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Validation of biphenyl degradation pathway by polymerase chain reaction, peptide mass fingerprinting and enzyme analysis

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ABSTRACT

Our previous studies showed, bacterium *Aquamicrobium* sp. SK-2 could degrade biphenyl and polychlorinated biphenyls (PCBs). In the present study, proteins involved in the biphenyl degradation was evaluated using various molecular biology methods. The gene *bphC* present in the strain SK-2 was identified using the polymerase chain reaction method. Further the key enzyme in biphenyl degradation, 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) was purified through anion exchange and gel filtration chromatography, subsequently the enzyme activity was measured. The N-terminal amino acid sequence of the purified enzyme showed 92% homology with BphC enzyme of Gram-negative bacteria (*Pseudomonas* sp. KKS102, *Comamonas testosterone*, *Burkholderiaceae bacterium*, *Delftia acidovorans*, and *Achromobacter denitrificans*). Fractions collected during protein purification were applied on SDS-PAGE gel. Significant bands were selected in SDS-PAGE gel, and the gel pieces were cut out to analyze the proteins using peptide mass fingerprinting (PMF) method. PMF method provided useful information about the proteins involved in biphenyl degradation. Apart from BphC, two other enzymes, benzoate dioxygenase and catechol 2,3-dioxygenase which were involved in biphenyl degradation process were identified. The results indicate that catechol can be degraded to 2-hydroxymuconic-semialdehyde and this result is in accordance with the results from our previous study. Based on all these results we can conclude that the strain SK-2 is a potential candidate for the bioremediation of biphenyl contaminated places.

1. Introduction

Polychlorinated biphenyls (PCBs) are insistent organic pollutants and creating serious environmental problems throughout the world (Pieper, 2005). Environmental pollution caused by PCBs is still abundant in Japan (Takashi et al., 2008). Cleanup of contaminated sites through physical methods is expensive. Bioremediation is a cost-effective and environment friendly method for the cleanup of contaminated soil and water (Chang et al., 2020a, 2020b, 2013a, 2013b). Various microorganisms which can aerobically degrade the PCBs were isolated in previous studies (Hatamian-Zarmi et al., 2009; Sakai et al., 2005). Interestingly, biphenyl degrading microorganisms can degrade PCBs with the same enzymes laboring in biphenyl catabolism (Abramowicz, 1990). Microorganisms can utilize monochlorobiphenyls as a sole source of carbon and energy. Compounds containing two or more chlorines are generally degraded via co-metabolism, and required biphenyl as a growth substrate. But, the compound biphenyl is toxic and difficult to disperse in the polluted soils or sludge. This problem can be solved by the isolation of organisms which can grow solely on PCBs without the need of biphenyl as co-substrate (Adebusoye et al., 2008). On this direction several PCBs degrading strains *i.e., Rhodococcus*, *Paenibacillus*, *Pseudoalcaligenes*, *Burkholderia xenovorans*, *Pseudomonas aeruginosa*, *Ralstonia* sp. and *Aquamicrobium* sp. SK-2 were isolated (Chang et al., 2013a, 2013b; Hatamian-Zarmi et al., 2009; Adebusoye et al., 2008; Jaysankar et al., 2006; Sakai et al., 2005).

Some of the above-mentioned organisms can use dichlorobiphenyls/or trichlorobiphenyls for their growth (Chang et al., 2013a, 2013b; Tu et al., 2011; Hatamian-Zarmi et al., 2009; Adebusoye et al., 2008; Kim and Picardal, 2001), but two bacteria named *Pseudomonas aeruginosa* TMU56 and *Aquamicrobium* sp. SK-2 are able to utilize 2,2′,4,4′,5,5′-hexachlorobiphenyl for their growth. Specially, the bacterium *Aquamicrobium* sp. SK-2 previously isolated in our laboratory can utilize the coplanar PCB (3,3′,4,4′-tetrachlorobiphenyl) in the presence of 3.0% NaCl or 500 mg L^{-1} KNO_3, so this salt tolerant bacterium can be applied

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for the bioremediation of high NaCl containing contaminated places. Also, the PCB utilization rate of this bacterium was higher than other PCB degrading bacteria.

