Detection, Cloning, and Expression of catechol 2,3 dioxygenase Genes from Novel Polychlorinated Biphenyl (PCB) Degraders

Hamdy A. Hassan

Environmental Biotechnology Department, Genetic Engineering and Biotechnology Research Institute, Sadat City University, Sadat city, Egypt
hamdy.hassan@gebri.usc.edu.eg

Abstract: 27 bacterial isolates were isolated from PCBs contaminated site. A partial 16S rRNA gene sequence revealed that the isolates belonged to the genus *Pseudomonas*, *Bacillus*, *Staphylococcus*, *Rhodococcus*, *Burkholderia*, and *Pandoraea*, all the isolates have the capability to use monochlorinated biphenyl as the sole carbon source, ten of these isolates were belonged to genus *Pseudomonas*, *Burkholderia, Bacillus* and *Rhodococcus* had also the capability to use dichlorinated biphenyl as the sole carbon source and only two strains *Burkholderia* sp. HA-OP24 and *Rhodococcus* sp. HA-30 were capable of metabolizing the highly recalcitrant congener 2,4,5,2′,4′,5′-chlorobiphenyl. Two catechol 2,3 dioxygenase genes termed $C23O^1$ and $C23O^2$ were identified and characterized from *Burkholderia* sp. HA-OP24 and *Pseudomonas* sp. HA-OP22 respectively. These genes may act as functional genes in the metabolic pathway of the PCBs. These findings may benefit in the bioremediation of PCBs by selecting the vital C23O for the mineralization of PCBS compounds.
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1. Introduction

PCBs are ubiquitous contaminants of public concern because of their persistence and bioaccumulation in the environment, as well as their potential toxicity to humans and ecotoxicology (Borlakoglu and Haegeles, 1991). One of the problems related to the persistence of PCBs can be explained by the inability of environmental microbial populations to degrade these compounds efficiently (Sylvestre, 2004). This problem could be solved by the introduction of specific PCBs degrading microorganisms into the environment. Cultivation of microorganisms isolated from PCBs contaminated sites or any variety of ecosystem samples by any chosen cultivation approach will inevitably favor the growth of some community members while others are inhibited or not cultured and allows only around 0.1 to 1% of the total viable bacterial cells present in a variety of ecosystems can be cultured (Amann *et al.*, 1995). It is likely to generate more cultivation methods allow more detections of the PCBs degraders in the contaminated sites.

Some aerobic bacteria capable of oxidizing PCBs have been reported (Pieper and Seeger 2008). Bacterial strains of *Pseudomonas*, *Burkholderia*, *Comamonas*, *Cupriavidus*, *Sphingomonas*, *Acidovorax*, *Rhodococcus*, *Corneybacterium* and *Bacillus* genera have been characterized (Furukawa and Fujihara, 2008; Seeger and Pieper, 2009) as well as some fungi, such as *Phanerochaete chrysosporium* (Beaudette *et al.*, 1998). The degradation of PCBs by microorganisms isolated thus far is started with transforming PCBs into vicinal diols by initial dioxygenase enzymes belong to a large family of Rieske non-heme iron oxygenases (Iwasaki *et al.*, 2007) by introducing two oxygen atoms into PCBs which dehydrogenated by a dehydrogenase to give chloro, 2,3' dihydroxybiphenyl which subjected to extradiol dioxygenase (Colbert, 2013), which can easily be identified due to their yellow pigmentation of the product (Pieper, 2005).

In this study identification and characterization new PCBs degraders were performed based on culture-dependent bacterial community in PCBs contaminated site, and identify, characterize, clone, and expression of new catechol 2,3 dioxygenase genes type I from the new isolates for contributing in dissolve the problem of degradation of PCBs compounds.

