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ISOLATION OF SALT AND NITRATE-TOLERANT BIPHENYL-DEGRADING BACTERIA AND THEIR BIPHENYL DEGRADATION PATHWAY

IZOLACJA BAKTERII ODPORNYCH NA ZASOLENIE I AZOTANY DEGRADUJĄCYCH BIFENYLE ORAZ ŚCIEŻKA DESTRUKCJI BIFENYLI TYMI BAKTERIAMI

Cztery szczepy bakterii degradujących bifenyle, ze zdolnością degradowania bifenyli przy obecności wysokich stężeń NaCl (3,0% w v^{-1}) lub KNO_3 (500 mg/l), zostały wyizolowane ze ścieków. Trzy szczepy (SK-1, SK-3 i SK-4) i zostały zidentyfikowane jako typ Rhodococcus, a jeden szczep (SK-2) przypisano do typu Aquamicrobium przy pomocy sekwencjonowania genów 16S rRNA. Wyizolowane szczepy mogą degradować szeroki zakres związków aromatycznych, w tym kongenery polichlorowanych bifenyli o wysokiej zawartości chloru (PCB) (zawierające cztery do sześciu podstawionych atomów chloru) w obecności bifenyli jako czynnika stymulującego wzrost, niezależnie od obecności NaCl. Poniższy artykuł sugeruje, że wyizolowane szczepy mogą być dobrymi kandydatami do bioremediacji gleby skażonej PCB, zwłaszcza ziem zasolonych.

1. Introduction

Biphenyl and polychlorinated biphenyl (PCBs), a synthetic biphenyl, are persistent organic pollutants in the environment and have become one of the most serious environmental problems around the world (Araki et al., 2011; Pieper, 2005). In Japan, the manufacture, import, and use of PCBs was prohibited in 1974. However, environmental pollution with PCBs is still ubiquitous in Japan (Takashi et al., 2008). PCB pollution has spread beyond common soil or rivers to coastal and offshore marine environments. PCBs can enter the marine environment through a number of routes, including direct deposition from the atmosphere, urban runoff and industrial discharge (Gevao et al., 2006). Fortunately, an increase in PCB pollution damage was not reported after the 2011

Tohoku earthquake and tsunami in Japan, as many condensers (about 40) and transmissions (about 157) under storage which contained PCBs suffered leakage (Hiroki, 2012). Thus, we need to be aware that the risk of PCB spills still exists. To understand local pollution levels and the spatial distribution of PCBs, The Ministry of the Environment in Japan has continuously analyzed PCBs in the sediment of water sources throughout Japan (Takashi et al., 2008). The survey results indicate that sediment contamination of rivers or the sea by PCBs is a very serious problem in Japan.

Estuaries and marine sediments are the ultimate global sinks for the worldwide accumulation of PCBs (Berkaw et al., 1996). Sediment is the main reservoir and a potential secondary source of various hydrophobic organic pollutants, especially some persistent organic pollutants (Liu et al., 2008). Once in the aquatic environment, PCBs, by virtue of their low-water solubility, are removed from the water column and associate with particulate matter. This association subsequently leads to accumulation in sediment, which may become a secondary contamination source.

One vigorous method to remediate PCB contaminated sediments is to dredge it from the contaminated site and treat it with proper remediation methods. However, the remediation of PCB contaminated sediments poses many technological and logistical problems. Furthermore, the treatment of heavily contaminated PCB sediment can be costly as there is the need to lease construction facilities.

Bioremediation by soil bacteria has been investigated extensively during the last few decades as an alternative and less expensive strategy for the degradation of PCB contamination (Furukawa and Matsumura, 1976). Bioremediation technology has countless advantages and is a highly attractive alternative for dealing with PCBs due to the high costs of transportation, incineration and other procedures of remediation that currently exist.