On the other hand, for more than three decades researchers focused on the microbial degradation of PCBs, biphenyl and its degradation intermediates such as benzoate and catechol (Koubek et al., 2013; Pieper and Seeger, 2008). On this aspect, we already reported the degradation pathway of biphenyl using *Aquamicrobium* sp. SK-2 (Chang et al., 2013a, 2013b) (Fig. S1). The putative degradation pathway indicates that strain SK-2 can metabolize biphenyl to 2-hydroxymuconic-semialdehyde via catechol. Some organisms have the upper pathway to produce metabolites like benzoate and catechol (Pieper and Seeger, 2008; Ohtsubo et al., 2000). Unfortunately, benzoate and catechol are toxic organic compounds, therefore it is necessary to know whether these toxic compounds are further metabolizing by the bacteria or not. Hence in our previous study, we evaluated the degradation of biphenyl, PCBs, and the resultant metabolites were determined using GC–MS analysis in the strain SK-2. Consequently, putative degradation pathway was postulated. Our aim is to find out whether the target gene *bphC* existed in the strain SK-2 or not. To find out this, continuation of our previous study, in the present study the gene *bphC* which encodes the important enzyme in biphenyl degradation, 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) was identified using polymerase chain reaction method. Further, other three enzymes involved in biphenyl degradation were identified by peptide mass fingerprinting (PMF) method. Furthermore, enzyme analysis for two enzymes, BphC and catechol 2,3-dioxygenase in SK-2 was carried out. Based on these results biphenyl degradation pathway in the strain SK-2 was validated.

2. Experimental methods

2.1. Chemicals

All chemicals used were of analytical grade and were purchased from Sigma. Biphenyl was purchased from Tokyo Chemical Industry, Japan.

2.2. DNA isolation and PCR amplification

Genomic DNA from the strain SK-2 was isolated using a NucleoSpin kit (Takara-bio, Japan). NCBI-BLAST was used to collect the nucleotide sequences of the *bphC* gene from existing aromatic compounds degrading bacteria. Four Gram-negative and four Gram-positive bacteria were selected. Primer synthesis was done and PCR amplification with a pair of (forward and reverse) primers was done. Conditions used in the PCR were stated in Table S1. PCR amplification was performed according to Chang et al. (2011). We selected the *bphC* gene in this study because this gene encodes the important enzyme 2,3-dihydroxybiphenyl-1,2-dioxygenase (BphC) which play a key role in biphenyl degradation. After conforming this gene through PCR amplification, we further purified the correspondent protein and enzyme activity was measured.

2.3. Protein partial purification

The bacterium *Aquamicrobium* sp. SK-2 was pre-cultured aerobically in mineral salt medium containing biphenyl (200 mg L^{-1}) for 48 hours, then collected by centrifugation (8000×*g*, 5 min, 4 °C) and the obtained pellet washed with potassium phosphate buffer (50 mM, pH 7.5) to remove the unwanted materials. The resulting cell suspension was inoculated in to 500-mL biphenyl containing mineral salt medium (OD_{600}: 0.02), and then flasks were aerobically incubated at 28 °C for 72 hours under shaking conditions. For enzyme purification, 72 h grown culture from four flasks was centrifuged for 10 min at 8000×*g*, the obtained pellet was suspended in 3–10 mL of potassium phosphate buffer (50 mM) and sonicated for 23 min for 15 cycles (60 seconds on and 30 s off) at 133 kW, 50 kHz with a SONIFIER250, BANSON sonicator. After sonication, the sample was centrifuged for 20 min at 6000×*g*, and the supernatant was filtered through 0.45 μm filter (Advantec, Dismic-25AS). The filtrate collected used as the crude enzyme (cell-free extract) and it was subjected to a cartridge prepacked with 5 mL of Bio-Scale Mini Macro-Prep HighQ Cartridge (BioRAD, USA) strong anion exchange media. The column was equilibrated with 50 mM Tris-HCl buffer at pH 7.5. The flow rate was maintained at 0.5 mL min^{-1}. A total of 60 fractions each of 1.0 mL was collected.

2.3. PMF analysis

For protein identification by mass spectrometry, protein spots in SDS-PAGE gel were enzymatically digested in a gel according to the previously described method (Shevchenko et al., 1996) with little modifications by adding porcine trypsin (Promega). Gel pieces were washed with 50% acetonitrile to remove SDS, salt and stain. Washed and dehydrated spots were then vacuum dried to remove solvent and rehydrated with trypsin (8–10 ng μL^{-1}) solution in 50 mM ammonium bicarbonate pH 8.7 and incubated 8–10 h at 37 °C.