2. Material and Methods

Isolation, purification and preservation of bacterial isolates

The samples were collected from the sediments of oxidation ponds which receives all the manufactories wastes of Sadat city – Egypt from the oxidation ponds 1 g of sediment was incubated in 1 L Erlenmeyer flasks containing 100 ml of mineral medium (MM) (Buffer:Na_2HPO_4 x12H_2O 14g, KH_2PO_4 2g, H_2O to 1L, pH 7.4;100x Salt-solution :Ca(NO3)2 x 4H_2O 5g, $MgSO_4$ x 7H2O 20g, Fe III-ammonium-citrate 1g (28 % Fe),Trace elements solution 100 ml, H_2O to 1L;

Trace elements (sterilized by filtration) : HCL (25%) 1.3ml, $ZnCl_2$ 70mg, $MnCl_2$ x $4H_2O$ 100mg, H_3BO_4 62mg, $CoCl_2$ x $6H_20$ 190mg, CuCl x $2H_2O$ 17mg, NiCl2 x $6H_2O$ 24mg, $NaMoO_4$ x $2H_2O$ 36mg, H_2O to 1L. Liquid medium was assembled by supplementing the buffer with 1% (vol/vol) of salt solution. Biphenyl was added as the sole source of carbon and energy in appropriate concentrations, usually 2mM. After one month of cultivation at 30°C with shaking, 10% of the culture was transferred to fresh medium and cultured for one more month. The culture was monitored for the presence of microorganisms until turbidity of the medium was observed by measuring at 600nm. Each isolated colony was recultured on solid MM supplemented with crystals of biphenyl in the lid of the plate as sole carbon source. All isolates were purified and stored in MM and glycerol added to a final concentration of 50% and stored at -80°C.

Screening of catechol 2,3-dioxygenase activity

Catechol 2,3-dioxygenase activity was screened by spraying with catechol. Positive clones could be easily identified due to yellow coloration around the colonies. Colonies turning yellow were purified by repeated subculturing and streaking on MM agar plates with Biphenyl as sole carbon source.

Growth on different PCBs compounds

Pure cultures were tested for their ability to grow on a variety of defined carbon sources. The tests were performed in MM supplemented with the tested compounds as a sole carbon source. Substrates were added to 6ml MM in tubes at a concentration of 100 ppm and inoculated with 10^5–10^6 cells/ml of phosphate buffer (pH 7.2). Tubes were sealed with Teflon-coated stoppers and incubated on shaker at 100 rpm. Tested substrates included monochlorobiphenyls (2-CB and 4-CB), dichlorobiphenyls (2,3CB, and 2,4CB), and polychlorinated biphenyls (2,4,5,2′,4′,5′-Chlorobiphenyl). Stock solutions of all substrates were autoclaved prior to use. Growth was evaluated by microscopy and visual monitoring of turbidity in conjunction with periodic HPLC analyses to measure test compound disappearance or appearance of products. In these substrate-screening studies, growth was scored as positive if turbidity was notably greater than in controls lacking the test compound, microscopic examination revealed an increase in cell numbers, and HPLC analysis showed loss of the test compound. Growth tests were conducted in triplicate for each substrate. In time course experiments, three replicate tubes were sacrificed at each time point.

Isolation and identification of PCBs degraders harboring Catechol 2,3 dioxygenase

Chlorobiphenyl-degrading bacteria were initially isolated by traditional enrichment culture method from the soil samples prepared using 1 g of soil in 1L Erlenmeyer flasks containing 100 ml of mineral medium with 2-Chlorobiphenyl (2-CB) and 4-Chloobiphenyl (4-CB) separately with concentration 2mM as the sole source of carbon and energy. Due to limited aqueous solubility, stock solutions of each 2-Chlorobiphenyl (2-CB) and 4-Chloobiphenyl (4-CB) were prepared in 2,2,4,4,6,8,8-heptamethylnonane (HMN), a non-degradable carrier, to provide an initial concentration of 2mM. For PCBs compounds mentioned below, which have limited aqueous solubility, the concentrations given represent the total mass in both the aqueous and HMN phases, divided by the aqueous volume. After one month of cultivation at 30°C, 10% of the culture was transferred to fresh medium and cultured for another month. Organisms in chlorinated biphenyl (CB) enriched cultures were obtained by a spray plate technique. Desired dilutions (0.1 ml) were spread on mineral medium agar. Immediately after, an ethereal solution of 2-CB or 4-CB was uniformly sprayed onto the surface of the agar. The plates were sealed with paraffin film and incubated for 4–6 weeks. CB-degrading microorganisms were identified by compound-cleared zones surrounding individual colony. Such colonies were purified on MM agar containing 2mM 2-CB or 4-CB was sprayed with filter sterilized catechol (10mM). Colonies turning yellow due to catechol dioxygenase were purified by repeated subculturing and streaking on MM agar plates supplemented with 2-CB and 4-CB as sole carbon source. The new PCBs degraders were identified partially using two primer sets targeting the 16S rRNA, the first primer set (16F8 5`-AGAGTTTGATCCTGGCTCAG 3`; 16R518 5`-CGTATTACCGCGGCTGCTGG-3`), and the second primer set (16F945 5`-GGGCCCGCACAAGCGGTGG-3` 16R1492 5`-TACGGYTACCTTGTTACGACTT-3`), the condition as described by Lane (Lane 1991) corresponding to position 1-518 and 945- 1492 respectively according to the *E. coli* numbering system were directly determined from PCR fragments after purification with GeneJET™ PCR Purification Kit (Fermentas)**.**

