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Expression, Purification and Characterization of *meta*-cleavage Enzyme CarBaBb from *Novosphiongobium* sp. KA1

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ABSTRACT

The *meta*-cleavage enzyme CarBaBb of carbazole-degrader *Novosphiongobium* sp. KA1 were cloned, expressed and purified to homogeneity in *Escherichia coli* strain. The enzyme was cloned with 6x histidine residues attached at the C-terminal of large subunit CarBb for purification using affinity chromatography method prior to gel filtration chromatography. The CarBaBb, a two-subunit *meta*-cleavage enzyme, approximately 30 kDa for CarBb dan 10 kDa for CarBa, was found to be $\alpha_2\beta_2$-heterotetrameric (*M*r 80,000), showed highest activity at pH 8.5 and temperature 30°C. CarBaBb showed highest catalytic activity towards 2,3-dihydroxybiphenyl with k_{cat}/K_m 4.1 $M^{-1}s^1$, and overall higher catalytic activities towards biphenyl-type substrates in comparison to catechol-type substrates. Based on the similarities, this *meta*-cleavage enzyme from *Novosphiongobium* sp. KA1 would also be a good candidate for protein crystallization and structural studies apart from CarBaBb from strain *P. resinovorans* strain CA10.

INTRODUCTION

Carbazole is a compound produced from impurities of fossil fuel which share the similar plane structure of the dioxin family and is also characterized to be a recalcitrant chemical and was reported to have carcinogen properties [1]. Isolation of bacteria capable of degrading carbazole as sole carbon and nitrogen source have been reported and genes responsible for degradation of aromatic compounds have been discovered and characterized extensively [2, 3, 4, 5].

Generally, in the carbazole degradation pathway, 2 molecules of oxygen are added to carbazole through the process of angular dioxygenation, which produces a diol derivative which is later converted to 2'-aminobiphenyl-2,3-diol through spontaneous ring cleavage [4, 5, 6]. Extradiol dioxygenase also known as the *meta*-cleavege enzyme then catalyzes the ring cleavage opening at the C-C bond next to the added hydroxy groups. In contrast, *ortho*-cleavage is the process of cleaving C-C bond between the two hydroxy groups. Product of *meta*-cleavage will be hydrolyzed by a *meta*-cleavage product hydrolase which will further transform substrates for downstream pathway before assimilation through TCA cycle [5].

The *car* genes of *Pseudomonas resinovorans* CA10 have been studied most extensively among other carbazole degrading bacteria. Extradiol dioxygenase of strain CA10 is a class III extradiol enzyme designated CarBaBb coded by carBa and carBb genes [7, 8]. Although most extradiol dioxygenase are homo-multimeric, CarBaBb is made of two proteins like LigAB enzyme [10]. CarBaBb is made of CarBb, a larger subunit which carries the catalytic site and CarBa, a smaller subunit which function remains unknown. CarBaBb from strain CA10 showed strong *meta*-cleavage activity towards biphenyl-type substrates but showed weak activity towards catechol-type substrates [11].

Both *carBa* and *carBb* genes were also found in car genes of *Novosphiongobium* sp. KA1 [9]. Significant differences between car_{CA10} and car_{KA1} gene cluster have been shown but the similarity of both CarBaBb is approximately 40% [12]. However, to date, the crystal structure of CarBaBb enzyme has not been reported [13]. Here, we report the expression, purification and characterization of *meta*-cleavage enzyme from *Novosphiongobium* sp. KA1.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and culture conditions. *Escherichia coli* strains DH5α (Takara Bio Co., Ltd., Kyoto, Japan), and BL21(DE3) (Novagen, Inc., Madison, USA) were used for expression plasmid construction and protein expression. Expression plasmids were constructed based on plasmids pUC119 and pET26b [14]. Relevant information regarding the strains and plasmids used for this study is as listed in **Table 1**.

