Cloning and Expression of Gene encoding *meta*-Cleavage Enzyme of BTEX Degradation Pathway from Haloalkaliphilic *Pseudomonas* sp. HA10

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Abstract: The potential degradation of haloalkaliphilic hydrocarbon degrading bacteria is an interesting class of extremophilic organisms that possess special adaptation strategies that make them interesting for the remediation of polluted sites. Haloalkaliphilic pure culture using BTEX as a sole source of carbon and energy was isolated from water samples from Mediterranean Sea, Abu Qir-coastline, Egypt. An analysis of the 16S rRNA gene sequence and morphological and physiological characteristics showed that this strain is a member of the genus *Pseudomonas*, and it was designated as strain HA10. Strain HA10 could grow at pH from 8 to 11 and salt concentration from 1.5% to 10%.Its optimal conditions for biodegradation of BTEX were pH 10 and 7% salt concentration. Catechol 2,3-dioxygenase (C23O) gene as a key catabolic genes in the degradation of BTEX compounds was cloned and sequenced. Analysis of C23O nucleotide sequence revealed a 912bp and encoded a polypeptide of molecular weight of 34.43KDa containing 303 amino acids residues. By comparison, the C23O of *Pseudomonas* sp. HA10 showed 81-100% identity in amino acids sequence with other C23Os. The C23O gene was overexpressed in E. coli DH5α and the gene product was identified by SDS-PAGE. To our knowledge, this is the first reported C23O gene cloned from haloalkaliphilic bacterium and successfully expressed and such finding is essential for developing *in situ* bioremediation technologies.

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1. Introduction

Benzene ring is among the most abundant chemical substructure in nature and many compounds (from both natural and anthropogenic sources) containing benzene in their structure as toluene, ethylbenzene and xylenes (BTEX), which are inevitably present in oil production and exploration operations spills. Large volumes of crude oil spills occur during transportation through seas and oceans, by leaking underground storage tanks and due to poor management. Contamination by crude oil results in the release of toxic and carcinogenic compounds such as BTEX, which are major environmental pollutants (Diaz, 2004). BTEX components are classified as priority pollutants by the U.S. Environmental Protection Agency due to their detrimental effect to human health and the environment (Tsao *et al*., 1998).

Most of the degradation activity, metabolism and genetics of BTEX degraders have come from *Pseudomonas* species (Arenghi *et al*., 2001; Brusa *et al*., 2001; Yu *et al*., 2001; Jahn *et al.*, 2005; Wang *et al.*, 2008) or closely related species such as *Ralstonia* and *Burkholderia* (Kato *et al.*, 1996). Where 86.9% of the bacterial species found in oil production such as BTEX were *Pseudomonas* species (Ridgeway *et al*., 1990).

Haloalkaliphilic bacteria have been isolated and studied from the extreme hypersaline sites (Singh, 2006), where haloalkaliphilic and their dual extremity of halophiles and alkaliphiles make them interesting from both, fundamental research and biotechnological points of view (Feng *et al.*, 2005; Joshi, 2006). The extreme conditions can kill or inhibit non-extremophilic species. There is growing interest in the isolation and identification of novel haloalkaliphilic bacterial strains adapted for the development and optimization of bioremediation processes to deal with haloalkaliphilic environments contaminated with aromatic compounds (Baraniecki *et al.*, 2002). Not only extreme haloalkaliphilies but also the moderate halophiles and alkaliphilies are also important group of microorganisms adapted to live in hyper saline and alkalinity habitats and constitute a heterogeneous group, which includes a great variety of bacteria (Manikandan *et al*., 2009; Ramesh *et al*., 2009; Hassan *et al*., 2012). The capability of moderately halophilic bacteria for exciting and promising application making it the most potential candidates, compared with other extremophiles. Although many

studies have been done on ecology, physiology, and taxonomy of halophilic and alkaliphilic organisms separately and revealed only to the impressive diversity, still limited attempts have been made to explore haloalkaliphilies and molecular basis of adaptation, specially its enzymatic potential either for halophilic or alkaliphilic or haloalkaliphilic bacteria (Manikandan *et al.*, 2009; Ramesh *et al*., 2009; Hassan *et al*., 2014).