Primer design and PCR conditions for catechol dioxygenase

Catechol 2,3 dioxygenase genes were amplified using the primer set C23O-ORF-F (AGGTGWCGTSATGAAMAAAGG) and C23O-ORF-R (TYAGGTSAKMACGGTCAKGAA) according to (Junca and Pieper, 2003) PCR amplification was carried out in a 50µl reaction

mixture containing 100 ng of pure strain DNA as templates, the PCR mixture contained 1.25 U *Taq* polymerase, 10 pmol of the forward primer, 10 pmol of the reverse primer, 200µM of each dNTP and 5µl of 10x *Taq* reaction buffer (20mM $MgCl_2$). Cycling program was as follows: hot start at 94°C for 5 min followed by 35 cycles of 94°C for 40 seconds, 50˚C annealing for 40 sec, 72°C for 1 min and a final step of 72°C for 8 min. The PCR products (4µl of the PCR reaction) were checked for integrity by agarose gel electrophoresis. 1% agarose gel was dissolved in 1x TBE buffer, melted and poured into an electrophoresis chamber, A volume of 10% (v/v) loading buffer (60% glycerin, 0.3% xylolcyanol 0,3% bromophenolblue) was added to the DNA samples. Samples were loaded onto the gel and the electrophoresis was performed at 50 Volt/cm in 1x TBE buffer. DNA was visualized by soaking in a dilute solution of ethidium bromide. DNA fragments were separated and their sizes examined and determined under UV light. The DNA extraction was processed by the GeneJET Gel Extraction Kit (#K0831).

Cloning Catechol 2,3 dioxygenase genes

The amplified $C23O^1$ and $C23O^2$ genes were ligated into pTz57R/T Vector (Thermo Scientific) each reaction was carried out in a total volume of 15µl, comprising 1.5µl Vector (55ng/µl), 3 µl of 5x ligation buffer, 1.5µl PCR product, 0.5µl T4DNA ligase(5u/µl, Thermo Scientific) and 8.5µl water nuclease-free. The ligation reaction mixture was incubated over night at 4°C. *E.coli* JM107 was used for preparation of competent cells using Transform Aid Bacterial Transformation Kit and its protocol (Thermo Scientific). 2.5µl of ligation mixture were added into new microcentrifuge tubes, and chilled on ice for 2min, 50µl of the prepared cells were added to each tube containing the ligation mixtured, mixed, incubated on ice for 5 min and plated immediately on pre-warmed LB-ampicillin X-Gal/IPTG agar plates. The plates were incubated overnight at 37°C.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS polyacrylamide gel were used for determining the molecular weight of denaturated protein subunits, to check the efficiency of the protein purifications, and to show overexpression of the three genes separately cloned in pTz57R/T vector and were prepared as follows: The proteins of the samples were prepared from cell extracts of *E.coli* JM107 harbor vector with the insert from ($C23O^1$ and $C23O^2$) and the control was *E.coli* JM107 with vector without insert. All preparations were performed aerobically grown in 10ml liquid media supplemented with IPTG, and ampicillin and incubated overnight. 2ml from each sample harbor $C23O^1$ and $C23O^2$ and control were centrifuged for 10min at 8944xg, discarded the supernatant, 200 µl of extraction buffer were added for each sample and was applied for sonications for 1 min at 40 pulses with a Sonicator, the samples were centrifuged for 10 min at 8944xg, 10µl of 2x treatment buffer were added to 20µl from supernatant protein solution, incubated 95°C for 5min in a water bath, and loaded 30µl of the samples into each well. The protein gels were stained overnight using a Coomassie brilliant blue stain and then were distained initially in an aqueous solution containing 30% methanol 10% acetic acid. Broad-Way Dual™ Prestained Protein Marker was used (iNtRON Biotechnology Co).