To date, several microorganisms belonging to the genera *Achromobacter*, *Burkholderia*, *Pseudomonas*, *Sphingomonas*, *Rhodococcus*, *Comamonas*, *Norcardia*, *Arthrobacter*, *Acinetobacter*, *Vibrio*, *Aeromonas*, *Micrococcus*, *Bacillus*, *Enterobacter*, *Streptomyces*, *Paenibacillus* and *Corynebacterium* have been isolated that can aerobically degrade PCBs (Hatamian-Zarmi et al., 2009; Sakai et al., 2005). One similarity among PCB-degrading microorganisms is that they utilize biphenyl and metabolize PCBs with the same enzymes employed in biphenyl catabolism (Abramowicz, 1990).

High salt concentrations (greater than 1%) may cause a decrease in endogenous respiration of cells and/or plasmolysis (Kargi and Dincer, 1999). Thus, it is difficult to treat marine sediments or high saline soils using biological methods. Therefore, it is important to isolate bacteria that can degrade PCBs in the presence of high concentrations of NaCl, which could prevent costly dilution to lower the salinity before biological treatment. To date, only a few strains capable of degrading PCBs in the presence of NaCl have been reported (Abdughafurovich et al., 2010; Jaysankar et al., 2006). However, the maximum NaCl concentration used in those studies was less than 1.8% (w v^{-1}). In addition, recent studies have suggested that a high concentration of nitrate can also inhibit the proliferation of cells in contaminated sites (Davidova et al., 2001; Russo, 1985)

The aims of the present study were to investigate the degradation capability of biphenyl and PCBs by isolated bacterial strains (*Rhodococcus* sp. SK-1, SK-3, and SK-4), including the first identified *Aquamicrobium* genus (strain SK-2) able to degrade PCBs. In addition, we wanted to identify the metabolic intermediates of biphenyl when those substrates were used as the sole carbon source, and describe the effects of high concentrations of NaCl and nitrate on biphenyl and PCB degradation.

2. Methods

2.1. Isolation of biphenyl-degrading bacteria

Biphenyl as the single carbon source was added (0.32 mM, final concentration) to the mineral salt culture medium. The mineral salts medium (MSM, pH 7.0) contained 1.0 g $(NH_4)_2SO_4$, 1.0 g K_2HPO_4, 0.2 g NaH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.05 g NaCl, 0.05 g $CaCl_2$, 8.3 mg $FeCl_3 \cdot 6H_2O$, 1.4 mg $MnCl_2 \cdot 4H_2O$, 1.17 mg $Na_2MoO_4 \cdot 2H_2O$ and 1 mg $ZnCl_2$ per liter of deionized water. To enrich biphenyl-degrading bacteria, wet activated sludge (5 g) was collected aseptically from a sewage treatment plant located in Seoul, Korea and was used as a source of inoculum for enrichment cultures by adding it to MS liquid medium. The MSM culture was cultured at 28 °C with shaking (120 rpm). The activated sludge contained a high concentration of NaCl (0.5–1.0%, w v^{-1}) because a major component of sewage from the plant originated from kitchen waste such as Kimchi, with a high NaCl content (Kim et al., 1995).

To enrich the culture to be capable of degrading a high concentration of biphenyl, after activation of the sludge (mixed liquor suspended solids (MLSS): 4500 mg L^{-1}), the sludge (wet weight, 5 g) was inoculated aerobically in fresh MS liquid medium containing 0.32 mM biphenyl, and cultured at 28 °C with shaking. The culture was further subcultured in fresh MS medium containing 0.65 mM biphenyl, and cultured at 28 °C with shaking. The enrichment cultures were maintained with a weekly subculture using the medium described above for two months. After approximately ten enrichment cultures, the biphenyl-degrading bacteria were successfully isolated using the traditional serial dilution method.

2.2. Growth and degradation experiments on biphenyl

The degrading ability and growth of the isolated strains on biphenyl were investigated. In liquid culture, 20 mL of medium was used in 50 mL serum bottles. Cells of the isolated strain were cultivated aerobically in MS-biphenyl (0.65 mM) for 48 h, then harvested by centrifugation (8000 × g, 5 min, 4 °C) and washed twice with 50 mM potassium phosphate buffer (pH 7.5). The cell suspension (200 μL) was inoculated in the medium to give an optical density of 0.02 at 600 nm (OD600). For aerobic cultivation, the cells were cultivated by rotary shaking. The cultures were incubated in the dark at 28 °C, and periodically sacrificed, at which time the cell density was determined.