Samples were analyzed using the Applied Biosystems 4700 proteomics analyzer with TOF/TOFTM ion optics. Both mass spectrometer (MS) and MS/MS data were acquired with a Nd:YAG laser with 200 Hz repetition rate, and up to 4000 shots were accumulated for each spectrum. MS/MS mode was operated with 2 keV collision energy; air was used as the collision gas such that nominally single collision conditions were achieved. Although the precursor selection has a possible resolution of 200, in these studies of known single component analyses a resolution of 100 was utilized. Both MS and MS/MS data were acquired using the instrument default calibration, without applying internal or external calibration. Sequence tag searches were performed with the program MASCOT (http://www.matrixscience.com).

2.4. Estimation of catechol cleaving enzyme

Estimation of catechol cleaving enzyme was carried out using crude extract (cell-free extracts), 0.6 mL of potassium phosphate buffer (50 mM), 0.2 mL of 0.01 M catechol solution, and 0.2 mL of the cell-free extract. The reaction was started at room temperature and the absorbance at a wavelength of 375 was measured with a UV–Vis spectrophotometer (UV-1800, Shimadzu, Japan) according to the method described in Nozaki et al. (1963). In principle, the increase of 2-hydroxymuconic-semialdehyde was recorded.

2.5. Purification of BphC enzyme

Purification of BphC was performed by an anion exchange column (Bio-scale Mini Macro-Prep HighQ Cartrige, Bio-Rad) using the above-mentioned crude enzyme. Protein concentration was determined for each fraction according to the protocol of Pierce™ Coomassie Plus (Bradford) Assay Kit (Thermo Fisher Scientific). The anion exchange column was equilibrated with 50 mM Tris-HCl (pH 7.5), and then the sample was added. The protein was eluted by linearly changing the concentration of sodium chloride (0.1 to 1 M). The flow velocity was 0.5 mL min^{-1} and the fraction size was 1 mL. Fractions with BphC activity were mixed and applied to a gel filtration column with Hi Prep 16/60 (Sephacryl S-200HR, GE Healthcare, Japan). The gel filtration column was

equilibrated with 50 mM Tris-HCl (pH 7.5). The protein was eluted with the buffer used for equilibration at a flow rate of 0.5 mL min^{-1}. The eluate was fractionated in the same manner as the anion exchange column.

After purification, enzyme activity was measured. To measure the enzyme activity, 970 μL of 50 mM Tris-HCl buffer (pH 7.5), 10 μL of 10 mM 2,3-dihydroxybiphenyl-acetone solution, and 20 μL of enzyme solution was added in the cuvette. Immediately the OD value at wavelength of 432 nm was measured for 5 minutes using the UV–Vis spectrophotometer. In principle, *meta*-cleavage of the substrate 2,3-dihydroxybiphenyl leads to formation of 2-h ydroxy-6-oxo-6-phenylhexa-2,4-dienote and this compound can be measured at OD_{432} (Catelani et al., 1973).

2.6. GC–MS analyses

The metabolites of 2,3-dihydroxybiphenyl was determined by GC–MS. The samples were acidified with 1 N HCl, shaken for 3 min with an equal volume of 1:1 (v v^{-1}) ethyl acetate, and centrifuged at 8,000 × g for 10 min. The organic layer was then extracted and analyzed directly by GC–MS. Prior to the GC–MS analysis, metabolite was dried under a nitrogen flow and derived by trimethylsilylation (TMS) using a BSTFA-acetonitrile solution at 60 °C for 1 hour. The GC–MS analysis was conducted on a Shimadzu GC–MS system (GCMS-QP2010) with an Rxi-5 ms capillary column (30 m, 0.25 mm ID, 1.00 lm df; Restek, Pennsylvania, USA).

3. Results and discussion

Genomic DNA from the strain SK-2 was isolated and the gene *bphC* that encodes the important enzyme (BphC) in biphenyl degradation was identified using polymerase chain reaction. Further, the enzyme BphC was purified from the strain SK-2 and enzyme assay was carried out. Other key enzymes involved in biphenyl degradation was identified by peptide mass fingerprinting method. All the results were discussed in the following sections.

