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Degradation of various toxic compounds at low and medium temperature conditions using the isolated fungi

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Abstract

In the present study, a fungal strain isolated from the Antarctic soil was identified as *Penicillium* sp. CHY-2 based on its 5.8S rRNA gene sequence analysis. Further, evaluated its biodegradation ability towards 13 different toxic compounds at low (4°C) and medium (15°C) temperature conditions using high pressure liquid chromatography (HPLC). Among 13 compounds, strain CHY-2 effectively degraded the 7 compounds at 15°C within one week, and 6 compounds at 4°C within three weeks. Among different carbon sources tested, glucose was found to be the most suitable and the growth at 4°C was slower than at 15°C. Addition of Tween 80 increased the growth and degradation ability of CHY-2 towards 4-butylphenol at 4°C and 15°C. The metabolites produced during the degradation of 4-butylphenol were identified by gas chromatography-mass spectrometry (GC-MS). Also, bacteria present in the Antarctic soil were determined by DGGE and the result showed presence of *Pseudomonas* and *Syntrophus* group of bacteria.

Keywords: 4-butylphenol; *Penicillium* sp. CHY-2; alkylphenol; Antarctic; cold regions

Abbreviations: **BPA,** bisphenol A; ***cis*-DCE,** *cis*-1,2-dichloroethylene; **DGGE,** denaturing gradient gel electrophoresis; **DDBJ,** DNA data bank of Japan; **GC-MS,** gas chromatography-mass spectrometry; **MGY,** mineral glucose yeast extract; **PAHs,** polycyclic aromatic hydrocarbons; **PCE,** tetrachloroethylene; **PDA,** potato dextrose agar; ***p*-t-OP,** *p-tert*-octylphenol; **RBBR,** Remazol Brilliant Blue R; **TCE,**

trichloroethylene; **VC,** vinyl chloride; **4-BP,** 4-butylphenol; **4-CB,** 4-chlorobiphenyl; **4-CP,** 4-chlorophenol; **2,4-DCP,** 2,4-dichlorophenol; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **4-HP**, 4-*n*-hexylphenol; **4-NP,** 4-nonylphenol; **4-s-BP,** 4-*sec*-butylphenol; **2-s-BP,** 2-*sec*-butylphenol; **2-t-BP,** 2-*tert*-butylphenol; **4-t-BP,** 4-*tert*-butylphenol; **2,4,5-TCP,** 2,4,5-trichlorophenol; **1,3,5-TCB,** 1,3,5-trichlorobenzene; **1,2,3,5-TCB,** 1,2,3,5-tetrachlorobenzene;

1. Introduction

Increased human activities conveyed contaminants and anthropogenic compounds in to the Antarctic ecosystems. The Antarctic continent can no longer be considered as pristine, because of the increased human activities [1]. Several studies already reported the presence of anthropogenic compounds such as metals [2], polycyclic aromatic hydrocarbons (PAHs) [3], chlorinated biphenyl, phenol, pesticides and their toxic effects on Antarctic region [4]. In addition, recently, the effect of toxic malachite green (MG) on the bacterial community in Antarctic soil and the physiology of MG-degrading *Pseudomonas* sp. MGO was reported [5]. Although several investigations were made for degradation of anthropogenic compounds, all those reports have been focused on bacteria, but not on fungi. Therefore, to extend our knowledge on *in-situ* remediation strategies in cold regions, not only bacteria but also degradation mechanism related to fungi needs to be investigated. Fungi play a central role in biodegradation at the intermediate temperature range. In particular, filamentous fungi play vital role in the

degradation of a wide range of aromatic hydrocarbons [6, 7]. In addition, the potential role of fungi as hydrocarbon degrader in Antarctic soil has been identified [8], very little information was available about the potentiality of fungi to degrade toxic compounds at low temperature conditions.

Phenol and phenolic compounds are widely distributed as environmental pollutants, and they are common constituents of many industrial wastewaters such as crude oil refineries and coal gasification plants [9, 10]. On the other hand, the widespread contamination of drinking water with alkylphenols has led to a concerted effort to find efficient treatment methods. Alkylphenols such as 4-nonylphenol, 4-*tert*-butylphenol, 4-*tert*-octylphenol, 4-butylphenol, 2-*sec*-butylphenol, and 4-chlorophenol have originated from the sources of detergents [11]. These chemicals are prevalent and persistent groundwater contaminants, which are all known or suspected human carcinogens, mutagens or toxins [11, 12]. These compounds have also been detected in industrial wastewater and causes microbial growth inhibition in activated sludge under the process of biological wastewater treatment. Therefore, alkylphenol-degrading microorganisms attracts attention as a cost effective pretreatment method for biological wastewater treatment.

In our previous studies, we reported the characterization of *Pseudomonas* sp. MS-1 isolated from a natural aquatic environment as the first 2-*sec*-butylphenol-utilizing bacterium [13]. However, the degradation of alkylpenol at low temperature conditions is still unknown. As a matter of fact, sometimes the temperature of wastewater becomes below 10°C in the cold areas of Japan. Thus, it is thought that development of microorganisms which are capable of degrading toxic organic compounds at low-temperature conditions could help the lowering of operational costs of wastewater treatment plants.

Although potential microorganisms with higher degradation capability were continuously isolated from the environment [14], always a concern arises about degradation at low-temperature regions. In the current study, numerous fungi were isolated from the Antarctic soils and evaluated their toxic organic compounds-degrading ability. In addition, selection and characterization of *Penicillium* sp. CHY-2 was done. Degradation ability of CHY-2 towards 13 different toxic organic compounds at low (4°C) and medium (15°C) temperature conditions were analysed by high pressure liquid chromatography (HPLC). Influence of carbon source on the growth of CHY-2 was evaluated using 8 different types of organic carbon sources. Effect of glucose, NaCl and Tween 80 concentration on degradation of 4-butylphenol was evaluated. The

metabolites produced during the degradation of 4-butylphenol were identified by gas chromatography-mass spectrometry (GC-MS). Furthermore, bacteria present in the Antarctic soils were identified by denaturing gradient gel electrophoresis (DGGE).

2. Materials and methods

2.1. Chemicals

All chemicals used were of analytical grade. The following chemicals: phenol, toluene, benzene, xylene, 4-chlorophenol (4-CP), 2,4-dichlorophenol (2,4-DCP), 2,4,5-trichlorophenol (2,4,5-TCP), 4-butylphenol (4-BP), 4-*n*-hexylphenol (4-HP), 4-nonylphenol (4-NP), 4-*sec*-butylphenol (4-s-BP), 2-*tert*-butylphenol (2-t-BP), 2-*sec*-butylphenol (2-s-BP), 4-*tert*-butylphenol (4-t-BP), *p*-*tert*-octylphenol (*p*-t-OP), bisphenol A (BPA), 2,4-dichlorophenoxyacetic acid (2,4-D), 4-chlorobiphenyl (4-CB), 1,3,5-trichlorobenzene (1,3,5-TCB), 1,2,3,5-tetrachlorobenzene (1,2,3,5-TCB), naphthalene, and phenanthrene, were purchased from Tokyo Chemical Industry (Tokyo, Japan). The chlorinated volatile organic compounds: tetrachloroethylene (PCE), trichloroethylene (TCE), *cis*-1,2-dichloroethylene (*cis*-DCE), and vinyl chloride (VC), were purchased from GL Science (Tokyo, Japan).