In the present study, we explore the biodegradation potential of nonoxygenated, low molecular weight fuel hydrocarbons including benzene, toluene, ethyl benzene and xylenes (BTEX) in water samples from Mediterranean Sea, Abu Qir-coastline, Egypt. We identified and characterized novel moderate halophilic alkaliphilic *Pseudomonas* strain that has the capability to degrade BTEX under dual of halophilic and alkaliphilic conditions. Also, further we tested the BTEX degradation pathways in this haloalkaliphic *Pseudomonas* strain HA10 by cloning, sequencing and expression of The ring-cleavage enzymes catechol 2,3-dioxygenase (C23O), which play central role in the degradation of a variety of aromatic compounds. This is important since not much is known about the phylogenetic diversity and metabolic capabilities of haloalkaliphic bacteria.

2. Material and Methods

2.1. Liquid sample

Water samples contaminated with BTEX were collected from the Mediterranean Sea, Abu Qir-coastline, Egypt. Samples were taken from the water surface with pH 9 and salinity 32% and placed in sterile bottles swabbed with 75% ethanol, and stored at 4 ◦C until they were used.

2.2. Enrichment culture and isolation of BTEX-degrading moderately haloalkaliphilic Strain

A moderately haloalkaliphilic strain was isolated by enrichment culture techniques from alkaline and saline water samples. Primary enrichment cultures were prepared in 100ml bottles by adding 1 ml of water sample to 19 ml of mineral medium (MM) (Dorn *et al.*, 1974) containing 3% M NaCl and pH 9 (adjusted by adding Na_2CO_3) with BTEX each compound separately benzene, toluene, ethyl benzene or xylene (2 mM) as the sole carbon and energy source. Following 1 month of cultivation at 30°C, 10% of the culture was transferred to fresh medium containing 5% NaCl and cultured for a further month and this step repeated on 7%, 10% and 12% NaCl. Dilutions of the culture were spread onto minimal medium agar plates supplemented with BTEX in end closed tips on the lid of the agar plates and incubated for 7 days after which, colonies appeared were sprayed with filter-sterilized catechol (10mM each). Yellow colonies due to extradiol cleavage of either catechol were purified on MM agar plates with BTEX as the sole carbon source. One predominant colony morphotype, which grew rapidly at 30°C, was selected for further studies.

2.3. Identification and characterization of the strain and phylogenetic analyses

Cell morphology and dimensions were observed using phase contrast microscopy. Colony morphology was determined using a Leica M8 stereomicroscope. Gram staining was carried out (Dussault, 1955), and the KOH test was confirmed (Halebian *et al.*, 1981). Physiological and biochemical characteristics were performed according to Bergey's Manual of Systematic Bacteriology (Palleroni, 2005). A 3 ml sample of the pure culture was centrifuged at 12,879xg for 15 minutes. The supernatant was decanted and the cells were washed twice with sterilized water. The cells were resuspended in 0.5 ml of sterilized water. Then, genomic DNA was extracted from the pure culture using a GeneJET Genomic DNA Purification Kit (Thermo Scientific). Three primers were used in the amplification of 16S rRNA. These include: 27f (5'-AGAGTTTGATCMTGGCTCAG-3'), 1492r (5'-TACGG(C/T)-ACCTTGTTACGACTT-3'), and Bact 1098r (5'-AAGGGTTGCGCTCGTTGCG-3') (Chang *et al.*, 2000). Theoretically, amplification with 27f-1492r should yield 1505bp and amplification with 27f - 1098r should yield 1108bp from the 16S rRNA. Amplifications with these two primer sets were used to obtain the nearly full-length sequence (1492bp) of the 16S rRNA of the isolate. PCR amplification was performed in a total volume of 50µl in Touch Screen Thermal Cycler / PCR Model: A100/A200 (Hangzhou LongGene Scientific Instruments Co., Ltd). Each PCR mixture contained 25 ng of template DNA, 0.6µM of each primer, 1.75mM MgCl2, 200µM of dNTPs, 1.25U of *Taq* polymerase in buffer A (Promega Chemicals, Madison, WI). Amplification of 16S rRNA using both primer sets consisted of an initial denaturation of the genomic DNA at 94ºC for 3minutes, followed by 30 cycles of denaturation at 94ºC for 1 minute, annealing at 53ºC for 1 minute, and extension at 72ºC for 2 minutes, and a final extension at 72ºC for 8 minutes. PCR products were checked for expected size on 1% agarose gels. The PCR product was purified by Gene JET^{TM} Gel Extraction kit (Thermo Scientific). After purification, a sample of the PCR product was sequenced in both directions, the determined 16S rRNA gene nucleotide sequences were entered for BLAST searching into the Web site of NCBI (http://www.ncbi.nlm.nih.gov/blast/), and aligned using Clustal W implemented in MEGA software version 3,1(Kumar *et al*., 2004) The phylogenetic tree was constructed using Phylogeny.Fr (Dereeper *et al.*, 2008).