3.1. bphC gene amplification

Genomic DNA from the strain SK-2 was isolated to analyze whether the target gene, *bphC* existed or not in the same strain. To find out this, nucleotide sequences of the *bphC* gene from already reported both Gram-positive and Gram-negative bacteria were collected, and primer synthesis was done. After PCR amplification the size and purity of the *bphC* gene was analyzed by agarose gel electrophoresis. Fig. S2 shows the gel images of *bphC* gene amplification done using the primers designed from Gram-negative and Gram-positive bacteria respectively. No bands were observed with the primers derived from the Gram-negative bacteria, but bands were observed with the primers derived from the Gram-positive bacteria. One clear band and a tint band with different sizes, *i.e.,* 800 bp and 1700 bp were observed in the first lane. The size of target bphC gene of *Rhodococcus equi* 103S in the first lane was 871 bp. Thus, band near 800 bp and band near 1700 bp is still unknown. In the second lane, the band near 810 bp was observed, this is not matched with *bphC* gene (size, 950 bp) of *Rhodococcus jostii* RHA1. On the other hand, in the third lane, a tint band near 600 bp was observed, but it is not matching with actual size of *bphC* gene (902 bp) of *Mycobacterium* sp. In the fourth lane, only smear was observed. The SK-2 strain is a Gram-negative bacterium; interestingly PCR amplification was unsuccessful with primers derived from the Gram-negative bacteria. This result indicates that *bphC* gene in the SK-2 strain may be different with the above mentioned *bphC* gene from reported both Gram-positive and Gram-negative bacteria.

3.2. Protein purification, PMF and enzyme analysis

Various fractions were collected during the process of protein purification. High BphC activity was observed with the fraction numbers 27, 34, 36, and 46 (Fig. S3). These fractions were applied in an SDS-PAGE gel (Fig. 1). Fraction number 47 was also applied as a reference of low activity sample. Various bands were observed in gel, among these, three protein bands (band-1, 2, 3) of the fraction number 36, which might be related to the biphenyl degradation, were cut out from the gel for identification of proteins through PMF analysis. As shown in Fig. 1, the molecular weight of protein allocated to band-1 was 77.0 kDa, and it shows the 38% protein homology with the enzyme catechol 2,3-dioxygenase present in the bacterium *Burkholderia cepacia* GG4. Hence the activity of catechol 2,3-dioxygenase in the strain SK-2 was analyzed in this study using cell free extracts. During the enzyme assay, optical density at the wavelength of 375 nm was increased after adding the crude enzyme to the reaction mixture which containing catechol and buffer (Fig. S4). With this enzyme assay, it is inferred that catechol degradation will occur in the biphenyl degradation process in the strain SK-2. It was also confirmed by PMF analysis by identifying the band-1 in MS spectrum (Fig. 2). Catechol 2,3-dioxygenase is an enzyme that degrades catechol into 2-hydroxymuconic-semialdehyde via a *meta*-pathway (Setlhare et al., 2019). The bacteria *Burkholderia cepacia* GG4 has the capacity for degradation of biphenyl and PCBs (Kyoto Encyclopedia of Genes and Genomes database).

As shown in Fig. 1, band-2 has a molecular weight less than 23 kDa and shows the highest protein homology with transcription

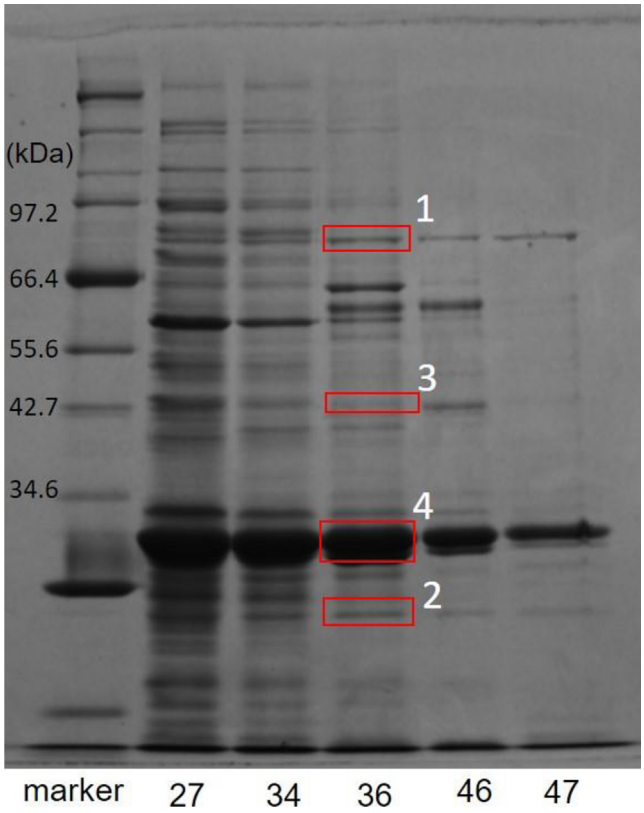

Fig 1. SDS-PAGE gel image of proteins purified through anion exchange and gel filtration chromatography.