2.2 Sample collection

Several surface soil samples including isolated fungi were listed in Table S1. Moss colony near Zucchelli Station at Terra Nova Bay, Antarctica were provided by Prof. Dr.

Satoshi Imura, National Institute of Polar Research, Japan. The information about sampling sites and tested fungal isolates were well described by Tsuji et al. (2013) [15].

2.3 Degradation of toxic compounds using enriched cultures

Organisms in soil/moss/sediment samples (sample number #1-5 indicated in Table S1) were enriched and cultivated under aerobic or anaerobic conditions by inoculating 3.0 g of samples into 20 ml of mineral glucose yeast extract (MGY) medium in 50-mL serum bottles. MGY medium contains the following composition (per liter of deionized water): K_2HPO_4, 1.0 g; NaCl, 0.05 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.05 g; $FeCl_3 \cdot 6H_2O$, 0.0083 g; $MnCl_2 \cdot 4H_2O$, 0.014 g; $NaMoO_4 \cdot 2H_2O$, 0.017 g; $ZnCl_2$, 0.001 g; yeast extract, 0.5 g; glucose, 5.0 g; at pH 6.0. Enrichment was done under aerobic and anaerobic conditions using five different groups of toxic compounds: 1) PCE, *cis*-DCE, and VC; 2) 4-t-BP, 4-HP, and BPA; 3) 4-CB, 1,3,5-TCB, and 1,2,3,5-TCB; 4) 2,4-DCP and 2,4,5-TCP; 5) naphthalene and phenanthrene. The final concentration of substrates in the medium was 50 mg/l. Enriched cultures were maintained with a monthly subculturing using the medium described above for 6 months.

2.3.1 Microbial diversity analysis using DGGE

To investigate the degradation of variety of toxic compounds, each enrichment culture was grown in MGY medium supplemented with 50 mg/l of toxic compounds under

aerobic and anaerobic conditions in separate serum bottles. The serum bottles were shaken at 110 rpm at 15°C for 10 days. Bacterial community present in the Antarctic soils were analyzed by DGGE analysis. DNA extraction, amplification of the 16S rRNA genes, and DGGE analysis were done as described by Martínez-Alonso et al. (2010) [16].

2.4 Identification of toxic compounds degrading fungi

Twelve isolated fungi (Table S1) were used as the source of inoculum for degradation of various toxic compounds. Decolorization test with Remazol Brilliant Blue R (RBBR) was used as rapid screening method for detection of toxic compound degrading fungi. Decolorization activity was tested on solid media containing MGY agar medium with RBBR at a final concentration of 0.5 g/l. Degradation experiments were carried out using several fungi which have the capacity to decolorize RBBR. The selected RBBR decolorizing fungi were tested in liquid medium. Liquid culture experiments were conducted in 50-ml serum bottles containing 10 ml of MGY liquid medium. The medium pH was adjusted to 6.0 with 1 N HCl and sterilized for 20 min at 121°C. Three disks (10 mm diam) obtained with a cork-borer from actively growing fungi on agar was placed into each serum bottle. The inoculated bottles were precultivated for 5 days to obtain similar radial growth and minimize the growth variation. Then 4-BP (as a final

concentration of 50 mg/l) was added to each bottle and the bottles were incubated for 10 days under dark condition at 15°C with agitation speed at 110 rpm. A non-inoculated control was used to quantify the loss of 4-BP during incubation and extraction. A killed inoculated control was also used to quantify the adsorption of 4-BP.

2.5 Degradation of variety of compounds by strain CHY-2

Degradation experiments were conducted in 50-mL serum bottles containing 10 ml of MGY liquid medium. The medium pH was adjusted to 6.0 with 1 N HCl and sterilized in autoclave for 20 min at 121°C. Five disks (10 mm diam) obtained with a cork-borer from actively growing fungi on agar were placed into each bottle. The inoculated bottles were precultivated for several days to obtain similar radial growth and minimize growth variation. Various toxic organic compounds (at a final concentration of 50 mg/l), such as 4-BP, 4-s-BP, 4-t-BP, 4-NP, 4-t-OP, 4-CP, phenol, BPA, benzene, toluene, xylene, naphthalene, and phenanthrene were injected to separate bottles, and the bottles were incubated under the dark condition with agitation speed of 110 rpm.

2.5.1 DNA isolation, PCR amplification and sequencing of CHY-2

The isolation of fungi was done as previously described by Tsuji et al. (2013) [15]. Fungal colonies were grown on the surface of potato dextrose agar plates (PDA, Difco). The procedure was repeated to ensure a pure culture. The purity of the isolated strain

was confirmed using an inverted microscope (Diaphot TMD300, Nikon, Tokyo, Japan) equipped for simultaneous recording of cell length. An isolated strain CHY-2 was sequenced. Total genomic DNA of CHY-2 was extracted from colonies using an Isoplant II kit (Wako Pure Chemical Industries, Osaka, Japan) in accordance with protocols provided by the manufacturer. Extracted DNA was amplified through PCR using PrimeSTAR HS DNA polymerase (Takara-bio Inc., Shiga, Japan). The ITS region of DNA samples was amplified using the primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG) and ITS4 (5'-TCCTCCGCTTATTGATATGC) [17]. Sequences were determined using an ABI prism 3130 Sequencer (Applied Biosystems, CA, USA). Sequence products were assembled with ChromasPro 1.5 (Technelysium Pty Ltd., Tewantin, AUS). ITS region sequences of isolate were deposited into the DNA Data Bank of Japan (DDBJ). Phylogenetic analyses with neighbor-joining analyses of ITS regions containing 5.8S rRNA gene were performed using MEGA software version 5.0 [18]. Nucleotide sequence of ITS region (609 bp) of *Penicillium* sp. CHY-2 was deposited into GenBank with accession number AB980801.

2.5.2 Optimization studies for 4-BP degradation

2.5.2.1 Carbon source utilization

A monohyphal culture of the isolate was obtained by single spore isolation. The cultures

were maintained on 2% MGY agar plates with initial pH 6.0. Nine different organic sources *i.e.,* glucose, fructose, glycerin, citric acid, starch, sucrose, lactose, and maltose were taken separately at a concentration of 5.0 g/l in the medium. Duplicates experiments were conducted for all the conditions. Uniform circular agar blocks (10 mm diam) containing mycelial mat were cut from a 10 days old culture plate and transferred into sterilized 25 ml of basal medium. The inoculated flasks were incubated at 15°C under dark condition with shaking at 110 rpm. After 10 days of incubation, the cultures were spread on petri plates containing different carbon sources. Glucose (1, 5, and 10 g/l), and NaCl (1, 3, and 5 g/l) at different concentrations were used for 4-BP degradation optimization studies. Furthermore, to know the optimum NaCl concentration for higher degradation of 4-BP, the combination of glucose and NaCl concentration was also examined.