Data deposition

The sequences reported in this study has been deposited in the GenBank database for the first primer with accession numbers (HQ918231- HQ918254) and for the second primer set with accession numbers (JF264732- JF264755), and the two catechol 2,3 dioxygenase genes with accession numbers JQ 015315 and JQ 015316.

3. Results

Isolation and characterization of strains exhibiting 2,3-dihydroxybiphenyl dioxygenase activity.

Dilutions of soil samples were spread on MM plates supplemented with monochlorinated biphenyl (2-CB and 4-CB). After incubation of plates from 4-7 days, Whereas there was no significant difference in the number of colony forming units from the four differently contaminated soil samples tested (approximately 3 x 10^6 CFU/g of soil), there were a correlation between presence of extradiol dioxygenase activity and the capability to grow on 2-CB and 4-CB contaminants, all colonies were analysed for the dioxygenase activity by spraying with 10mM catechol solutions. A subset of colonies with different colony morphotypes and exhibiting yellow coloration upon spraying, which exhibited dioxygenase activity could be isolated. 27 bacterial isolates were isolated; the isolation was achieved by selective enrichment and subsequent plating on MM containing the PCBs substrate (2-CB and 4-CB) as the sole source of carbon. 27 isolates were characterized by sequencing 16S rRNA partially with two primer sets. One primer set (16F8, 16R518) corresponding to position 1-518 according to the *E. coli* numbering system and the other primer set (16F945, 16R1492) corresponding to position 945-1492 according to the *E. coli* numbering system. Analysis using the RDPII database revealed that 18 isolates HA-OP1, HA-OP2, HA-OP3, HA-OP4, HA-OP5, HA-OP6, HA-OP7, HA-OP8, HA-OP9, HA-OP10, HA-OP13, HA-OP14, HA-OP15,

HA-OP18, HA-OP19, HA-OP20, HA-OP21 and HA-OP22, were belonged to the genus *Pseudomonas*; two isolates HA-OP25 and HA-OP30 belonged to genus *Rhodococcus;* two isolates HA-OP29 and HA-OP31 belonged to *Bacillus*, another isolates HA-OP17, HA-OP24, HA-OP26 and HA-OP28 were belonged to genus *Staphylococcus*, *Burkholederia*, *Brevibacillus and Pandoraea* respectively. Two phylogenetic relationships between isolated strains with related aromatic compounds degraders taxa were determined by a neighbor-joining distance analysis of their 16S rRNA gene sequences for the first primer set (Figure 1) and for the second primer set (Figure 2) *Pseuudomonas* sp., Strains HA-OP3, HA-OP4, HA-OP5, HA-OP6, HA-OP7, HA-OP9, and HA-OP10 showed highest similarity with each other and grouped into one cluster either for the first primer set (Fig. 1), or for the second primer set (Fig. 2). Some *Pseuudomonas* strains as *Pseuudomonas* sp. Strains HA-OP13, HA-OP14, HA-OP15, HA-OP18, HA-OP19, HA-OP20, HA-OP21 and HA-OP22 were clustered together and have high similarity in the first primer set with *Pseudomonas* sp. C16w, *Pseudomonas* sp. ARDRA PS2, *Pseudomonas* sp. CT-1, and *Pseudomonas* sp. DK2009-3a which have previously been reported to be able to grow on hydrocarbon, BTEX, naphthalene and fenamiphos & oxamyl respectively (Figure 1) (Junca and Pieper 2004; Viñas et al. 2005). In case of the second primer with *Pseudomonas veronii* UFZ-B54 and *Pseudomonas* sp. BZ27 which have previously been reported to be able to grow on chlorobenzene and hydrocarbons respectively. The remained *Pseudomonas* isolates *Pseudomonas* sp. HA-OP1 showed high similarity in case of the first and second primer set with *Pseudomonas aeruginosa* W3, which has been reported as anthracene degrader (Figures 1-2). *Brevibacillus* sp. HA-OP26 showed high similarity with the polyaromatic hydrocarbon degrader *Brevibacillus brevis* BEA. *Rhodococcus* sp. HA-OP25 showed high similarity with *Rhodococcus* sp. CH9 as phenol degrader and *Rhodococcus* sp. HA-OP30 with high PCBs substrate diveristy (Table 1) has high similarity with *Rhodococcus erythropolis*, which has been reported as dibenzofurane degrader. *Burkholderia* sp. HA-OP24 showed high PCBs substrate diveristy (Table 1) has high similarity with *Burkholderia* sp. isolate N2P5 (Mueller, 1997) for the first primer set and *Burkholderia fungorum* DBT1 for the second primer set as a polyaromatic hydrocarbon and dibenzothiophene degrader, whereas *Bacillus* sp. Strains HA-OP29, and HA-OP31 and *Staphylococcus* sp. Strain HA-OP17 showed highest similarity with the type strain of *Staphylococcus* sp. PN/Y which has previously been reported as phenanthrene degrader (Somnath *et al.*, 2007).