E. coli strains were grown on LB medium or 2xYT medium containing appropriate antibiotics (final concentration of 100 μg/ml ampicillin) at 37˚C with shaking (300 strokes/min for glass tubes or 120 rpm for flasks). For plate cultures, media solidified with 2.0% agar (wt/vol) were used. Appropriate amount of isopropyl-B-D-thiogalactopyranoside (IPTG) was added to the medium to induce expression [15].

Table1. Bacterial strains and plasmids used in this study.

	Relevant characteristics	Source or reference
Strain		
E. coli DH5α	F⁻, 80d*lacZ*ΔM15, Δ(*lacZYA–argF*)U169, *deoR*, *recA1*, *endA1*,*hsdR17*(r_K^-, m_K^+), *phoA*, *supE44*, *λ*⁻, *thi–1*, *gyrA96* , *relA1*	Takara Bio
E.coli BL21(DE3)	F⁻, *ompT*, *hsd*S_B (r_B^- m_B^-), *gal*, *dcm*, (DE3)	Novagen
Plasmid		
pT7Blue	Ap^r, *lacZ*	Novagen
pUC119	Ap^r, *lacZ*	Takara Bio
pBKA102	Ap^r, pBluescript II SK(-) with 8.7-kb *Hind*III fragment of strain KA1 DNA	[9]
pUCA525	Ap^r, pUC119 with carries *carBaBb*	This study

DNA manipulation

Plasmid DNA was prepared from the *E. coli* host strain by alkaline lysis method [15, 16]. Restriction endonucleases and DNA Ligation Kit version 2 (Takara Bio, Kyoto, Japan) were used according to manufacturer's instructions. DNA fragments were fractionated by 0.9% agarose electrophoresis and extracted using Gel Extraction Kit (Roche, WA, USA) as instructed by manufacturer.

Polymerase chain reaction (PCR)

For expression plasmids construction, *carBa* and *carBb* genes were amplified from pBKA102 plasmid as the template. Each gene was amplified separately. Gene *carBa* was amplified using primers with sites *Xba*I at its 5'-end and *Bgl*II at its 3'-end. Gene *carBb* was amplified with sites *Bgl*II at its 5'-end and *BamH*I at its 3'-end. A Shine-Dalgarno (SD) sequence [17] was added in between the 5'-end restriction enzyme sites and the start codon of each gene. Codons coding for 6x His were also added before the end codon of *carBb* gene. Amplified PCR products were cloned into pT7Blue and then ligated once more into vector pUC119 between site *BamH*I and *Xba*I to construct vector pUCA525.

The primers used for amplifying *carBa* were 5'- TCTAGA-AT**AAGGAGG**TGTTCATATGGGCACGACCGCCTCCTAT-3' (underlined sequences are *Xba*I sites, bolded sequence is Shine-Dalgarno (SD) sequence) and 5'-AGATCT-TCAGCCATTGCGATCGACCATTTTCCGGTA-3' (underlined sequence is *Bgl*II site). For *carBb* amplification, the primers used were 5'-AGATCT-T-**AAGGAGG**TGGCTTGATGGCTGAAATCGTT-3' (underlined sequence is *Bgl*II site, bolded sequence is Shine-Dalgarno (SD) sequence) and 5'- GGATCC-CTA**GTGGTGGTGGTGGTGGTG**AACGATTGCTATTCC GCC-3' (underlined sequences is *Bam*HI site, bolded sequence is coding 6X His). PCR was conducted with Ex *Taq* polymerase (Takara Bio, Kyoto, Japan) using GeneAmp PCR System 9700 (PE Biosystems, Urayasu, Japan).

Expression and Preparation of cell extract

E. coli BL21(DE3) transformed with pUCA525 plasmid harboring genes encoding carBaBb was cultured first in 5 ml of 2xYT medium containing ampicillin at 37˚C with reciprocal shaking (300 strokes/min) until the OD_{600} is above 1.5. Two mililitres of the culture then was inoculated to 200 ml of the same medium in Sakaguchi flasks (500 ml) at 37˚C with reciprocal shaking (120 rpm). IPTG was added to a final concentration of 1mM when the OD_{600} reached above 1.5 and culture was continued for 12 h.