2.5.2.2 Culture conditions

Effect of different conditions *i.e.,* anaerobic, aerobic, static, and shaking (at 110 rpm) on degradation of 4-BP were evaluated using MGY medium with 4-BP at 50 mg/l concentration. For anaerobic cultivation, the bottles were sealed with a butyl rubber septum and aluminum crimp seals. The headspace above the liquid phase was replaced with N_2 gas and cultivation was performed by rotary shaking. Control experiments were

performed without inoculation of culture.

2.5.2.3 Effect of Tween 80

Liquid culture experiments were conducted in 50-mL serum bottles containing 10 ml of MGY media with (0.25% (w/v) and without Tween 80. Three disks (10 mm diam) of actively growing fungi on agar were placed into each liquid medium. 4-BP at 50 mg/l concentration was added to each bottle and the bottles were incubated for 21 days under the dark condition at 4°C with agitation speed at 110 rpm. The net hyphal growth of the fungus was determined in terms of mycelial dry weight. Adhered agar medium from the mycelial mat was removed by straining through a filter paper (Whatman No. 1). The mycelial mat was rinsed 3-4 times with distilled water to remove traces of basal medium and it was dried at 80°C for 2 hr, and the fungal biomass was weighed.

2.6 Analysis

The chlorinated ethylenes, alkylphenols, and other toxic organic compounds were identified and quantified as described by Chang et al. (2013ab) [19, 20]. The chlorinated ethylenes were identified and quantified in a 250 μL headspace sample using a model GC-8A gas chromatograph (Shimadzu Co., Japan) equipped with a flame ionization detector (FID). The alkylphenol and chlorophenol concentrations were determined by HPLC (Table S2). The detection limit was 0.03 mg/l. Recovery of samples was 99.5%

in percent. For the HPLC analysis, the samples were acidified with 1N HCl, shaken for 3 min with an equal volume of 1:1 (v/v) ethyl acetate:*n*-hexane, and centrifuged at 8000×g for 10 min. The organic layer was then extracted and analyzed directly by HPLC.

The metabolites produced during 4-butylphenol degradation were determined by GC-MS. The samples with an equal volume of 1:1 (v/v) ethyl acetate were shaken for 3 min and centrifuged at 8000 × g for 10 min. The organic layer was then extracted and analyzed directly by GC-MS. Prior to the GC-MS analysis the extract was dried under a nitrogen flow and derived by trimethylsilylation (TMS) using a BSTFA-acetonitrile solution at 60°C for 1 h. The GC-MS analysis was conducted on a Shimadzu GC-MS system (GCMS-QP2010) with an Rxi-5ms capillary column (30 m, 0.25 mm ID, 1.00 lm df; Restek, Pennsylvania, USA).

3. Results and Discussion

3.1. Toxic compounds degradation using enriched cultures

In this study, we did not find any significant results for degradation of toxic compounds using enriched cultures. Bacteria present in the Antarctic soil were unable to degrade various toxic compounds. It is not clear that low degradation activity of enriched cultures was due to the poor bacterial communities present in Antarctic soil or due to

any other reasons. So, in order to identify the bacterial community present in the soil samples, DGGE analysis was conducted for three individual samples *i.e.,* sample 1, sample 2, and sample 3 (Fig. 1). Majority of the bands were almost similar in three conditions (Fig. 1). In all the samples, two different types of genus were found, *i.e., Pseudomonas* and *Syntrophus*. Bacteria belong to *Pseudomonas* genus were found to be dominant (Fig. 1). After that the bacteria belong to syntrophus genus were found in the soil samples. Auburger and Winter, (1996) reported that *Syntrophus buswellii* strain GA has the capacity to degrade benzoate, 3-phenylpropionate, and crotonate [21]. Tuan et al., (2011) reported that the two strains, *Pseudomonas putida* TX2 and *Pseudomonas* sp. TX1, contained catechol 1,2-dioxygenase, catechol 2,3-dioxygenase genes and were able to degrade long-chain alkylphenols [22]. Toyama et al., (2010a) isolated the *Pseudomonas* sp. strain MS-1 from fresh water sediment, and reported that strain MS-1 degraded 2-alkylphenols with various sized and branched alkyl chain, also dialkylphenol [13]. The DGGE results were coordinated with the previous studies conducted by Chong et al. (2012) using Antarctic soil. It has been reported that most of the Antarctic soil environments contained a narrow range of bacterial species [23]. Hence to make bioremediation successful, introduction of microorganisms which have the capacity to degrade toxic compounds is more effective methodology [24, 25].

Fig. 1

3.2 Identification of strain CHY-2

After 10 days of incubation on RBBR agar plates, four fungal strains named CHY-1, CHY-2, CHY-3, and CHY-4 showed the zone of color change at 15°C and 20°C. Based on these results, those four fungi were selected for degradation studies using 4-BP. We selected the toxic compound 4-BP, because it is a serious aquatic pollutant and also due to its widespread use considerable amount has been released directly or through wastewater into the aquatic environment [12]. Among the four fungal strains, only one fungal strain named as CHY-2 was able to degrade 4-BP. Fungal colonies on the surface of nutrient agar plate were bluish-green, velvet-shaped, and the back side of the colonies were yellowish brown (Fig. 2ab). Microscopic image of strain CHY-2 was shown in Fig. 2c. Morphological characters of strain CHY-2 were in accordance with those described for *Penicillium polonicum* by Frisvad and Samson (2004) [26]. Result of BLAST homology search for Apollo DB-FU and ITS-5.8S rDNA nucleotide sequence obtained from strain CHY-2 showed 100% homology with the nucleotide sequence of *Penicillium polonicum* NRRL 995^{T} (AF033475) (Fig. 2d). Phylogenetic tree constructed based on BLAST homology search for Apollo DB-FU and the International Nucleotide Sequence Databases. CHY-2 strain belongs to the family of *Viridicada* section in *Penicillium* subgenus, and it formed the same branch as *Penicillium polonicum* in the phylogenetic tree, so the strain CHY-2 was identified as *Penicillium* genus.