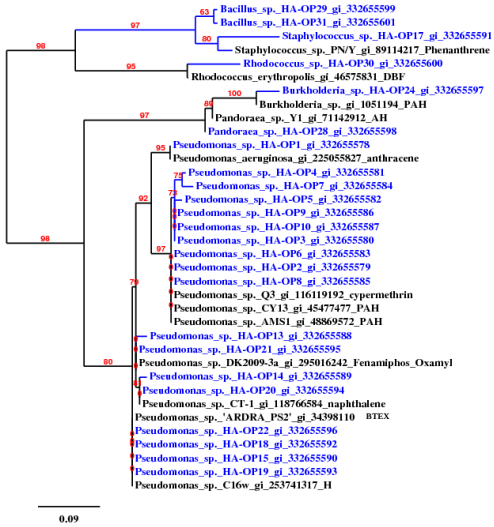

Figure 1. Phylogenetic tree based on the comparison of partial 16S rRNA gene fragments, corresponding to position 1-518 according to the *E. coli* numbering system, showing the relationship of the new PCBs degraders strains (with blue colour and the GI numbers after the strain name) with the nearest strain for each, that have already reported before as aromatic degraders. "H" hydrocarbon, "DBF" dibenzofurane, "PAH" Polyaromatic hydrocarbon, "AH" aromatic hydrocarbon.

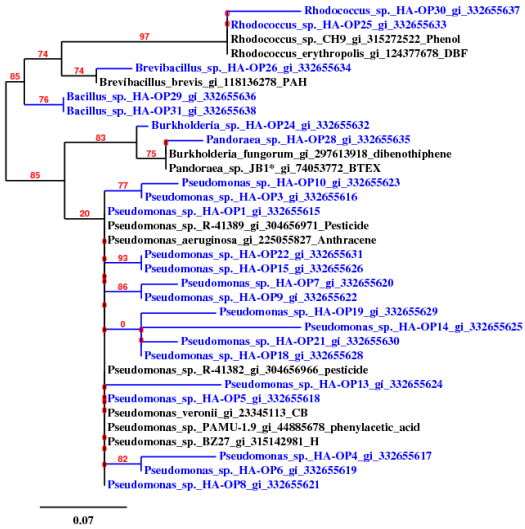

Figure 2 Phylogenetic tree based on the comparison of partial 16S rRNA gene fragments, corresponding to position 945-1492 according to the *E. coli* numbering system, showing the relationship of the new PCBs degraders strains (with blue color and the GI numbers after the strain name) with the nearest strain for each, that have already reported before as aromatic degraders. "H" hydrocarbon, "DBF" dibenzofurane, "PAH" Polyaromatic hydrocarbon, "AH" aromatic hydrocarbon, "DBT" dibenzothiophene.