Cells were harvested by centrifugation (3,000 × g, 10 min, 4˚C), washed with buffer containing 20 mM Tris-HCl 200 mM NaCl 10% glycerol (pH 7.5) and resuspended in the same buffer with 1 μM phenylmethylsulfonyl fluoride (PMSF) added. Harvested cells were disrupted thoroughly by ultrasonication conducted on ice for 10 min. Crude extracts were obtained by centrifugation at 25,000 × g for 60 min.

Enzyme Purification

All purification procedures were conducted at 4˚C. Buffers were made with distilled water purified on a MilliQ apparatus (Millipore Corp., MA, USA). Chromatography was done on a ÄKTA FPLC system (GE Healthcare, WI, USA). The crude extracts were filtered with Millipore and then injected into a HisTrap affinity column (GE Healthcare). After equilibration, enzymes were eluted with a linear gradient from 25 mM to 250 mM of imidazole.

Fractions were inspected with SDS-PAGE [18] using 15% w/v tricine minigel slabs. Enzymes were pooled and concentrated with 10k MWCO Vivaspin centrifugal device (Vivascience, Hannover, Germany) and then further purified with HiLoad Superdex 200 26/60 column (GE Healthcare) equilibrated with buffer containing 20 mM Tris-HCl 200 mM NaCl 10% glycerol (pH 7.5). The purity of purified enzymes was determined by SDS-PAGE.

Proteins concentrations

Protein concentrations were determined by method of Bradford using Bio-Rad Protein Assay (Bio-Rad Laboratories, CA, USA) according to manufacturer's protocol. Bovine serum albumin (BSA) was used as the standard for protein concentration [19].

Estimation of molecular weights of enzymes

The native molecular weight of CarBaBb was determined by size exclusion chromatography using HiLoad Superdex 200 26/60 column (GE Healthcare) equilibrated with 20mM Tris-HCl (pH 7.5) 200mM NaCl. The column was calibrated with thyroglobulin (M_r 669,000), ferritin (M_r 440,000), catalase (M_r 232,000), lactate dehydrogenase (M_r 140,000) and albumin (M_r 66,000) as references. SDS-PAGE was used to estimate the subunit molecular weights with phosphorylase *b* (M_r 94,000), albumin (M_r 66,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000), trypsin inhibitor (M_r 20,100), and lactalbumin (M_r 14,400) as references (LMW standards; GE Healthcare).

Enzymatic *meta*-cleavage activity assays

Enzymatic *meta*-cleavage activity assays were conducted using spectrophotometer (GE Healthcare) equipped with thermo-jacketted cuvette holder and a circulating water bath. CarBaBb enzyme was reactivated by incubating with ammonium iron (II) sulfate (5 μM) and sodium ascorbate (5 μM) for 5 min on ice prior to assays [11].

Optimal pH, temperature and temperature stability

Optimal pH for CarBaBb activity was determined by using 50 mM citrate buffer (pH 3.0-6.0), 50 mM 2-(N-morpholino) ethanesulfonic acid (MES)-NaOH buffer (pH 5.5-7.0), 50 mM 3-(N-morpholino) propanesulfonic acid buffer (MOPS)-NaOH buffer (pH 6.5-8.0), 50 mM Tris-HCl buffer (pH 7.5-9.0), and 50 mM glucine-NaOH buffer (pH 8.5-10.5) as reaction buffer [20]. The optimal temperature for CarBaBb was determined by measuring *meta*-cleavage activity from 5˚C to 70˚C. Enzyme temperature stability was tested by measuring the remaining activity after 30 min of incubation temperatures ranging from 5˚C to 70˚C.

Subtrate specificity

Substrates 2,3-dihydroxybiphenyl, catechol, 3-methylcatechol, 4-methylcatechol and protocatechuate used for determining the specificity of CarBaBb were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and of the highest purity available. 2'-aminobiphenyl-2,3-diol and 2,2',3-trihydroxybiphenyl for assaying *meta*-cleavage activity were prepared from carbazole and dibenzofuran respectively as described previously.