Fig. 2

3.3 Degradation of thirteen different toxic compounds

Among the four fungal strains, only one fungal strain named as CHY-2 was able to degrade 4-BP. So further studies were carried out using strain CHY-2. Thirteen different toxic compounds degradation was investigated using CHY-2 at 15°C and at 4°C (Table 1). At 15°C, among all the compounds CHY-2 showed highest degradation with 4-t-BP (100%) followed by 4-BP (98%), 4-s-BP (97%), 4-NP (71%), 4-t-OP (70%), phenol (68%) and 4-CP (65%). CHY-2 showed moderate degradation with phenanthrene (48%) and xylene (23%), and lower degradation with toluene (10%), naphthalene (12%), BPA (12%) and benzene (18%). At 4°C, among all the compounds CHY-2 showed highest degradation with 4-t-BP (75%) followed by 4-s-BP (70%), 4-CP (62%), phenol (60%), 4-BP (60%), 4-NP (55%) and 4-t-OP (35%) (Table 1). On the other hand BPA, benzene, toluene, xylene, naphthalene, and phenanthrene were not degraded by the strain CHY-2 at 4°C. Degradation rate was measured at 7th day at 15°C and at 21st day at 4°C. White rot fungi are well known for not only as decomposers of lignin but also for their ability to degrade a wide variety of organopollutants, such as polychlorinated biphenyls, polycyclic aromatic hydrocarbons (PAHs), pesticides, synthetic polymers or synthetic dyes [27]. Syed et al., (2011) reported *Phanerochaete chrysosporium*, which belongs to the white rot group of the wood decaying fungi has the ability to mineralize a broad

range of environmental toxicants, including PAHs and alkyl-substituted aromatics [28]. Toyama et al., (2010b) isolated three *Sphingobium fuliginis* strains from *Phragmites australis* rhizosphere sediment that were capable of utilizing 4-t-BP as a sole carbon and energy source [29]. They reported that degradation experiments with different alkylphenols showed that strain TIK-1 could degrade 4-alkylphenols with variously sized and branched side chains (ethyl, *n*-propyl, isopropyl, *n*-butyl, *sec*-butyl, *tert*-butyl, *n*-pentyl, *tert*-pentyl, *n*-hexyl, *n*-heptyl, *n*-octyl, *tert*-octyl, *n*-nonyl, and branched nonyl) via meta cleavage pathway.

Table 1

3.4 Optimization studies using CHY-2

3.4.1 Glucose identified as good carbon source

Selection of inexpensive and effective organic carbon source is the key to reducing the cost in production of useful materials as well as in implementation of bioremediation [30, 31]. Various carbon sources (glucose, fructose, glycerin, citric acid, starch, sucrose, lactose, and maltose) were screened for the growth of strain CHY-2 at 15°C. The growth results are provided in Table 2. Basal media without carbon source (the control) showed little growth (data not shown). Although the growth efficiency and growth rates were varied based on carbon source. The growth of *Penicillium* sp. CHY-2 was observed in all liquid cultures containing each 2.0 and 5.0 g/l of carbon source. Among all, glucose and sucrose were found to be the most suitable carbon sources for the growth of strain

CHY-2 and the growth was found to be equally good as like PDA medium. Based on these results it was concluded that glucose was suitable organic carbon source on the basis of cost-performance for the growth of CHY-2.

Table 2

3.4.2 Higher degradation observed under aerobic condition

To determine the optimum conditions for 4-BP degradation using CHY-2, four different culture conditions were examined *i.e.,* static, shaking, aerobic, and anaerobic. Higher degradation was observed with aerobic conditions than anaerobic conditions. Slightly faster degradation rate was observed under shaking conditions compared with the static conditions (data not shown).

3.4.3 Temperature influence on 4-BP degradation

To investigate the effect of temperature on 4-BP degradation by *Penicillium* sp. CHY-2, batch experiments were carried out for 7 days under aerobic condition using 50 mg/l of 4-BP in MGY medium at different temperatures, *i.e.,* 4°C, 10°C, 15°C, and 20°C. Among different temperature conditions, CHY-2 grown at 15°C and 20°C showed complete removal (100%) of 4-BP within 7 days (Fig. 3a). Strain CHY-2 was unable to show complete removal of 4-BP within 7 days, it showed 29% removal of 4-BP at 10°C and 7% removal of 4-BP at 4°C. Biomass growth was also followed similar trend. CHY-2 showed high dry cell weight at higher temperature conditions and low dry cell

weight at lower temperature conditions. It showed higher dry cell weight at 20°C (5.81 g/l) and 15°C (5.79 g/l), lower dry cell weight at 10°C (1.65 g/l), and 4°C (0.95 g/l) (Fig. 3b). Strain CHY-2 showed lowest degradation of 4-BP at 4°C at 7th day, so further experiments were carried out for 21 days. CHY-2 showed 60% removal of 4-BP at 21st day. The degradation rate at 4°C was very low compared to 15°C and 20°C due to slow growth of CHY-2. This results indicates that the degradation of 4-BP in the culture of *Penicillium* sp. CHY-2 was strongly affected by cell growth.

Fig. 3

3.4.4 Glucose and NaCl concentration influence

High salt concentrations may cause a decrease in endogenous respiration of cells and/or plasmolysis [32]. Thus, it is difficult to treat marine sediments or high saline soils using biological methods. Therefore, it is important to isolate strain that can degrade the toxic organic compounds 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 biodegrading toxic organic compounds in the presence of NaCl have been reported [20, 33-34]. After the carbon source utilization test, optimum concentrations of glucose and NaCl for 4-BP degradation were examined. The results are shown in Fig.4. High degradation ratio was obtained when glucose (5 g/l or 10 g/l) was added to the cultures without NaCl. The high degradation rate was also observed in

the PDA medium cultures. NaCl (1.0 g/l) addition to the medium containing glucose did not show effect on the degradation rate. However, addition of NaCl (1.0 g/l) promoted degradation activity when 1.0 g/l of glucose was existed. On the other hand, when the NaCl concentration was increased from 1.0 g/l to 3.0 g/l, removal efficiency decreased and prominent inhibition was observed at a concentration of 5.0 g/l regardless of glucose concentrations.

Fig. 4

3.4.5 Tween 80 addition enhanced 4-BP degradation

Tween 80 is a non-ionic surfactant with less toxicity to bacteria than anionic and cationic surfactants [35]. Moreover, Tween 80 is biodegradable and widely used in PAHs biodegradation and bioremediation [36]. Previously, it has been reported that the enhancement of the biodegradation of fluorine was observed by *Doratomyces stemonitis* (46–62%) and *Penicillium chrysogenum* (28–61%) in the presence of Tween 80 (0.324 mM) [37]. In addition, recently significant improvement in the degradation rate of PAHs has been reported using Tween-80 [38, 39]. Wang et al. (2014) reported that the addition of Tween 80 under existing fungal strain (*Lasiodiplodia theobromae*) enhanced benzo[a]pyrene degradation in soil [38]. Chen et al. (2013) also reported that positive effect of Tween 80 on biodegradation of pyrene by PAH-degrading bacteria *Burkholderia cepacia* [39]. Brar et al. (2005) reported addition of Tween 80 to

non-hydrolyzed sludge resulted in increase in cell and spore count of *Bacillus thuringiensis* by 1.67 and 4 times respectively, maximum specific growth rate (μmax) increased from 0.19 to 0.24 h^{-1} [40]. They indicated that Tween 80 improved *B. thuringiensis* growth in non-hydrolyzed sludge which will have tremendous impact on its use as raw material for fermentation and finally bio-pesticide formulations. Budde et al. (2011) conducted experiments to know the effect of Tween 80 at 0.2% concentration on the growth of *Ralstonia eutropha* [41]. They reported that Tween 80 cultures showed considerably higher optical densities than the controls (without Tween-80), and Tween 80 can serve as an effective carbon source for *R. eutropha*. Tween molecules contain a fatty acid group. The enzyme esterase cleave the fatty acid from Tween, allowing it to be used as a carbon source by many bacteria. Tween 80 might interfere in membrane permeability, and also affect taking of nutrients. In fact, promotion of spore formation has been observed (data not shown). As mentioned above, although previous studies have focused on the effect of Tween 80 for the degradation of PAHs, but till date there are no reports about the influence of Tween 80 on degradation of 4-BP at low temperature conditions.