2.4. Effect of culture conditions on growth of strain HA10 and BTEX degradation

Bacterial pure culture was grown at 30˚C with shaking (150 rpm) in MM containing 2mM of BTEX until growth reached late exponential phase. Cells were harvested by centrifugation, washed twice in sterile MM and resuspended in one-tenth volume of medium. This concentrated cell suspension was used as inoculum for subsequent experiments. The effect of salt on BTEX biodegradation was determined at various concentrations of NaCl (0–12%) in methods described above. The effect of pH on BTEX biodegradation was determined by using 50 mM - $NaHCO_3$ (pH 7.0–9.0), 50mM Na_2CO_3 (pH 9–11). Abiotic loss was monitored in sterile BTEX-containing medium, and the residual BTEX concentration each compound from BTEX separately was measured at regular intervals over an incubation period.

2.5. Growth condition and Growth curve

To quantify growth rate and substrate disappearance, *Pseudomonas* sp. HA10 was grown as described above and cultures harvested during late exponential growth phase by centrifugation at 4383 xg for 10 min. Cells were washed twice with 50 mM - $NaHCO_3$ - Na_2CO_3 (pH 7–11) and salinity (0-12%) and resuspended in liquid MM to give an OD600nm of 0.1. Degradation of BTEX were tested in sterilized glass tubes containing 2ml cell suspension (OD600nm = 0.1) and 2mM of BTEX as sole carbon source. The test tubes were incubated at 150 rpm and 30˚C. At each time point, 2 test tubes were analyzed by HPLC for residual substrates as required. For the estimation of the colony forming units (CFU) aliquots were serially diluted, 100µl aliquots were plated on solid LB medium and the CFUs counted after 2 days incubation at 30°C. Uninoculated tubes and tubes without substrate served as controls.

2.6. Detection, identification, cloning and characterization of catechol 2,3-dioxygenase gene

Genomic DNA was extracted from *Pseudomonas* sp. HA10 grown on MM + Na_2CO_3 (pH 10) and salinity (NaCl 7%) in the presence of BTEX as a sole carbon source using the method described earlier. The isolated DNA was screened for the presence of key ring-cleaving genes including catechol 2,3-dioxygenase (C2,3O). The following degenerate primer set was used to amplified the above gene using the PCR conditions described in the corresponding reference: C23O-ORF-F (5'- AGG TGW CGT SATGAA MAA AGG -3'), and C23O-ORF-R (5'- TYAGGT SAK MAC GGT CAK GAA -3') (Junca *et al*., 2004). To validate that the correct PCR fragments had been amplified, suspected band was purified, ligated into pGEM®-T Easy Vector system 1 (Promega) and cloned into *E. coli* DH5*α* prepared as competent cells by transform aid bacterial transformation Kit and its protocol (Thermo Scientific). The transformed cells were plated on Luria-Bertani (LB) agar plates containing ampicillin (50µg/ml) and the plate incubated at 37˚C overnight. Colonies on the agar plates were sprayed with 10mM catechol for screening for extradiol dioxygenase activity. The positive colonies plasmids were isolated using The GeneJET Plasmid Miniprep Kit (Thermo Scientific) and the extradiol dioxygenase gene was amplified by PCR using the M13f (5'-AGCGGATAACAATTTCACACAGGA -3' and M13r (5'- CATTTTGCTGCCGGTCA –3`). The purified DNA was sequenced and the nucleotide sequences determined in this study were compared with existing sequences in GenBank by performing a BLASTn and BLASTp search.

2.7. SDS polyacrylamide electrophoresis (SDS-PAGE)

The positive transformed clones carrying pGEM®-T Easy Vector harboring C23O gene and negative control carrying only pGEM®-T Easy Vector without any insert were cultivated in 2 ml of LB medium supplemented with 100 µg/ml ampicillin overnight at 37 ˚C. A 100 µl aliquot of the pre-culture was transferred to 9ml fresh LB medium with ampicillin and further incubated to an OD600 of 0.6-0.7. Induction was brought about at 30˚C for 3 h by addition of isopropyl-b-D-thiogalactopyranoside (IPTG) to a final concentration of 1.0 mM. Then, cells were harvested by centrifugation. Total cell extracts were prepared by resuspending *E. coli* cells in 200µl of extraction buffer for each sample and applied for sonication for 1min at 40 pulses with a sonicator. (keep samples in ice during sonication), the samples were centrifuged for 10 min at 8944xg, 10 µl of 2x treatment buffer were added to 20µl from supernatant protein solution, incubated at 95˚C for 5min in a water bath, and loaded 30 µl from the control with different dilution (1:4 and 1:1) and 30 µl from the positive protein samples with different dilutions (1:4, 1:3, 1:2, and 1:1) 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).