The extinction coefficients values for *meta*-cleavage product of 2,3-dihydroxybiphenyl (434 nm; 13,200 cm^{-1} M^{-1}), 2'-aminobyphenyl-2,3-diol (403 nm; 9,100 cm^{-1} M^{-1}), 2,2',3-trihydroxybiphenyl (438 nm; 22,100 cm^{-1} M^{-1}), catechol (375 nm; 33,400 cm^{-1} M^{-1}), 3-methylcatechol (388 nm; 13,800 cm^{-1} M^{-1}), 4-methylcatechol (382 nm; 28,100 cm^{-1} M^{-1}) and protocatechuate (410 nm; 34,000 cm^{-1} M^{-1}) were used to determine the relative *meta*-cleavage activities.

RESULTS AND DISCUSSION

Expression of CarBaBb

CarBaBb was expressed under the influenced of *lac* promoter from pUC119. Expression of CarBaBb using vectors pUCA525 produced a band at near 30 kDa and this band was not observed when using vector pUC119 as a negative control (not shown). It is predicted to be the subunit of CarBb protein expressed due to its molecular weight close to the calculated molecular weight from amino acid sequences. However, the band representing subunit CarBa was observed weakly on the same gel.

Despite this fact, crude cell extract from host expressing from vector pUCA525 showed strong activity indicating CarBa subunit was co-expressed in the same amount but only faintly visible from the used concentration of SDS-PAGE gels. Among various growth parameters tested, CarBaBb was expressed most at growth temperature 37˚C.

Purification

CarBaBb was purified by affinity chromatography and gel filtration chromatography. No significant activity was detected from the flow through samples collected downstream of the affinity column indicating the enzyme was well bounded. In both purification processes, only one major peak was eluted, and SDS-PAGE showed both enzymes were the major proteins from the collected elution although other light bands were still visible (**Fig. 1**). Summary of purification for CarBaBb is shown in **Table 2**.

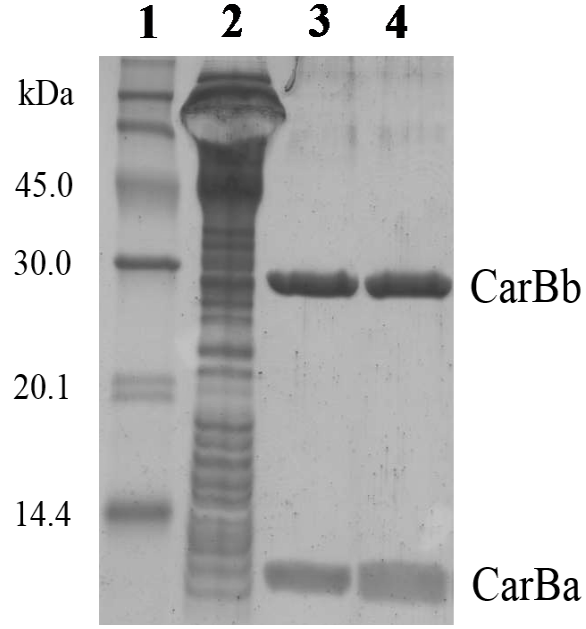

Fig. 1. SDS-PAGE for CarBaBb at different purification steps. Lane 1, low molecular weight standards; lane 2, crude extract; lane 3, after HisTrap affinity chromatography; lane 4, after HiLoad Superdex 200 gel filtration chromatography.

Molecular weight estimation

SDS-PAGE results showed that molecular weight of small subunit CarBa and large subunit CarBb of CarBaBb enzyme are approximately 10,000 and 29,000 Da. This result corresponded well to the expected molecular weight from amino acid sequences deductions. The relative molecular weight of native CarBaBb estimated with gel filtration was approximately 80,000 Da suggesting that CarBaBb is a heterotetramer ($\alpha_2\beta_2$) natively (**Fig. 2**).