For high performance liquid chromatography (HPLC) analysis, the samples were acidified with phosphoric acid (10%, w v^{-1}) to stop the biological reaction, shaken for 3 min with an equal volume of 1:1 (v v^{-1}) ethyl acetate and centrifuged at 8000 × g for 10 min. The organic layer was then analyzed directly by HPLC. All experiments were performed in triplicate.

2.3. Effect of NaCl and KNO_3 on biphenyl degradation

All isolated strains were grown to the mid-log phase on 0.65 mM biphenyl, and 200 μL of cell suspension was inoculated into the fresh MSM medium to give an optical density of 0.02 at OD600. NaCl was added at final concentrations of 0, 1.0, 2.5, 3.5% (v w^{-1}). For other experiments, potassium nitrate was added at 10, 50, 100, 200, or 500 mg L^{-1}. The cultures were incubated in the dark at 28 °C and 120 rpm. Controls consisted of autoclaved cells.

2.4. Degradation of other aromatic compounds

Each isolated strain was grown in biphenyl-MSM (0.65 mM) for 48 h. Cells were harvested by centrifugation (8000 × g, 4 °C, 10 min), washed twice with 50 mM potassium phosphate buffer (pH 7.5), and inoculated at an OD600 in 2.0 to 50 mM potassium phosphate buffer (pH 7.5) supplemented with the following aromatic compounds at 0.5 mM, as the sole carbon source: benzoic acid, catechol, phenol, toluene, 4-chlorophenol, aniline, nitrobenzene, naphthalene, diphenylamine, and 1,3,5-triphenylbenzene. Cultivation was performed at 28 °C and 120 rpm.

2.5. Co-metabolic degradation of PCB congeners

SK-1 or SK-2 were cultivated aerobically in MSM and biphenyl (1.95 mM) for 48 h, then harvested by centrifugation (8000 × g, 5 min, 4 °C) and washed twice with 50 mM potassium phosphate buffer (pH 7.5). The cell suspension (200 μL) was transferred to a 50 mL vial containing 10 mL fresh MS medium to give an optical density of 0.02 at OD600 and 0.65 mM of biphenyl and 0.6 mg L^{-1} Congener calibration set (C-CSQ-Set, AccuStandard, Inc., USA) were added. The headspace of each vial was aseptically flushed with oxygen, sealed with a Teflon-lined cap, and then cultivated by rotary shaking at 28 °C. Background PCB-degradation activity was measured by using control cells which were inactivated by autoclaving at 121 °C for 15 min. After 3 days incubation, an internal standard (^{13}C labeled PCB congeners "cleanup spike") was injected to the culture, and the culture was centrifuged (8000 × g, 5 min, 4 °C). The supernatant was then extracted with 15 mL of n-hexane three times (final volume: 45 mL). To recover PCB congeners absorbed in the cells and to dissolve the cells, 50 mL of 1N KOH/ethanol solution was added to the debris and kept static at room temperature for 16 h. Then the aqueous solution was extracted with 15 mL of n-hexane three times (final volume: 45 mL), and pooled with the supernatant extracts, and the extracts were washed with 45 mL of concentrated sulfuric acid. This process was repeated until the color of the sulfuric acid layer turned pale. The n-hexane layer was washed with n-hexane extracted water until the pH reached neutrality. The washed hexane layer was purified with 4 Layer Cleanup Cartridge (GL Sciences, USA), dried over anhydrous sodium sulfate and concentrated by an evaporator and N_2 stream. Internal standards ([U-^{13}C]-2,3',4,4',5,5'-hexachlorobiphenyl "syringe spike") in 20 μL of n-nonane were added. This solution was analyzed by high-resolution gas chromatography-high-resolution mass spectrometry (HRGC-HRMS) in the selected ion-monitoring mode on a JMS-700 mass spectrometer (JEOL) fitted with a model HP6890 gas chromatograph (Hewlett Packard).

2.6. Nucleotide sequence accession number

The 16S rRNA gene sequences of the isolates (strains SK-1, SK-2, SK-3, and SK-4) determined in this study was deposited in the DDBJ under accession nos.: AB612122, AB612121, AB612123, and AB612124.