To enhance the 4-BP degradation activity of strain CHY-2 at low temperature conditions, supplementation of Tween 80 could be useful. Addition of Tween 80 enhanced the

degradation activity of CHY-2 towards 4-BP at both 15°C and 4°C owing to increase of cell growth (Fig.5ab). Predominant promotion of 4-BP degradation rate by CHY-2 was observed after addition of Tween 80 at 4°C. Interestingly, under this condition, enhancement of solubilization of 4-BP by addition of Tween 80 was not observed. This result suggests that Tween 80 at the test concentration is not toxic to strain CHY-2. In addition, experimental results showed that Tween 80 could serve as a readily available carbon source, thus increasing the biomass concentration in the medium. Through the studies, it was found that Tween 80 not only enhance the oxygen consumption by removing precipitates such as lipid and protein substances attached to cell surface, also increases the cell growth. By observing these results, it has been concluded that Tween 80 may play an important role in a degradation of toxic compounds in cold regions also. The degradation of 4-BP was coincidence with proliferation of *Penicillium sp.* CHY-2. However, under the conditions, strain CHY-2 did not utilize 4-BP as the sole carbon/energy source for the growth (data not shown). Therefore, this result indicates that strain CHY-2 could be applied for the treatment of wastewater contaminated with 4-BP at low-temperature conditions.

Fig. 5

3.5 Determination of 4-butylphenol metabolites

The peak area of 4-butylphenol seen at retention time 12 min decreased with

progression of the reaction time and several metabolites were observed. However, only two peaks (I and II) from mass spectrography were identified as 2-*n*-butylhydroquinone and hydroquinone (Fig. 6a). The mass spectrometry of peak I was shown in Fig. 6b. The maximum molecule ion at m/z=310 was presumed to be a TMS derivative of 2-*n*-butylhydroquinone. The ms spectrum corresponded to that of previous report [13].

Fig. 6

4. Conclusions

A novel fungal strain *Penicillium* sp. CHY-2 isolated from Antarctic soil was effective in the degradation of 4-BP, 4-s-BP, 4-t-BP, 4-t-OP, 4-NP, phenol and 4-chlorophenol at lower (4°C) and middle temperature (15°C) conditions. Glucose was found to be the most suitable carbon source for the growth of CHY-2. Tween 80 addition enhanced the growth and degradation ability of CHY-2 towards 4-butylphenol. DGGE result showed the presence of *Pseudomonas* and *Syntrophus* group of bacteria in Antarctic soil. Our results indicated that the strain CHY-2 can be used for in situ bioremediation of alkylphenols contaminated water in cold regions.

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The authors have declared no conflict of interest.

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Table 1. Biodegradation of toxic compounds by strain CHY-2

Sample No	Toxic compounds	Degradation ratio (%)	
		15°C	**4°C**
1	4-Butylphenol	98.2	60.2
2	4-*sec*-Butylphenol	97.4	70.1
3	4-*tert*-Butylphenol	100.0	75.3
4	4-Nonylphenol	71.3	55.6
5	4-*tert*-Octylphenol	70.5	35.7
6	4-Chlorophenol	65.6	62.8
7	Phenol	68.3	60.2
8	Bisphenol A	12.7	10.8
9	Benzene	18.4	6.3
10	Toluene	10.2	3.4
11	Xylene	23.6	11.2
12	Naphthalene	12.6	3.3
13	Phenanthrene	48.4	18.5

Table 2. Effect of carbon source on the growth of strain CHY-2.

Carbon source	Concentration (g/l)	Cell growth (Relative, %)
Glucose	2.0	100.0
	5.0	100.0
Fructose	2.0	75.23 ± 3.12
	5.0	75.33 ± 3.76
Glycerin	2.0	74.7± 3.68
	5.0	78.47 ± 3.65
Citric acid	2.0	50.2 ± 2.51
	5.0	48.2± 0.345
Starch	2.0	55.2 ± 3.26
	5.0	75.6± 5.24
Sucrose	2.0	98.6± 7.24
	5.0	98.4 ±8.24
Lactose	2.0	50.3 ±5.26
	5.0	75.6 ± 6.32
Maltose	2.0	75.3± 7.24
	5.0	71.1 ±4.15

Supplementary

Table S1. The list of samples collected from Antarctic region

Sample No	Source of species (Fungi)
1	Soils on East Ongul Island in Queen Maud Land
2	Lake sediment in Skarvsnes in Lützow-Holm Bay
3	Moss colonies on East Ongul Island
4	Moss colonies near Terra Nova Bay
5	Soil around penguin's nest from East Ongul Island
6	*Cryptococcus* sp. (fungus)
7	*Cryptococcus* sp. (fungus)
8	*Penicillium* sp. (fungus)
9	*Penicillium* sp. (fungus)
10	*Penicillium* sp. (fungus)
11	*Mucor hiemalis* f. *hiemalis* (fungus)
12	*Trichoderma* sp. (fungus)
13	Unidentified fungus
14	*Dioszegia* sp. (fungus)
15	*Rhodotorula* sp. (fungus)
16	*Leucosporidum* sp. (fungus)
17	*Mrakia* sp. (fungus)

Table S2: Conditions used for analysis of different toxic compounds using HPLC

Sample No	Compound name	HPLC conditions		
		Detector (nm)	Mobile phase (% ratio)	Retention time (min)
1	4-Butylphenol	277	Acetonitrile: water (8:2)	3
2	4-*sec*-Butylphenol	277	Acetonitrile: water (8:2)	3
3	4-*tert*-Butylphenol	277	Acetonitrile: water (8:2)	3
4	4-*tert*-Octylphenol	277	Acetonitrile: water (8:2)	5.3
5	4-Nonylphenol	277	Acetonitrile: water (8:2)	7.2
6	Phenol	277	Acetonitrile: water (8:2)	3
7	4-Chlorophenol	280	Acetonitrile: 1% Ethylacetate (1:1)	3
8	Naphthalene	254	Acetonitrile: water (8:2)	2.1
9	Phenanthrene	254	Acetonitrile: water (8:2)	5.5
10	Bisphenol A	280	Acetonitrile: water (1:1)	4.3

Figure legends

Fig. 1. Denaturing gradient gel electrophoresis profile of PCR amplified V3 region of 16S rDNA in the Antarctic soil microbial communities. Sample 1: soil sample from the Antarctic region; Sample 2: culture enriched under aerobic condition; Sample 3: culture enriched under anaerobic condition. a: *Syntrophus* sp. (89% sequence identity with *Syntrophus buswellii* DM-2). b: *Pseudomonas koreensis* (98% sequence identity with *Pseudomonas koreensis*). c, d, e: *Pseudomonas gessardii* (100% sequence identity with *Pseudomonas gessardii*). f, g, h, i, j were not determined due to the low concentration of DNA.