Substrate diversity of bacterial strains

The growth of the isolates on 2CB; 4CB; 2,3CB; 2,4CB and 2,4,5,2′,4′,5′-Chlorobiphenyl as sole carbon sources is summarized in Table 1. All the strains were able to utilize 2CB; 4CB quite rapidly, though its growth patterns varied which for different isolates. Interestingly, growth was observed on some diCBs including 2,3-, 2,4-DCB for ten strains, seven strains were belonged to genus *Pseudomonas*; one strain was belonged to genus *Burkholderia* (*Burkholderia* sp. HA-OP24); one strain was belonged to genus *Rhodococcus* (*Rhodococcus* sp. HA-OP30)*;* and one strain was belonged to genus *Bacillus* (*Bacillus* sp. HA-OP31). Furthermore, growth were observed on 2,4,5,2′,4′,5′-Chlorobiphenyl only with two strains *Burkholderia* sp. HA-OP24 and *Rhodococcus* sp. HA-OP30. There was a colour change from colourless to yellow observed in culture media of *Pseudomonas* sp. HA-OP2, *Pseudomonas* sp. HA-OP4, and *Pseudomonas* sp. HA-OP20. It is noteworthy that this *meta*-cleavage product persisted throughout the incubation period.

Isolated strains	MCB		DCB		PCB
	2CB	4CB	2,3CB	2,4CB	2,4,5,2′,4′,5′-Chlorobiphenyl
Pseudomonas sp. HA-OP1	+	+	-	-	-
Pseudomonas sp. HA-OP2	+[1]	+[2]	+	+	-
Pseudomonas sp. HA-OP3	+	+	-	-	-
Pseudomonas sp. HA-OP4	+[2]	+[1]	+[2]	+	-
Pseudomonas sp. HA-OP5	+	+	+	+	-
Pseudomonas sp. HA-OP6	+	+	-	-	-
Pseudomonas sp. HA-OP7	+	+	-	-	-
Pseudomonas sp. HA-OP8	+	+	-	-	-
Pseudomonas sp. HA-OP9	+	+	+	+	-
Pseudomonas sp. HA-OP10	+	+	-	-	-
Pseudomonas sp. HA-OP13	+	+	-	-	-
Pseudomonas sp. HA-OP14	+	+	+	+	-
Pseudomonas sp. HA-OP1 5	+	+	+	+	-
Pseudomonas sp. HA-OP18	+	+	-	-	-
Pseudomonas sp. HA-OP19	+	+	-	-	-
Pseudomonas sp. HA-OP20	+	+	+[3]	+[3]	-
Pseudomonas sp. HA-OP21	+	+	-	-	-
Pseudomonas sp. HA-OP22	+	+	-	-	-
Staphylococcus sp. HA-OP17	+	+	-	-	-
Burkholderia sp. HA-OP24	+	+	+	+	+
Rhodococcus sp. HA-OP25	+	+	-	-	-
Rhodococcus sp. HA-OP30	+	+	+	+	+
Bacillus sp. HA-OP27	+	+	-	-	-
Pandoraea sp.HA-OP28	+	+	-	-	-
Bacillus sp. HA-OP29	+	+	-	-	-
Bacillus sp. HA-OP31	+	+	+	+	-
Brevibacillus sp. HA-OP26	+	+	-	-	-

Table 1. PCB-degraders and its Substrate utilization spectrum +, Growth; –, no growth. Culture supernatant fluid turned a yellow color that was; 1permanent; 2disappeared with time and; 3occasional

PCR amplification of two genes

For isolation of the two ORFs C23O1and C23O2 from *Burkholderia* sp. HA-OP24 and *Pseudomonas* sp. HA-OP22 respectively a primer set C23O-ORF-F/ C23O-ORF-R with expected 934bp fragment, (Figure 3). the predicted protein of catechol 2,3 dioxygenase from *Burkholderia* sp. HA-OP24 was highly similar with the catechol 2,3 dioxygenase *Pseudomonas* sp. 1YB2 and catechol 2,3-dioxygenase from *Pseudomonas putida* CSV86, while the predicted protein of catechol 2,3-dioxygenase from Pseudomonas sp. HA-OP22 showed highly similarity with catechol 2,3 dioxygenase from Pseudomonas sp. 1YXyl1 and catechol 2,3-dioxygenase from *Pseudomonas stutzeri* CCUG (Figure 4).