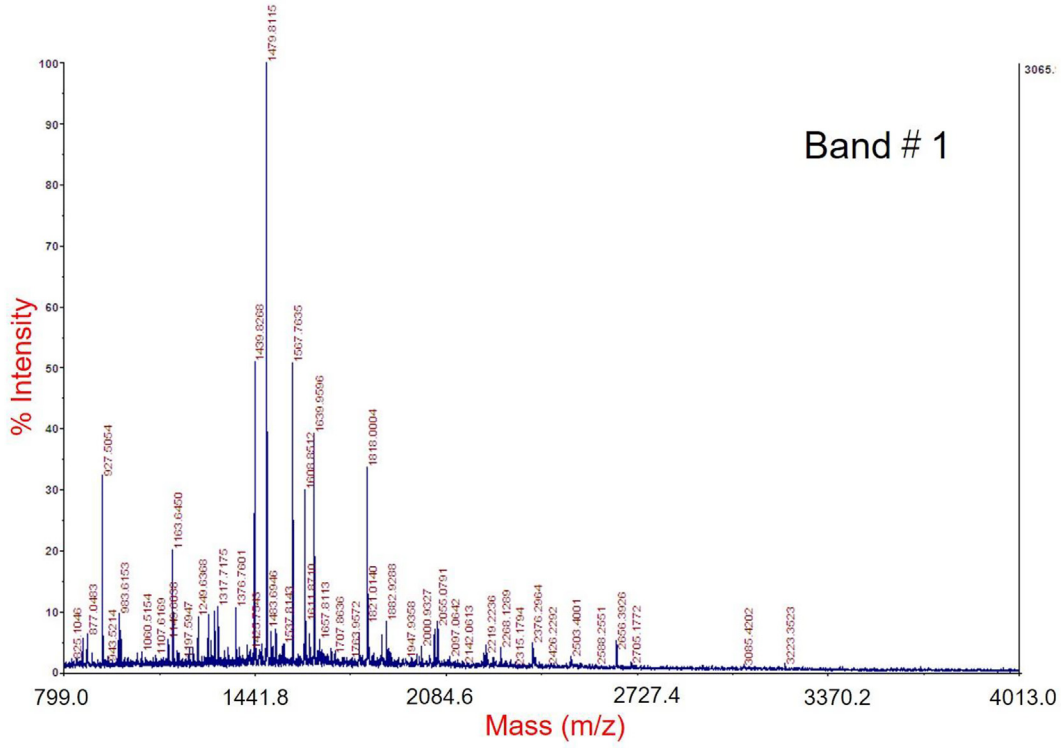

Fig 2. MS spectrum of band number 1. MS data obtained with the instrument default calibration without using internal or external calibration. Sequence tag searches done with the MASCOT program.