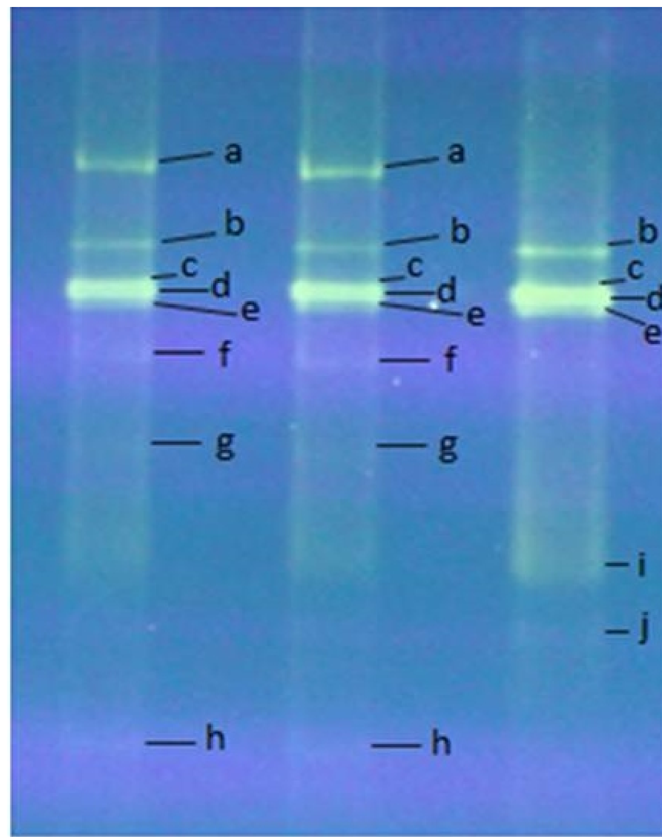
Fig. 2. Morphological characters of strain CHY-2. a) Surface of the PDA plate, b) Back shot of the plate, c) a microscopic image of *Penicillium* sp. CHY-2. d) A neighbour-joining tree showing the phylogenetic relationship of the ITS-5.8S rDNA nucleotide sequence of CHY-2 strain with related organisms. Bootstrap values of 100 analyses are shown at the branch point. CHY-2 strain is shown in orange color. Accession numbers at the GenBank of National Center for Biotechnology Information (NCBI) are shown in parenthesis.

Fig. 3. Effect of temperature on a) 4-BP degradation; b) cell growth using CHY-2. Sample were collected and residual concentration of 4-BP was analysed by HPLC. Data represent means of triplicate experiments. Error bar indicate 95% confidence intervals.

Fig. 4. Effect of glucose and NaCl concentration on 4-BP degradation using CHY-2.

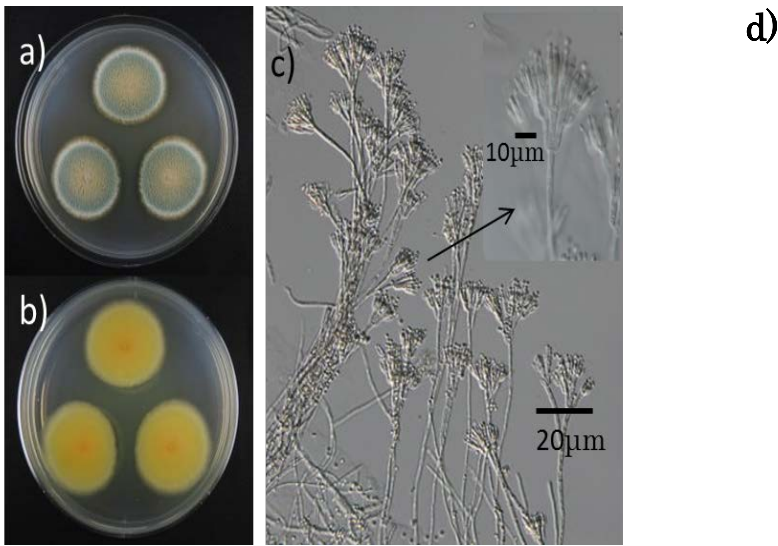
Fig. 5. Dry cell weight and 4-BP degradation using CHY-2 at a) 4°C; b) 15°C.
Symbols: ●: dry cell weight with addition of Tween 80, ▲: dry cell weight without addition of Tween 80, ■: 4-BP degradation without addition of Tween 80, ♦: 4-BP degradation with addition of Tween 80.

Fig. 6. a) Gas chromatogram of the intermediates produced during 4-BP degradation; b) Mass spectra of the peak I. Metabolites I and II were identified as 2-*n*-butylhydroquinone and hydroquinone.