3. Results and Discussion

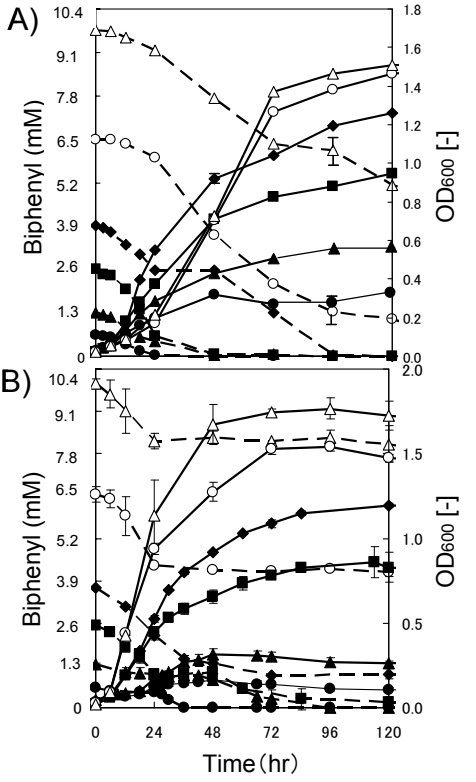
3.1. Identification and phylogenic characteristics of biphenyl-degrading bacteria

We isolated four biphenyl-degrading bacteria (strains SK-1, SK-2, SK-3, and SK-4) from a sewage treatment plant. Strains SK-1, SK-3, SK-4 were Gram-positive and strain SK-2 was Gram-negative and rod-shaped. The isolated strains were not able to assimilate glucose and did not produce indoles, proteases, or β-glucosidase except strain SK-1 (data not shown). The reduction of nitrates to nitrogen was only observed with SK-2. The phylogenic characteristics between the *Rhodococcus* genus (SK-1, SK-3, and SK-4) were slightly different as only SK-1 and SK-3 displayed positive urease activity.

The 16S rDNA sequence analysis identified SK-1, SK-3, and SK-4 as *Rhodococcus* erythropolis LG12 EU852376 (100% identity), *Rhodococcus* zopfii AF191343 (100% identity), and *Rhodococcus* rubber TOY7 FJ554666 or X80625 (100% identity), respectively (data not shown). Interestingly, SK-2 was identified as *Aquamicrobium defluvii* DSM11603 Y15403 (100% identity) which has not yet been reported as a biphenyl or PCB degrading microorganism.

3.2. Biphenyl degradation and growth experiments

The results of biphenyl degradation and growth experiments using SK-2 (*Aquamicrobium* sp.) or SK-3 (*Rhodococcus* sp.) are shown in Fig. 1A and B. SK-2 completely degraded 0.65 mM and 3.9 mM of biphenyl within 24 h and 96 h, respectively. Moreover, the growth of SK-2 correlated to the initial biphenyl concentration. When the initial biphenyl concentration was 6.5 mM, approximately 1.95 mM of biphenyl remained in the culture medium after 120 h-cultivation (Fig. 1A). SK-1 and SK-4 also had biphenyl degradation capability, and utilized biphenyl as a single carbon source (data not shown).



Solid line indicates growth curve, dotted line indicates biphenyl. Different concentrations of biphenyl are indicated as follows: △ 9.75mM; ○ 6.5 mM; ◆ 3.9 mM; ■ 2.6 mM; ▲ 1.3 mM; ● 0.65 mM.

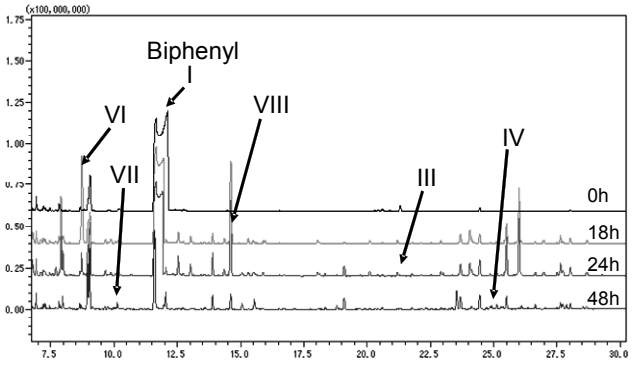
Fig. 1. The time courses of the ratios of residual to initial biphenyl concentrations (A), and the cell density (B)