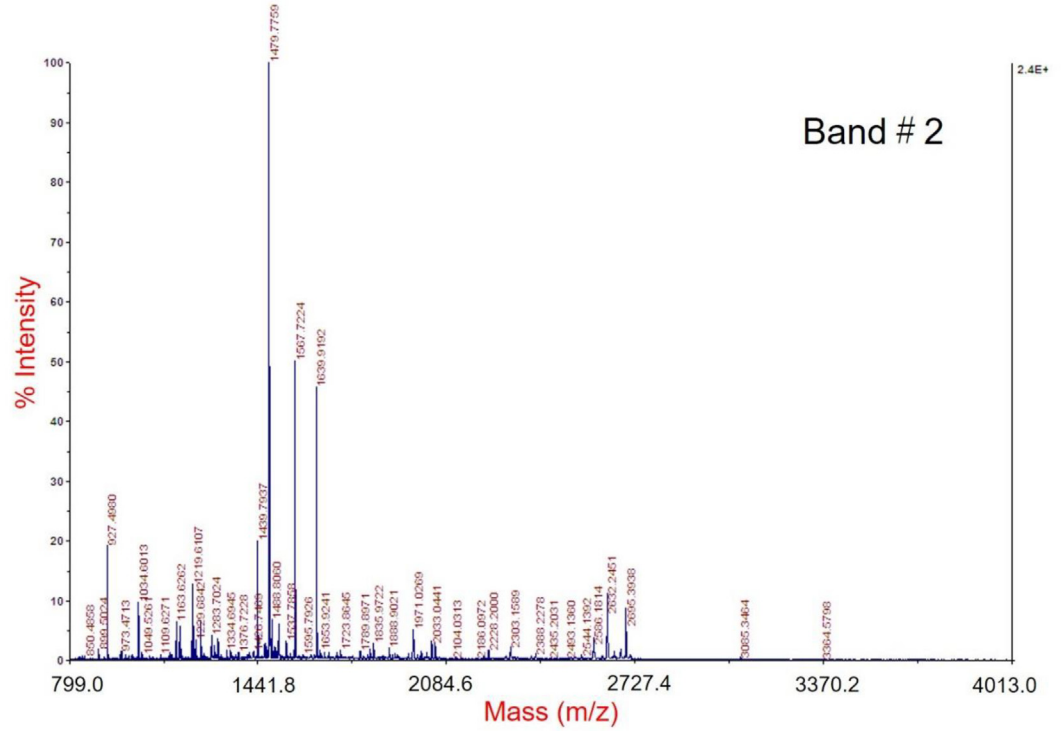

Fig 3. MS spectrum of band number 2. MS data obtained with the instrument default calibration without using internal or external calibration. Sequence tag searches done with the MASCOT program.

elongation factor NusA from *Burkholderia cenocepacia* AU 1054. The bacteria *Burkholderia cenocepacia* can degrade several aromatic compounds such as styrene, chlorocyclohexane and chlorobenzene (Kyoto Encyclopedia of Genes and Genomes database). No reports were available about involvement of this protein (present in band-2) in biphenyl degradation; hence, no further studies were carried

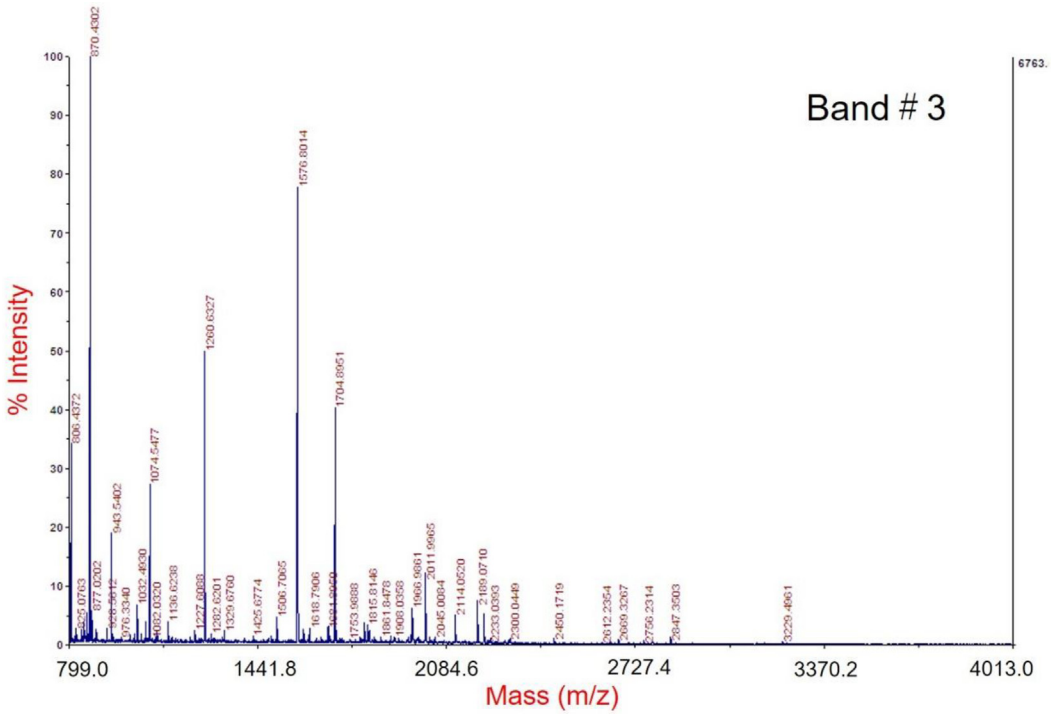

Fig 4. MS spectrum of band number 3. MS data obtained with the instrument default calibration without using internal or external calibration. Sequence tag searches done with the MASCOT program.