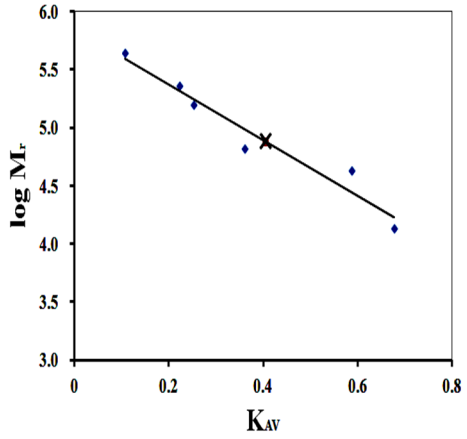

Fig. 2. Estimation of molecular weight of CarBaBb using gel filtration calibrated with several standard proteins. Round points show standard proteins and cross point shows CarBaBb.

Table 2. Summary of purification steps for CarBaBb.

Purific-ation step	Volume (ml)	Total protein (mg)	Total activity (U)	Spe-cific activity (U/mg)	Purifica-tion fold
Crude cell extract	60	824	3076	3.73	-
Affinity chromatogr-phy	25	26	830	32.22	8.6
Gel filtration	25	20	1015	50.94	13.6

Note: One unit (1 U) of activity was defined as the amount of enzyme needed to form 1 µmol of *meta*-cleavage compound per minute at 25 °C.

Effects of pH and temperature on activity and stability of CarBaBb

The CarBaBb enzyme showed more than 50% relative activity in the pH range of 7.0-9.5 and showed the highest activity at pH 8.5 (**Fig. 3**). The optimal temperature for CarBaBb activity was approximately 30˚C and retained more than 50% of activity in the temperature range of 15 to 40˚C. CarBaBb activity was stable after 30 min incubation at temperatures from 5 to 20˚C but decreases gradually for temperatures over 20˚C before completely inactivated at over 50˚C (**Fig. 4**).

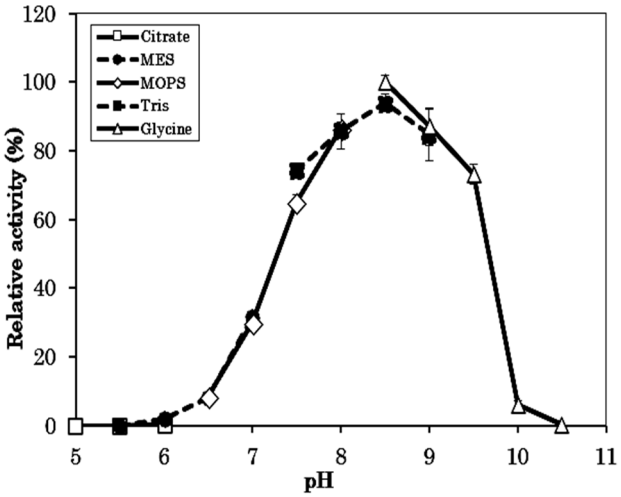

Fig. 3. CarBaBb activity at various pH. Activities were measured at 25°C using 100 µM 2,3-DHBP.

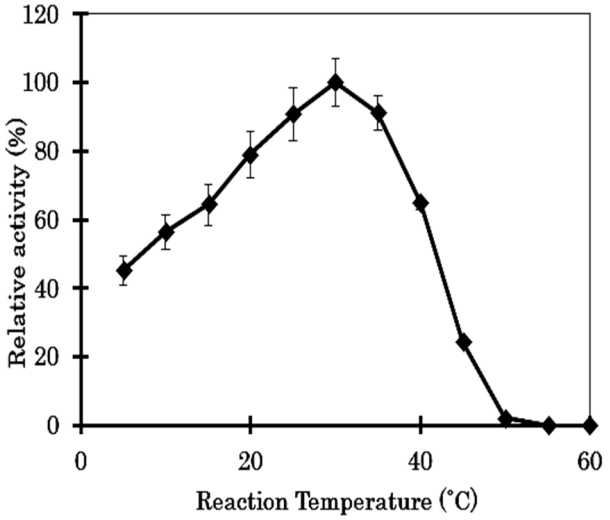

Fig. 4. CarBaBb activity at various temperatures. Activities were measured in 50 mM Tris-HCl pH 7.5 buffer using 100 µM 2,3-DHBP.