Sample 1 Sample 2 Sample 3

Figure 1



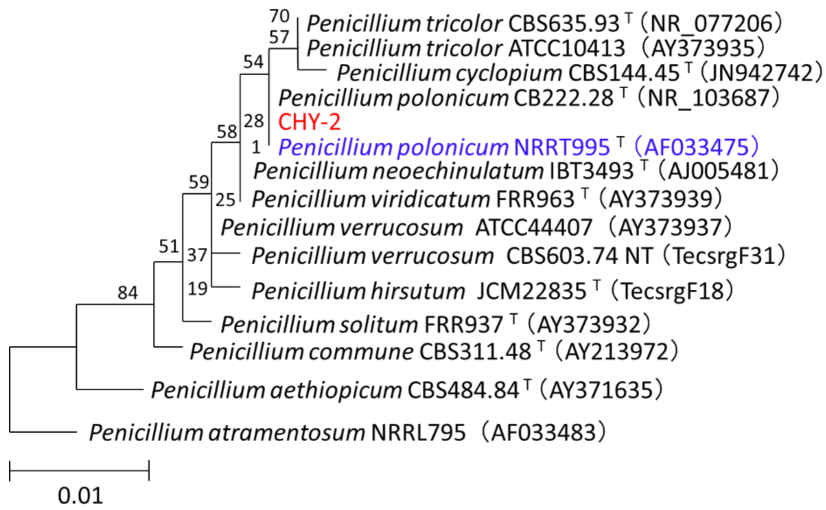


Figure 2

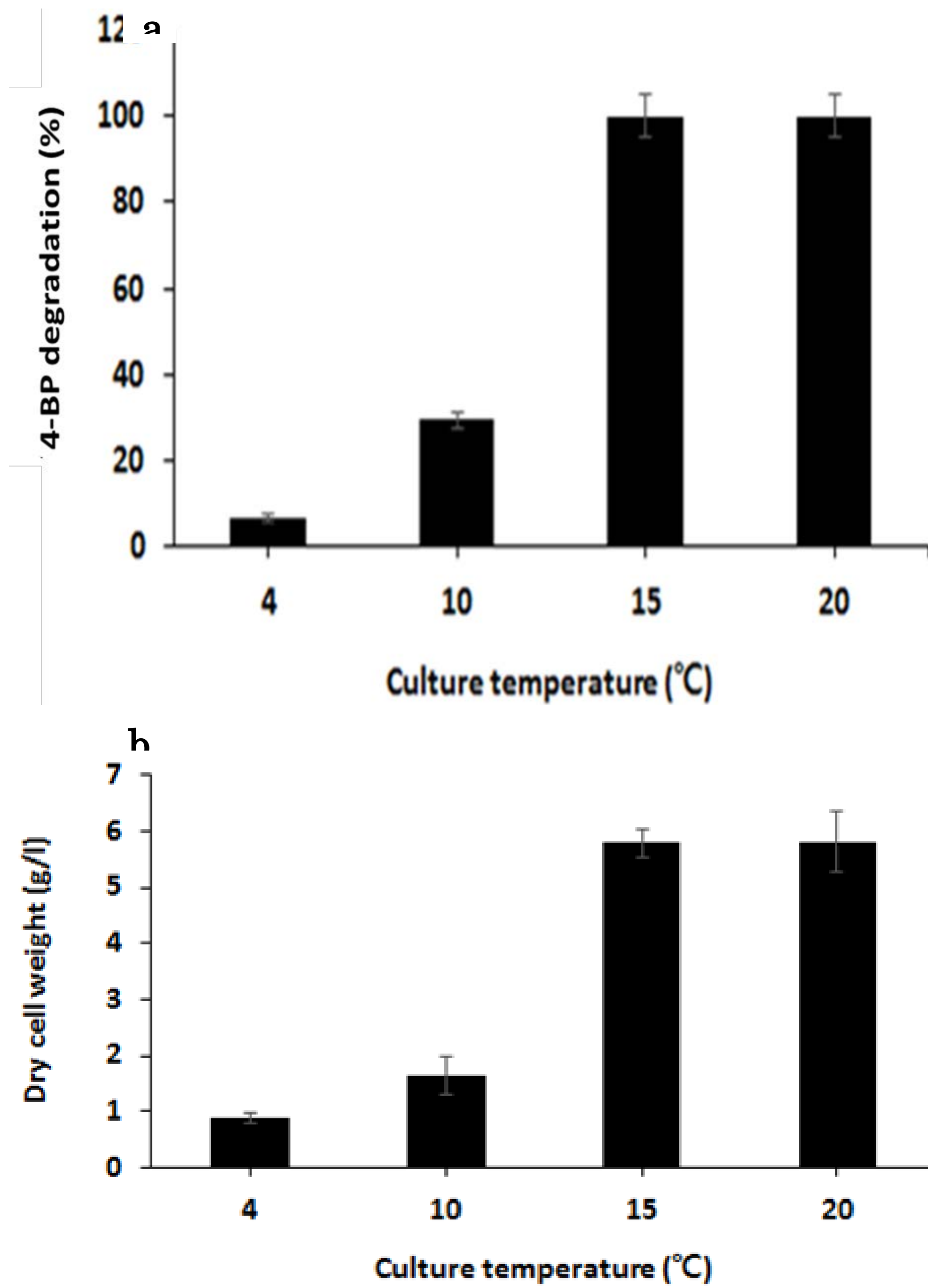


Figure 3

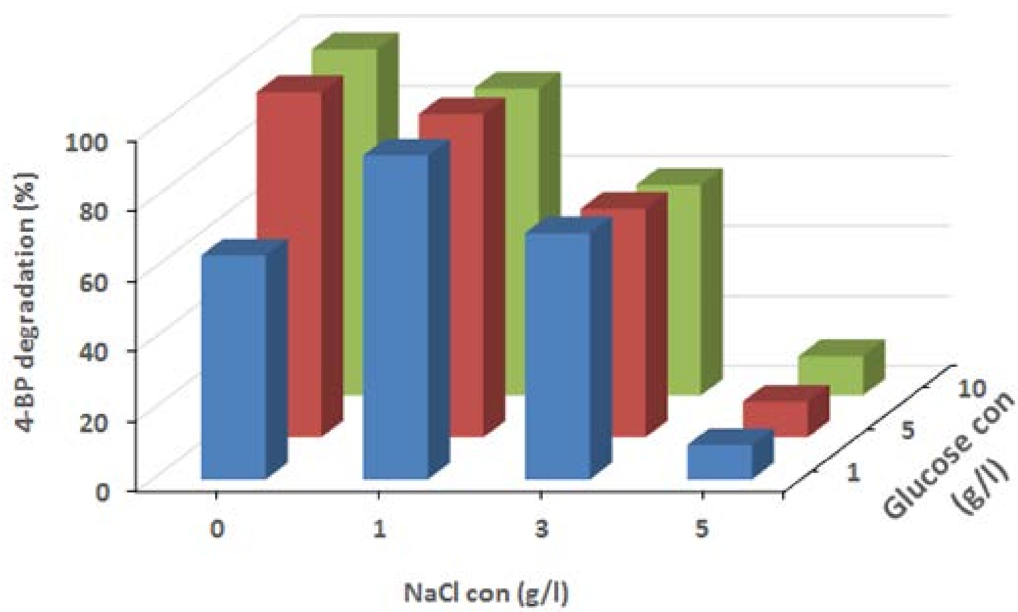
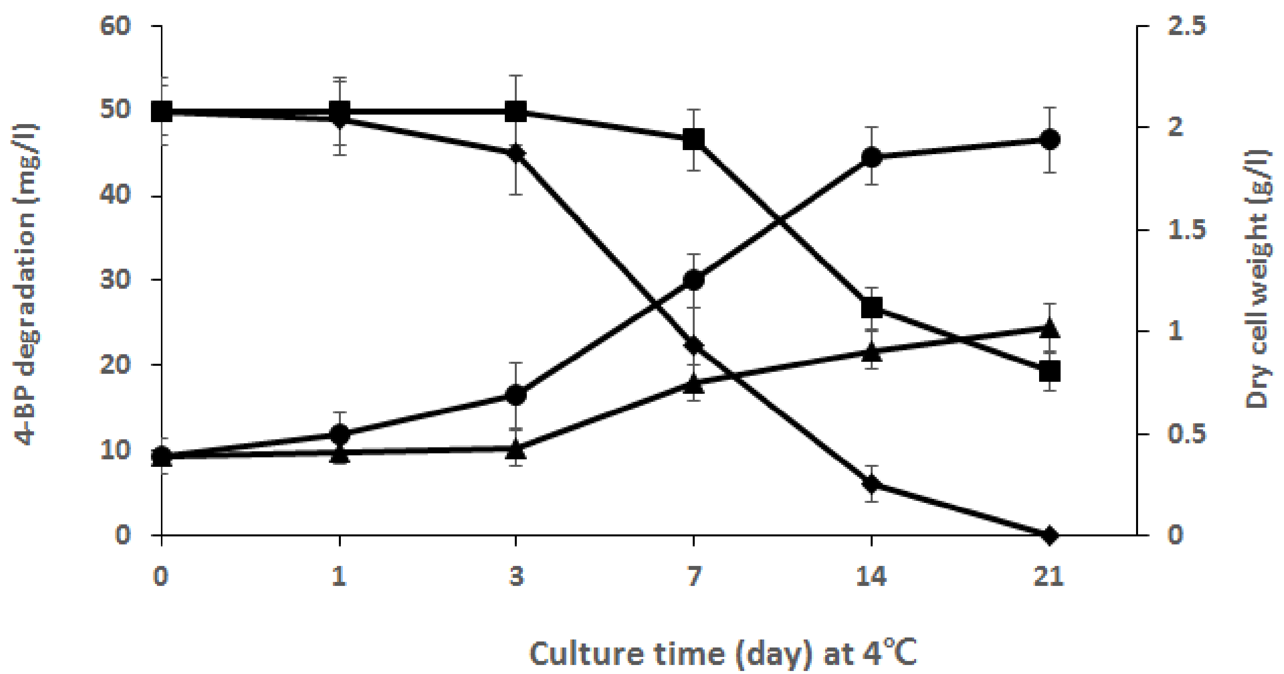