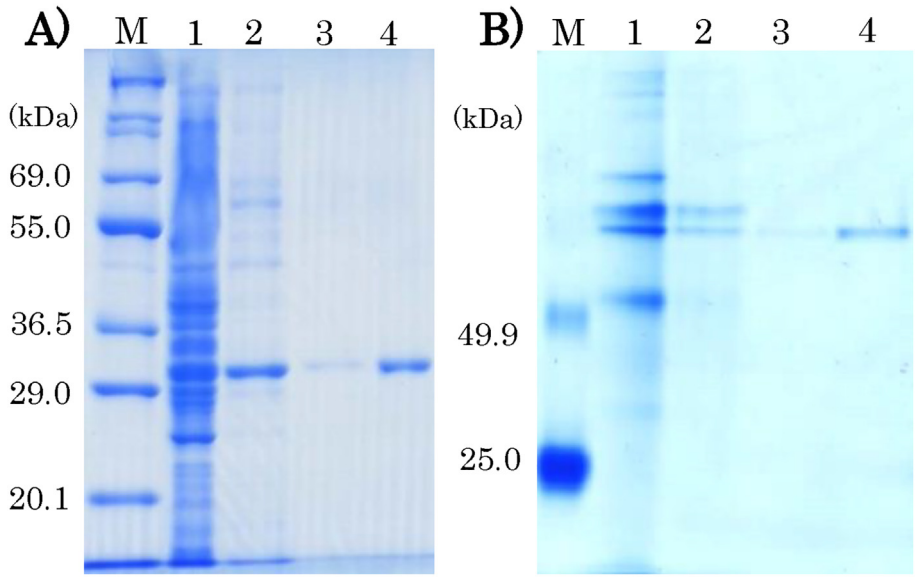

Fig 5. SDS-PAGE and Native-PAGE analysis of BphC enzyme. M: maker; 1: crude enzyme; 2: fraction sample after ion exchange; 3: fraction sample after gel filtration; 4: concentrated enzyme after gel filtration. Concentration was performed using Amicon® Ultra-4 Centrifugal Filter Units– 10,000 NMWL.

out to investigate about this protein. MS spectrum of band-2 was shown in Fig. 3. Protein present in band-3 has a molecular weight of 45.6 kDa and shows the 41% protein homology with the enzyme benzoate dioxygenase from *Burkholderia vietnamiensis* G4 strain. According to literature studies, benzoate dioxygenase is an enzyme that degrades benzoate into 1,6-dihydroxycyclohexa-2,4-diene-1-carboxylate. The strain *Burkholderia vietnamiensis* G4 is capable of degrading benzene, *o*-cresol, *m*-cresol, *p*-cresol, phenol, toluene, trichloroethylene, naphthalene and chloroform (O'Sullivan and Mahenthiralingam, 2005). MS spectrum of this protein was shown in Fig. 4.

The protein present in band-4 was further purified since the band was predicted as BphC (Fig. 1). So, BphC was purified by two step purification process using ion exchange and gel filtration chromatography columns. A purified band was confirmed at 32 kDa from the analysis of SDS-PAGE electrophoresis (Fig. 5A). However, it is possible that protein molecules form multimers and subunit structures in vivo. Therefore, Native-PAGE electrophoresis was performed. A single band was confirmed around 65 kDa (Fig. 5B), which was consistent with the estimated molecular weight of the protein using the gel filtration column (data not shown). Furthermore, since this molecular weight is twice that of

Table 1
The bacterial names and N-terminal amino acid sequences of *BphC*.

Bacterial names	N-terminal amino acid sequences of *BphC*	Identification (%)
Aqumicrobium sp. SK-2	**SXERLGYLGFAV**	-
Pseudomonas sp. KKS102	**SIERLGYLGFAV**	92
Comamonas testosteroni	**SIERLGYLGFAV**	92
Burkholderiaceae bacterium	**SIERLGYLGFAV**	92
Delftia acidovorans	**SIERLGYLGFAV**	92
Achromobacter denitrificans	**SIERLGYLGFAV**	92
Nocardioides gansuensis	**SVQRLGYLGFEV**	75
Cupriavidus lacunae	**SIQRLGYLGFEV**	75
Pseudomonas sp. KF707	**SIRSLGYMGFAV**	67
Burkholderia sp. LB400	**SIRSLGYMGFAV**	67
Dyella sp. LA-4	**SVKNLGYMGFSV**	59

