP19 identified not only indole-3-acetic acid and AAME[4], and 2'-aminobiphenyl-2,3-diol[2], but also indirectly identified the formation of 2-aminophenol as the condensation product 2-amino-phenoxazin-3-one[20]. The N-containing metabolites detected in our study might correspond to molecules such as 2'-aminobiphenyl-2,3-diol, 2-hydroxy-6-oxo-(2'-aminophenyl)hexa-4-enoic acid, AAME or anthranilic acid.

The present study detected almost no N-containing intermediate metabolites, suggesting that the metabolism of carbazole with *Novosphingobium* sp. strain NIY3 was too rapid to enable accumulation of intermediates in the culture. *Sphingomonas subarcticum*, which is closely related to *Novosphingobium* sp. strain NIY3, can degrade polychlorophenol, and *Sphingomonas paucimobilis* can degrade polycyclic aromatic hydrocarbons[21]. Clearly, *Sphingomonas* strains exhibit certain functions for degrading polycyclic aromatic compounds, which might be also the case for the synonymic *Novosphingobium* sp. strain NIY3.

Some carbazole degrading bacteria strains were previously reported[1)~12)]. Many of these strains belong to two genera, *Pseudomonas*[2),4),5),8),11),12)] or *Sphingomonas*[3),6),9),10)], in good agreement with the present study, as *Sphingomonas* and *Novosphingobium* may be synonymous[19]. However, the culture conditions varied, so the studies cannot be directly compared. *Sphingomonas* GTIN11 pre-grown on nutrient-rich media metabolized carbazole[10], whereas *Pseudomonas resinovorans* CA10, which can grow on carbazole as a sole source of carbon, nitrogen and energy[2], did not metabolize carbazole after pre-growth on the same nutrient-rich media[10].

Sphingomonas GTIN11, which is also used for degrading carbazole at low concentrations[10], exhibited interesting degradation rates compared to those obtained with *Novosphingobium* sp. strain NIY3 in the present study. Nevertheless, no indication was given on the quantities of cells used, so direct comparison is difficult. However, if large quantities of cells were inoculated, *Novosphingobium* sp. strain NIY3 as well as *Sphingomonas* GTIN11[10] should degrade low concentrations of carbazole rapidly and completely.

4. Conclusion

A carbazole degrading bacterium, strain NIY3, isolated by an enrichment culture technique, could efficiently metabolize very low concentrations of carbazole in the presence of yeast extract. DNA base sequence analysis revealed that strain NIY3 belongs to genus *Novosphingobium* sp., closely related to *Novosphingobium subarcticum* (98.3% similarity). About 0.77 g/*l* (dry weight) of *Novosphingobium* sp. strain NIY3 degraded 75-80% of carbazole (100 ppm, 0.6 mM) within 6 h in the presence of yeast extract. The initial rate of carbazole degradation corresponds to 1.7 $\mu mol \cdot min^{-1}$ (g dry cell)$^{-1}$, which is comparable to that achieved by *Sphingomonas* CDH-7, which degraded carbazole 2.99 mM at 10 $\mu mol \cdot min^{-1} \cdot g^{-1}$ [6].

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Novosphingobium sp. NIY3株を用いたカルバゾールの分解反応

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本研究では，我々は水素化処理反応ではなく，バイオ脱窒素反応を検討した。すなわち，化石燃料の超深度脱窒素を行うことを目的として，カルバゾールを分解する微生物の探索を行った。六つの異なる土壌から採取した土を用いて，カルバゾールを窒素源として集積培養を行った。その中の一つの土壌から低濃度のカルバゾールを分解することができるNIY3株を単離することができた。

NIY3株はコロニーが淡黄色，非運動性で細胞の大きさが約0.7×0.7～1.0 μmのグラム陰性桿菌である。16S rRNA塩基配列解析および相同性検索を行った結果，NIY3株は*Novosphingobium*属に属し，*Novosphingobium subarcticum*と最も高い相同性（98.3%）を示した。*Novosphingobium* sp. NIY3株は100 ppmのカルバゾールを3日間で95% 分解した。

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