Kinetics analysis and substrate specificity

The relationship of CarBaBb activity and all substrate concentrations was observed as a typical Michaelis-Menten curve and values of K_m and V_{max} were obtained by least square fitting the measured curves. Values of kinetics analysis of CarBaBb are shown in **Table 3**. K_m values showed that CarBaBb has high substrate preferential towards 2,3-dihydroxybiphenyl (2,3-DHBP). Results of catalytic activity for biphenyl-type substrates more than 100 fold than for catechol-type substrates indicated that CarBaBb has high preference towards biphenyl-type substrates Enzyme activity was also notably higher towards the 3- and 4- substituted catechol derivatives in comparison to catechol.

This preference suggested large substitute group near the 3- or 4- position of catechol derivative plays a vital role in the interaction of substrate and this *meta*-cleavage enzyme. Because CarBaBb was isolated as a *meta*-cleavage enzyme from a carbazole-degrading bacteria *Novosphiongobium* sp. KA1, its native substrate is 2'-aminobiphenyl-2,3-diol.

However, variation in the 2'-position of 2,3-DHBP revealed significant changes in the substrate affinity to CarBaBb enzyme. In this study, both 2,3-DHBP and 2,2',3-trihydroxybiphenyl showed increased affinity than the native substrate. Similar results were also reported for other 2 subunit *meta*-cleavage enzyme CarBaBb from strain CA10.

Table 3. Kinetics values of $CarBaBb_{KA1}$ with different substrates.

Substrate	K_m (M)	K_{cat}/K_m ($M^{-1}s^{-1}$)	K_{cat}/K_m ($M^{-1}s^{-1}$) ref*
2,3-dihydroxybiphenyl	2.3	4.1	3.3
2,2',3-trihydroxybiphenyl	1.9	3.4	1.4
2'-aminobiphenyl-2,3-diol	7.9	1.2	2.5×10^{-1}
catechol	9.3×10^{2}	9.2×10^{-3}	3.6×10^{-3}
3-methylcatechol	1.4×10^{2}	1.9×10^{-2}	1.6×10^{-2}
4-methylcatechol	1.7×10^{2}	3.9×10^{-2}	1.9×10^{-2}
protocatechuate	1.4×10^{2}	2.4×10^{-2}	1.5×10^{-2}

*Values of CarBaBb from strain CA10 referred from Iwata et al. [11].

CONCLUSION

In conclusion, the CarBaBb from strain KA1 showed close similarity in characteristics and biochemistry with the homologous *meta*-cleavage enzyme from the previously reported strain CA10. To date, apart from LigAB [10], no crystal structure of type III extradiol dioxygenase has been reported, leaving gaps in our understanding of the working mechanisms and dynamics of these heterotetrameric enzymes. Furthermore, in recent years, several of homologous genes have been isolated from marine environment indicating more important roles these degradative genes play in the degradation of aromatic compounds [21, 22]. From our study, we found that CarBaBb from strain KA1 could also be a good candidate for protein crsytallography and structural studies in the future.