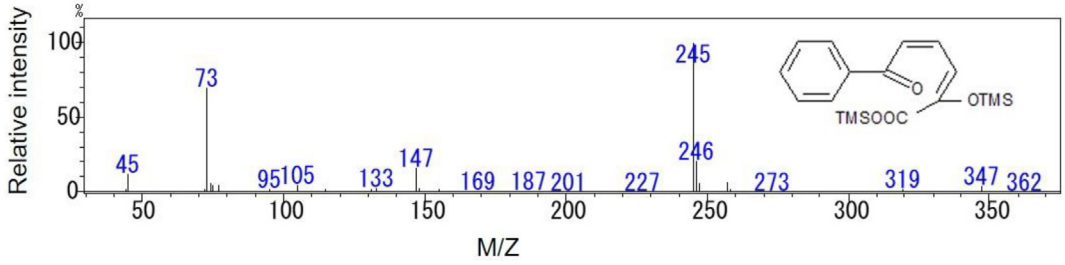

Fig 6. The gas chromatogram of the metabolite, 2,3-dihydroxybiphenyl.

SDS-PAGE electrophoresis, BphC was considered to be a dimer. The enzyme showed activity at wide range of temperatures, and from a neutral to alkali pH, it showed highest activity, at 30 °C and pH 8, respectively (Fig. S5). Previous reports also denoted that this enzyme in various organisms showed higher activity at basic pH conditions. The enzyme showed better activity at pH 8.0 in *Rhodococcus* sp. K37, at pH 8.5 in *Burkholderia* sp. JF8, and at pH 9.0 in *Rhodococcus* sp. R04 (Bian et al., 2012; Fei et al., 2011; Yang et al., 2008).

N-terminal amino acid sequence of BphC was further analyzed. The amino acid sequence of 12 residues (SXERLGYLGFAV) were compared with the previously reported N-terminal amino acid sequences of BphC derived from aromatic compounds-degrading bacteria using the NCBI-BLAST search. Sequencing results denoted that the enzyme BphC from strain SK-2 had high homology (92%) with the amino acid residues of *Pseudomonas* sp. KKS102 (Table 1).

On the other hands, degradation of aromatic compounds was examined using purified enzyme BphC. The aromatic compounds used in the experiment were selected in consideration of the fact that BphC is an extradiol type enzyme. The aromatic compounds used were 2,3-dihydroxybiphenyl, benzoic acid, phenanthrene, 4-chlorophenol, catechol, diphenylamine, nitrobenzene, toluene, aniline, 1,3,5-triphenylbenzene, naphthalene, *o*-xylene hydroquinone, resorcinol, 4-chlorocatechol, and 3,5-dichlorocatechol (Table S2). As a result, only two aromatic compounds (2,3-dihydroxybiphenyl, catechol) could be degraded by BphC. Further BphC enzyme assay in strain SK-2 was carried out using the substrate 2,3-dihydroxybiphenyl, buffer and purified enzyme. Degradation of the substrate with simultaneous generation of the metabolite, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid, was determined by GC–MS analysis (Fig. 6).

4. Conclusions

In our previous study, we reported that *Aquamicrobium* sp. SK-2 is involved in the degradation of biphenyl and PCBs. To validate this, present study focused on the identification of genes and enzymes involved in this process. A key enzyme (BphC) involved in biphenyl degradation was purified, and the enzyme activity was recorded. Through PCR method, it was denoted that *bphC* gene in the SK-2 strain could be horizontally transmitted from the *Rhodococcus* genus. Metabolites produced during biphenyl degradation were previously analyzed based on GC–MS analysis and now the responsible enzymes involved in the process were confirmed by PMF analysis. PMF analysis results indicated that the toxic metabolite catechol could be degraded to 2-hydroxymuconic-semialdehyde by the action of enzyme catechol 2,3-dioxygenase which already existed in the strain SK-2. Based on all these results we can conclude that the strain SK-2 possess genes and enzymes which are required for the degradation of biphenyl.

CRediT authorship contribution statement

Young-Cheol Chang: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Validation, Writing - original draft, Writing - review & editing. **Hideto Sugawara:** Data curation, Formal analysis. **M. Venkateswar Reddy:** Data curation, Methodology, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wen.2021.04.001.

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