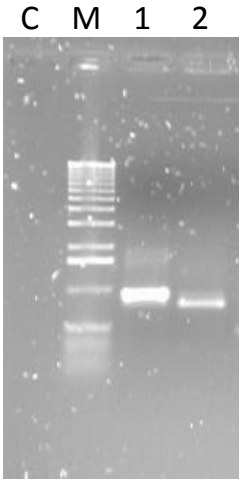

Figure 3. Agarose gel electrophoresis of PCR products obtained C23O-ORF-F/ C23O-ORF-R. lane c is the negative control: Lane 1, using DNA from *Burkholderia* sp. HA-OP24, Lan 2 using DNA from *Pseudomonas* sp. HA-OP22, and M is molecular weight markers X (Roche).

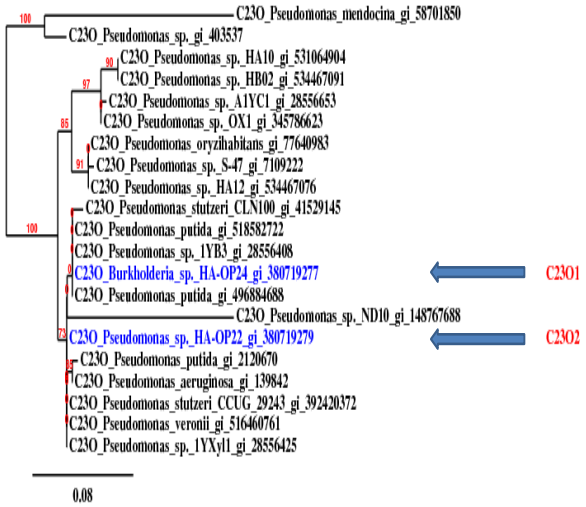

Figure 4. Phylogenetic tree shows the two catechol 2,3 dioxygenaes C23O1 and C23O2 from *Burkholderia* sp. HA-OP24 and *Pseudomonas* sp. HA-OP22 respectively pointed by two arrows and its similarities with the other catechol 2,3 dioxygenase from other strains.

Expression of C23O1 and C23O2 genes in *E. coli* JM107

The analyze of $C23O^1$ and $C23O^2$ encode functional extradiol dioxygenase amplified fragments were separately cloned into pTz57R/T Vector. *E.coli* JM107 harboring only vector without insert was applied as control. Screening of colonies carrying $C23O^1$ and $C23O^2$ by spraying with catechol, colonies exhibited extradiol dioxygenase activity turned yellow encoded functional extradiol dioxygenase proteins.

Analysis of the two extradiol dioxygenases activity by SDS PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the overexpression of $C23O^1$ and $C23O^2$ which were separately cloned on pTz57R/T were expressed and translated to polypeptides with the predicted sizes. The results explained the expression of $C23O^1$ gene was observed at approximately ~ 26KDa (Figure 5; lane2), $C23O^2$ was observed approximately ~ 29KDa (Fig. 5; lane3)

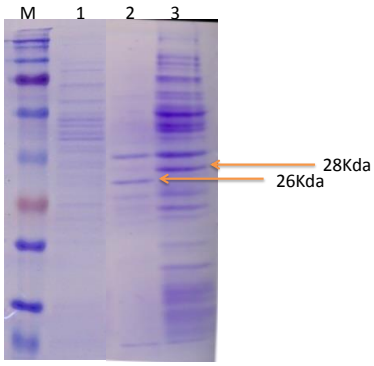

Figure 5 SDS-PAGE analysis of cell extracts of *E. coli* JM107 with $C23O^1$ insert lane 2 (26kda), and $C23O^2$ lane 3 (28kDa), and its control lane1 and M Broad-Way Dual™ Prestained Protein Marker was used (iNtRON Biotechnology Co).