REFERENCES

1. Tsuda H, Hagiwara A, Shibata M, Ito N, Carcinogenic effect of carbazole in the liver of (C57BL/6NxC3H/HeN)F1 mice. J. Natl. Cancer Inst., 1982; 69: 1393-1398.
2. Ouchiyama N, Kimura T, Omori T, Kodama T. Biodegradation of carbazole by *Pseudomonas* spp. CA06 and CA10. Biosci. Biotechnol. Biochem., 1993; 57:455-460.
3. Sato S, Ouchiyama N, Kimura T, Nojiri H, Yamane H, Omori T Cloning of genes involved in carbazole degradation of *Pseudomonas* sp. strain CA10; nucleotide sequence of genes and characterization of *meta*-cleavage enzyme and hydrolase. J. Bacteriol., 1997; 179:4841-4849.
4. Nojiri H, Habe H, Omori T. Bacterial degradation of aromatic compounds via angular dioxygenation. J. Gen. Appl. Microbiol., 2001; 47:279-305.
5. Nojiri H, Omori T. Molecular bases of aerobic bacterial degradation of dioxins; involvement of angular dioxygenation. Biosci. Biotechnol. Biochem., 2002; 66:2001-2016.
6. Bressler DC and Ferdorak PM. Bacterial metabolism of fluorene, dibenzofuran, dibenzothiophene, and carbazole. Can. J. Microbiol., 2000; 46:397-409.
7. Harayama S and Rekik, M. Bacterial aromatic ring-cleavage enzymes are classified in two different gene families. J. Biol. Chem. 1989; 264:15328-15333.
8. Eltis L and Bolin J. Evolutionary relationships among extradiol dioxygenases. J. Bacteriol., 1996; 78:5930-5937.
9. Inoue K, Widada J, Nakai S, Endoh T, Urata M, Ashikawa Y, Shintani M, Saiki Y, Yoshida T, Habe H, Omori T, Nojiri H. Divergent structures of carbazole degradative *car* operons isolated from Gram-negative bacteria. Biosci. Biotechnol. Biochem., 2004; 68:1467-1480.
10. Sugimoto K, Senda T, Aoshima H, Masai E, Fukuda M, Mitsui Y. Crystal structure of an aromatic ring opening dioxygenase LigAB, a protocatechuate 4,5-dioxygenase, under aerobic conditions. Structure, 1999; 7:953-965.
11. Iwata K, Nojiri H, Shimizu K, Yoshida T, Habe H, Omori T. Expression, purification and characterization of 2'-aminobiphenyl-2,3-diol 1,2-dioxygenase from carbazole degrader *Pseudomonas resinovorans* strain CA10. Biosci. Biotechnol. Biochem., 2003; 67(2):300-307.
12. Maeda, R, Ito Y, Iwata K, Omori, T. Comparison of marine and terrestrial carbazole-degrading bacteria. Curr Res Technol Educ Top Appl Microbiol Microb Biotechnol, 2010; 2:1311-1321.
13. Iwata K, Noguchi H, Usami Y, Nam JW, Fujimoto Z, Mizuno H, Habe H, Yamane H, Omori T, Nojiri H., Crystallization and preliminary crystallographic analysis of the 2'-aminobiphenyl-2,3-diol 1,2-dioxygenase from the carbazole-degrader *Pseudomonas resinovorans* strain CA10. Acta Crystallogr. D Biol. Crystallogr., 2004; 60:2340-2342.
14. Yanisch-Perron C, Vieira J, Messing J. Improved M13 phage cloning vectors and host strains; nucleotide sequences of the M13mp18 and pUC119 vectors, Gene, 1985; 33:103-119.
15. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. 1989.
16. Hanahan, D. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol., 1983; 166:557-580.
17. Shine J and Dalgarno L. Determination of cistron specificity in bacterial ribosomes. Nature, 1975; 254:34-38.
18. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London), 1970; 227:680-685.

19. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 1976; 72:248-254.
20. Stoll VS and Blanchard JS. Buffers; principles and pratice. Methods Enzymol., 1990; 182:24-38.
21. Maeda R, Nagashima H, Zulkharnain AB, Iwata K, Omori T. Isolation and characterization of a car gene cluster from the naphthalene, phenanthrene, and carbazole-degrading marine isolate *Lysobacter* sp. strain OC7. Current microbiology, 2009; 59(2):154-159.
22. Nagashima H, Zulkharnain AB, Maeda R, Fuse H, Iwata K, Omori T. Cloning and nucleotide sequences of carbazole degradation genes from marine bacterium *Neptuniibacter* sp. strain CAR-SF. Current microbiology, 2010; 61(1):50-56.