Rys. 1. Przebieg czasowy stosunków pozostałych do wyjściowych stężeń bifenyli (A) I zagęszczenia komórek

3.3. Determination of metabolites of biphenyl

As previously reported, biodegradation of biphenyl is initiated by a reaction which introduces a hydroxyl group into biphenyl and generates 2,3-dihydroxybiphenyl in the first reaction, and meta-cleaves the dihydroxylated ring to produce (chlorinated) 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (the ring meta-cleavage yellow compound). The yellow compound is hydrolyzed to (chlorinated) benzoic acid or catechol. Most biphenyl-utilizing bacteria isolated to date cannot degrade the chlorobenzoic acids produced any further. The gas chromatogram of the biphenyl degradation intermediates of SK-2 was shown in Fig. 2.

The peak area of biphenyl seen at retention time 11.5 min decreased with progression of the reaction time and the metabolites were observed. Peaks VI, VII, and III from mass spectrography were identified as benzoic acid, catechol, and 2,3-dihydroxybiphenyl (data not shown). The peak VIII was also presumed as a TMS derivative of 2-hydroxymuconic-semialdehyde which is a meta-cleaved catechol, or *cis*-muconic-semialdehyde which is *ortho*-cleaved. To confirm this, the metabolite catechol *meta*-cleavage enzyme (catechol 2,3-dioxygenase) activity in SK-2 was measured by the well documented method of Nozaki et al., (1963). Then, the peak was confirmed as 2-hydroxymuconic-semialdehyde. The maximum molecule ion mass of peak IV was 362, and the molecule ion mass of the base peak was 245 (data not shown). This result is in agreement with 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid from *Pseudomonas paucimobilis* Q1 that has been reported by Furukawa et al. (1983). Furthermore, the pathway of biphenyl degradation of SK-1, SK-3, and SK-4 was predicted to be the same as SK-2 since the same metabolic compounds were detected (data not shown). Therefore, all isolated strains metabolized biphenyl to 2-hydroxymuconic-semialdehyde via 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid.



I: biphenyl, III: 2,3-dihydroxybiphenyl, IV: 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid, VI: benzoic acid, VII: catechol, VIII: 2-hydroxymuconic semialdehyde

Fig.2. The gas chromatogram of the biphenyl degradation intermediates of strain SK-2.

Rys. 2. Chromatogram gazowy produktów przejściowych degradacji bifenyli przy pomocy szczepu SK-2

3.4. Effect of NaCl and KNO_3 on biphenyl degradation

As mentioned previously, the main purpose of this study was to gather basic information for the biodegradation of biphenyl and PCBs containing high concentrations of NaCl and nitrates. In addition to high concentrations of NaCl, nitrates may be a cell-growth inhibition factor in or on contaminated soils, including sediment. Several reports suggest that nitrate inhibited the growth of soil microorganisms (Davidova et al., 2001; Russo, 1985). Therefore, it is necessary to understand the influence of high concentrations of NaCl and nitrates on the growth and biphenyl degradation of the isolated strains.

The influence of NaCl on biphenyl degradation of SK-3 was shown in Fig. 3B. In the control (concentration of NaCl contained in MS culture medium: 0.05 g L^{-1}), biphenyl was degraded completely within 24 h. Although the degradation of biphenyl was delayed, biphenyl could be degraded even in the presence of 3.5% NaCl. However, the cell growth of SK-3 was reduced as NaCl concentrations increased (data not shown). Similar degradation and cell growth patterns were observed with SK-1 (Fig. 3A) and SK-4. However, SK-2 could not degrade biphenyl in the presence of 3.5% NaCl due to a lack growth of the cells. However, biphenyl degradation activity was observed at a NaCl concentration of 3.0% (data not shown). In contrast, the biphenyl degradation activities of the isolated strains (SK-1, SK-2, SK-3, and SK-4) were not affected in the presence of high concentrations of KNO_3 (up to 500 mg L^{-1}) (data not shown). At 500 mg L^{-1} of KNO_3, SK-2 proliferated well and degraded biphenyl (0.65 mM) completely within 36 h (Fig. 4). This suggests that the isolated strains are salt-tolerant biphenyl-degrading bacteria and may be tolerant to other salts such as nitrate.