4. Discussion

One of the challenges for successful PCBs bioremediation is the development of effective methods to analysis bacterial community structures and encourages the growth of selected microorganisms that are capable of transforming PCBs. Many PCB-degrading bacteria have been isolated and characterized (Bopp 1986; Bedard *et al.*, 1987; Ahmad *et al.*, 1990). In the present study, We described the isolation and characterization of 27 PCBs degraders, 27 isolates were capable to transform the monochlorinated biphenyl and only 10 isolates can transform dichlorinated biphenyl, whereas the rest of strains unable to transform higher chlorinated biphenyl, It is generally believed that biodegradation of PCBs decreases with the increase in chlorine substitution (Furukawa *et al.*, 1979). Based on the analysis of various biphenyl degrading isolates it could be deduced that lower chlorinated congeners are more easily transformed compared to higher chlorinated congeners and PCB congeners with chlorines on one aromatic ring only are more easily degraded than those bearing chlorine substituents on both aromatic rings (Pieper, 2005). From the 27 strains only two strains that have the ability to transform the higher chlorinated biphenyls *Burkholderia* sp. HA-OP24 and *Rhodococcus* sp. HA-OP30 (Table 1), behave like *Burkholderia* sp. strain LB400 and *Rhodococcus jostii* RHA1, which transform up to hexachlorinated biphenyls (Bopp 1986; Kohler *et al.*, 1988; Bedard and Haberl 1990; Warren *et al.*, 2004; Mary *et al.*, 2006; McLeod *et al.*, 2006), this may be explained by the highest activities, which were observed with the two strains against 2,3DHB and catechol due to the high induction of *meta*-cleavage enzymes 2,3 dihydroxybiphenyl dioxygenase or catechol 2,3 dioxygenase in both upper and lower pathways respectively, this indication may be a good marker for isolation of PCBs degraders with broad spectrum based on the highest activity of *meta*-cleavage in both the upper and lower pathway. However, each isolate exhibited a particular activity spectrum with regard to the type and extent of PCB congeners metabolized, with some strains having a narrow spectrum and other bacteria like *Burkolderia xenovorans* LB400 is able to degrade a broad range of PCBs (Haddock *et al.*, 1995; Seeger *et al.*, 1995a,b; 1997; 1999; 2001) and is a model bacterium for PCB degradation

Extradiol dioxygenases play a key role in the metabolism of PCBs and various other aromatic compounds. These enzymes utilize non-heme ferrous iron to cleave the aromatic nucleus *meta* (adjacent) to the hydroxyl substituents, incorporating both atoms of dioxygen into the product. The ferrous iron of these enzymes is coordinated by two histidines and one glutamate (Han *et al.*, 1995) in which has been termed the 2-His-1-carboxylate facial. $C23O^1$ and $C23O^2$ were cloned and showed as function genes transformed catechol to the yellow *meta*-cleavage product 2-hydroxymuconic semialdehyde, this means that all the three edos belong to the first domain type I Catechol 2,3-dioxygenase, act on a range of related substrates.

A critical step in improving the microbial catabolic activities for the degradation of PCBs is understanding the reactivities of the catabolic biphenyl pathway for PCB metabolites. 2,3-Dihydroxybiphenyl 1,2-dioxygenase (DHBD; EC 1.13.11.39) is the fourth enzyme of the biphenyl pathway and has also been identified as an important

determinant of PCB degradation, as it is competitively inhibited by some chlorinated (Cl) metabolites (Seah *et al.,* 2000, 2001). It utilizes a mononuclear nonheme iron (II) center to cleave 2,3-dihydroxybiphenyl (DHB) in an extradiol fashion. Most biphenyl-degrading bacteria included all the strains in this study can metabolize biphenyl by *meta*-cleavage reaction product (Colbert, 2013), which is yellow in color providing an easy colorimetric test for a rapid screening of bacterial colonies correlating with the contamination levels and carrying 2,3-dihydroxybiphenyl 1,2-dioxygenase activity, and or catechol 2,3 dioxygenase (Happe *et al.,* 1993; Hamdy *et al.,* 2008) as a result of the transformation of 2,3-dihydroxybiphenyl to the yellow *meta*-cleavage product 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid in the upper pathway and in case of using catechol as indicator for the lower pathway, it transformed into yellow color 2-hydroxymuconic semialdehyde, we can summeraize that the environmental conditions and, probably, the contamination level were selective for strains possessing 2,3-dihydroxybiphenyl 1,2-dioxygenase and catechol 2,3 dioxygenase activity.

This study identifies that PCBs are degraded primarily via the *meta*-pathway using the 2,3 dihydroxybiphenyl dioxygenase or catechol 2,3 dioxygenase at PCBs contaminated site. These observations are important because those functional genes have been reported that play an important role in the degradation of PCBs compounds (Pieper, 2005; Pieper and Seeger, 2008).

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