2.8. Data deposition

The 16S rRNA sequence reported in this study has been deposited in the GenBank database under accession number KF811028, catechol 2,3 Dioxygenase gene under accession number KC987072.

3. Results and Discussion

3.1. Isolation and characterization of BTEX-degrading strain HA10

A moderately halophilic alkaliphilic bacterial culture capable of growing on and degrading BTEX as a sole source of carbon and energy was isolated from alkaline and saline water samples from Mediterranean Sea, Abu Qir- coastline- Egypt. The isolate HA10 could grow at pH 8-11, with optimum growth at pH 10, and in the presence 1.5-10% NaCl, with optimum growth at 7% NaCl. The strain couldn't grow at lower pH 7 or lower salinity1.5%.The morphological and physiological properties isolate designated HA10 were examined according to Bergey's Manual of Systematic Bacteriology as follow: Gram- negative, KOH stirring test positive. Colonies are circular and white, does not produce a fluorescent pigment and showed blue color as a positive oxidase test. The cultured strain in phenol red glucose (Dextrose) broth with Durham tube showed hot pink in the presence of bases/alkali (indicating a negative test). To determine the phylogenetic position of the isolate, 16S rRNA sequence (1242bp) of strain HA10 was determined and deposited under GenBank accession number KF811028. When comparing the 16S rRNA sequence of strain HA10 with those in the NCBI database, results suggested that strain HA10 was phylogenetically most closely related to the *Pseudomonas* species and formed a cluster with *Pseudomonas alcaliphila* strain L8 (KF358253) with a relatively high bootstrap resampling value of 85% (Fig. 1). Strain HA10 exhibited levels of 16S rRNA identity of 99% with type strains of *Pseudomonas alcaliphila* strain L8 (KF358253). Identification of strain HA10 as a *Pseudomonas* isolate is consistent with the physiological properties and habits of such organisms, since other *Pseudomonas* strains have been isolated from marine environments not only have been reported to degrade a variety of aromatic compounds pollutants (Kahng and Nam, 2002; Liu *et al*., 2010; Maeda *et al*., 2010), but also have been successfully applied in the bioremediation of contaminated sites (Atlas, 1985; Mishra *et al*., 2001; Gray *et al*., 2010). According to biological and biochemical characteristics as well as 16S rRNA gene analysis, the isolate was classified as *Pseudomonas* sp. and designated as strain HA10.

3.2. Growth of strain HA10 and BTEX degradation

Given that growth of the isolated strain HA10 was verified by demonstrating an increase in the tested BTEX concentrations. To determine the capacity of strain HA10 to degrade BTEX compounds in media with different salinities, a series of tests using salt concentrations ranging from 0 to 12 % were performed. Results (Fig. 2) showed that strain HA10 fully degraded 2mM of BTEX compounds in media containing NaCl concentrations 7% for 2 days, where the optimal NaCl concentration was 7%. Strain HA10 did not degrade BTEX in the absence of NaCl, indicating that BTEX degradation by strain HA10 had an absolute requirement for sodium ion of at least 1%. No degradation occurred at NaCl concentrations above 10 % for 4 days, even after a longer incubation time, indicating that biodegradation inversely correlated with salinity at higher salt concentrations. This observation is consistent with those noted in an extremely halotolerant bacteria (li *et al*., 2006; Sonal *et al*., 2012). The bioremediation of BTEX from environments with variant salinities can only be accomplished by maintaining the growth of indigenous microorganisms capable of degrading BTEX or through the bioaugmentation of halophilic or halotolerant organisms.