Figure 4



b

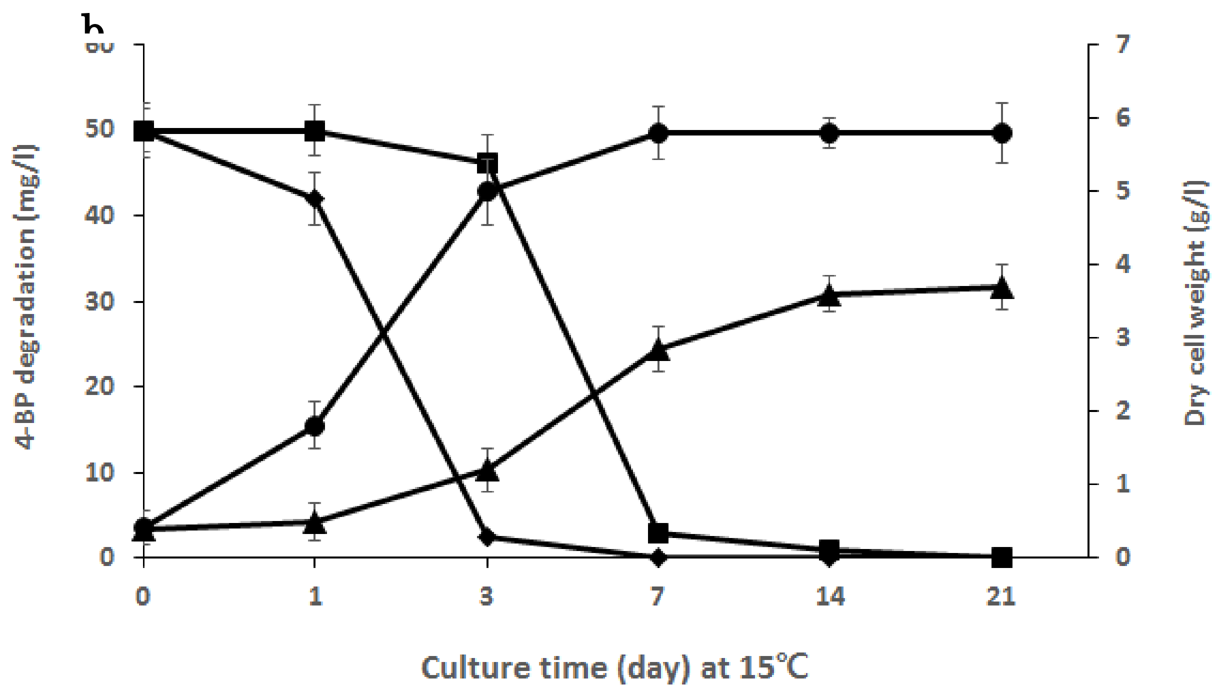


Figure 5

a

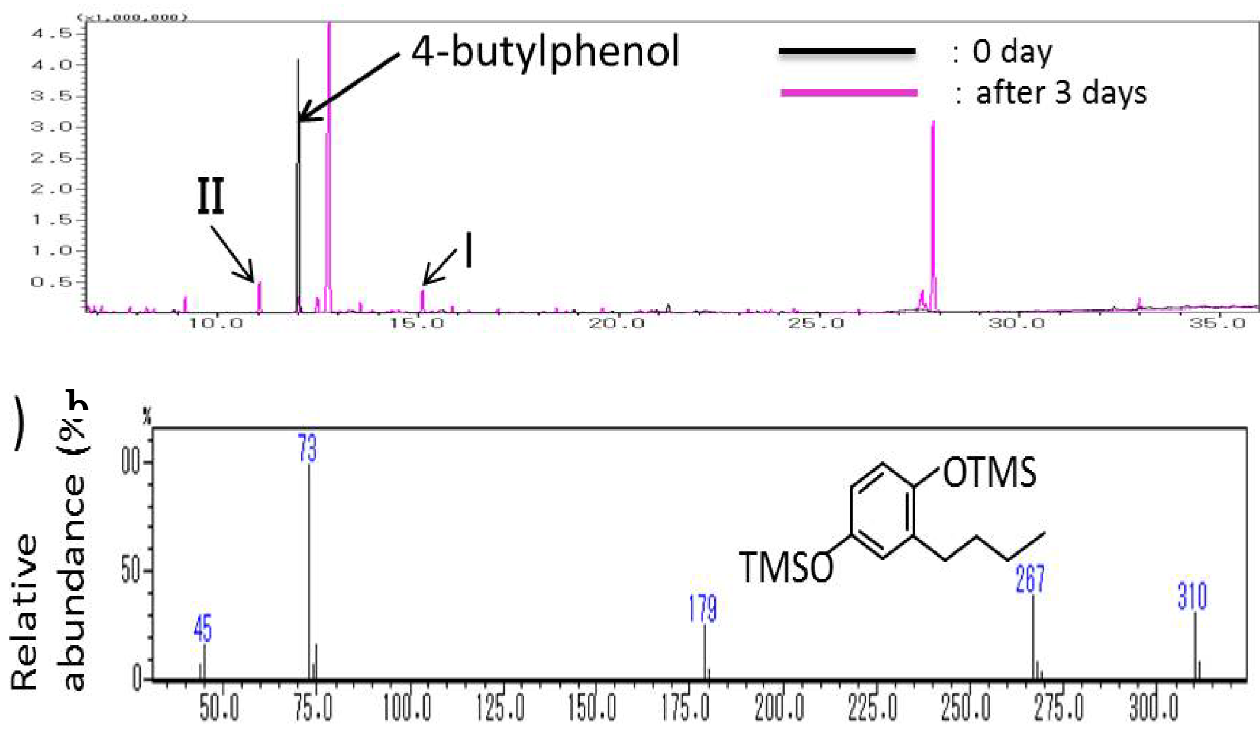


Figure 6