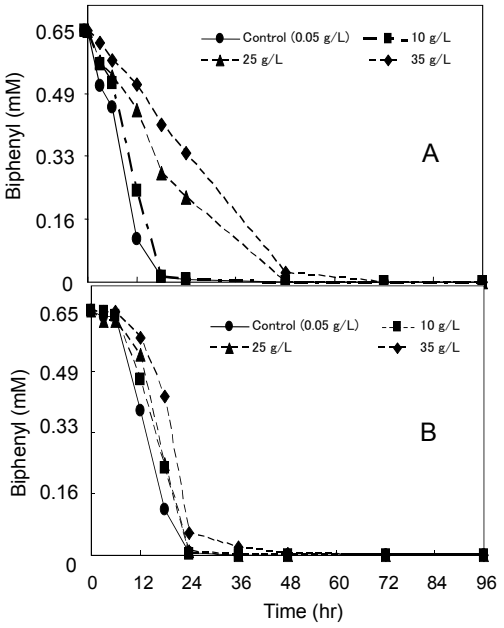


Fig.3. The influence of NaCl on biphenyl degradation

Rys. 3. Wpływ NaCl na degradację bifenyli

3.5. Degradation of other aromatic compounds

The degradation of other aromatic compounds by the isolated strains was tested. SK-1 degraded benzoic acid, catechol, and phenol completely. Moreover, 4-chlorophenol, aniline, and toluene were degraded up to 50%, 13%, and 90% (w w^{-1}), respectively.

Nitrobenzene and naphthalene were degraded up to 20% and 6.0%, respectively. However, during the incubation period the degradation activity of diphenylamine and 1,3,5-triphenylbenzene was not observed. Similar degradation patterns were observed with SK-2. SK-3 degraded benzoic acid, catechol, and phenol completely. Naphthalene, diphenylamine and nitrobenzene were degraded up to 30%, 20%, and 14%, respectively.

However, 4-chlorophenol, aniline, toluene and 1,3,5-triphenylbenzene were not degraded. Similar degradation activity and patterns were observed with SK-4. These results indicate that all of the isolates could degrade several other aromatic compounds.

3.6. Co-metabolic degradation of PCB congeners

Degradation by aerobic bacteria is easier for PCB congeners (with few chlorine substituents), and PCB congeners (with less than 4 chlorine substituents) are especially

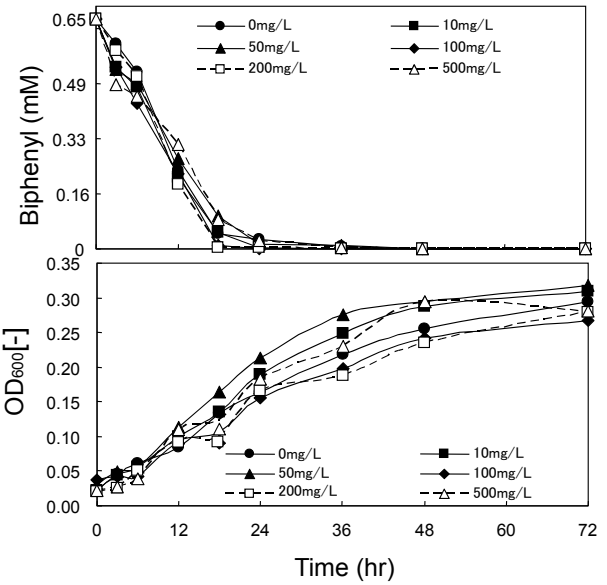


Fig.4. The influence of KNO_3 on biphenyl degradation

Rys. 4. Wpływ KNO_3 na degradację bifenyli

susceptible to degradation (Furukawa and Fujihara, 2008). Table 1 summarizes the results of PCB degradation by the isolated strains in the presence of biphenyl. SK-1 degraded various PCB congeners with six or less chlorine substituents. The degradation characteristic was similar to other *Rhodococcus* genus, which is a representative PCB degrading bacterium (Seto et al., 1995). A similar degradation pattern was observed when SK-3 and SK-4 were inoculated in the culture.

In contrast, SK-2, belonging to the *Aquamicrobium* genus, also degraded six or less chlorine substituted congeners. However, the degradation activity was lower than those of SK-1, and the degradation activity of PCB congeners with chlorines at position ortho-substituted especially was low. In addition, many recalcitrant PCB congeners (with seven chlorine substituents) were observed. PCB congeners with eight or more chlorine substituents were not degraded. Although the degradation rate was slightly different, the same degradation pattern was observed in the presence of high concentrations of NaCl (3.0%, w v^{-1}) (data not shown).

To date, few studies have described PCB degradation in the presence of NaCl. *Pseudomonas* sp. CH07, a marine bacterium, degraded PCB congeners with 4 chlorine substituents in the presence of approximately 1.75% (w v^{-1}) NaCl (Abdughafurovich et al., 2010). In addition, the *Bacillus* genus is capable of degrading PCB in the presence of 0.05% (w v^{-1}) NaCl (Jaysankar et al., 2006). However, these PCB degradation experiments were limited as they contained less than 1.8% (w v^{-1}) NaCl in the culture medium. In contrast, SK-1, SK-3, or SK-4 were able to degrade various PCB congeners in the presence of NaCl (3.5%, w v^{-1}; the NaCl concentration of sea water), and SK-2 was able to degrade PCB congeners in the presence of NaCl (3.0%, w v^{-1}) (data not shown).

Tab. 1. Degradation of various PCB congeners by isolated strains

Tab. 1. Degradacja różnych kongenerów PCB przez wyizolowane szczepy

Substrates	SK-1	SK-2	Substrates	SK-1	SK-2
	Residual (%)			Residual (%)	
2-Mo-PCB	60	90	2,4,4',5-Te-PCB	54.8	79.7
3-Mo-PCB	35.6	NA	2,2',3,3',4-Pe-PCB	ND	ND
4-Mo-PCB	34	68.6	2,2',3,4,5'-Pe-PCB	ND	ND
2,2'-Di-PCB	85.8	65.3	2,3,3',4',6-Pe-PCB	75	88
2,3'-Di-PCB	66.3	65.2	2,2',4,4',5-Pe-PCB	88	88.3
2,5-Di-PCB	76.4	NA	2,2',3,4,4',5'-Hx-PCB	78.8	66
2,4'-Di-PCB	80	78.7	2,2',3,4',5,5'-Hx-PCB	ND	78.1
2,2',6-Tr-PCB	75.5	84.5	2,2',3,4',5,6-Hx-PCB	78	81.3
2,4,4'-Tr-PCB	78	84.2	2,2',4,4',5,5'-Hx-PCB	81.5	68.2
2,2',3-Tr-PCB	84.1	63	2,2',3,3',5,6,6'-Hp-PCB	69.5	83
2,2',5-Tr-PCB	60	NA	2,2',3,4',5,5',6-Hp-PCB	73	83
2,3,4'-Tr-PCB	77	83.8	2,2',3,3',4,5,6-Hp-PCB	84	ND
2,3',4-Tr-PCB	43	62.2	2,2',3,3',4,5,6'-Hp-PCB	ND	ND
2,2',3,5'-Te-PCB	69	NA	2,2',3,3',4',5,6-Hp-PCB	ND	ND
2,2',5,5'-Te-PCB	71	63	2,2',3,4,4',5,5'-Hp-PCB	ND	ND
2,3,3',4'-Te-PCB	52	75	2,2',3,3',4,4',5,5'-Oc-PCB	ND	ND
2,3',4,4'-Te-PCB	64	77	2,2',3,3',4,5,5',6-Oc-PCB	ND	ND
2,3',4,5-Te-PCB	65.5	52	2,2',3,4,4',5,5',6-Oc-PCB	ND	NA
2,3',4',6-Te-PCB	43	44	2,2',3,3',4,4',5,5',6-No-PCB	ND	ND

ND: not detected NA: not available

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