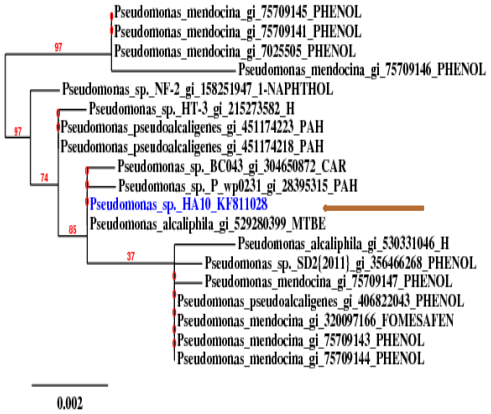

Fig. 1 Phylogenetic tree based on 16S rRNA sequences, constructed by the neighbor-joining method, showing the position of strain HA10 and representatives of some related taxa.GI number is given after the strain followed by the substrate. Bootstrap values (1000 replications) are shown as percentages at each node only if they are 37% or greater. Bar, 0.002 substitutions per nucleotide position.

The optimal pH for biodegradation of BTEX by strain HA10 was 10 (Fig. 3), degradation was observed at pH 8 and11, but not at pH less than 8.0 or more than 11, indicating that strain HA10 is an alkaliphilic bacterium (Romano *et al.*, 2005; Li *et al.*, 2006). From the obtained results concerning the optimal salinity concentration and pH for the biodegradation and growth conditions, strain HA10 classified as moderately haloalkaliphilic bacterium, strain HA10 grew and degrade on toluene and xylene better than benzene and ethylbenzene. Although much research has centered on the degradation of BTEX in terrestrial environments has been studied extensively under oxic conditions ((Leahy and Colwell, 1990; Arenghi *et al.*, 2001; Van Hamme *et al.*, 2003; schneiker *et al.*, 2006; Patzelt, 2007; Pérez-Pantoja *et al.*, 2010) little is known about BTEX degradation in hypersaline environments. Bioremediation of polluted

hypersaline waste waters and other environments with non-halophilic microorganisms is difficult because salt inhibits their growth and the degradation of BTEX (Nicholson and Fathepure, 2004; Li *et al.*, 2006; Dalvia *et al.*, 2012).

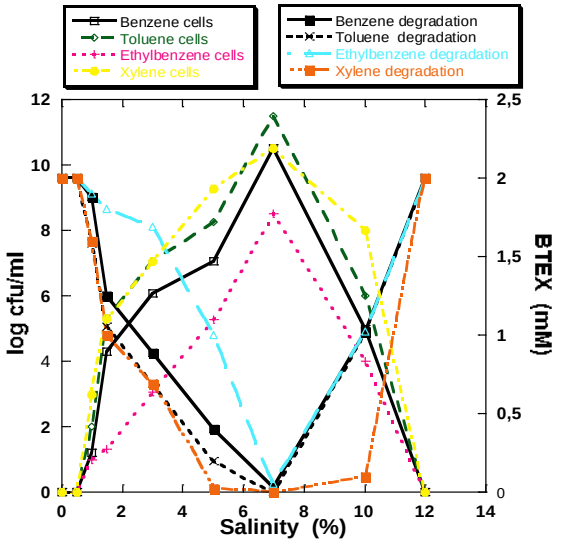

Fig. 2 Biodegradation of BTEX by strain HA10 at different salinity (0-12%). The strain was grown at 30°C with shaking (150 rpm) in MM containing 2mM of BTEX with 5% concentrated cell suspension inoculum pregrown on BTEX, after 2 days incubation the CFUs were counted and BTEX degradation were measured.

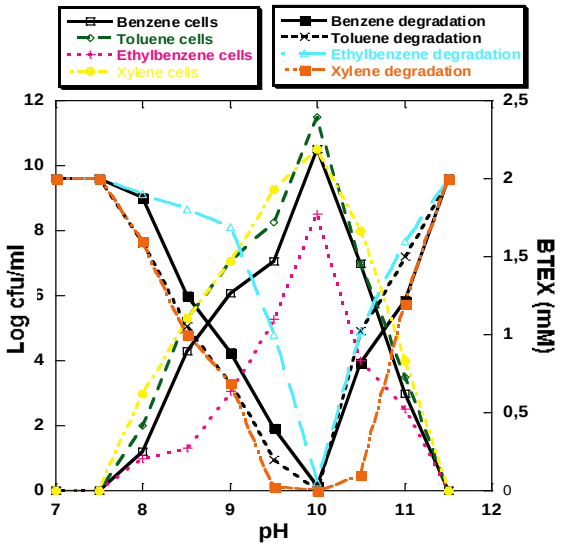

Fig. 3 Biodegradation of BTEX by strain HA10 at different pHs (7- 12). The strain was grown at 30°C with shaking (150 rpm) in MM containing 2mM of BTEX with 5% concentrated cell suspension inoculum pregrown on BTEX, after 2 days incubation the CFUs were counted and BTEX degradation were measured.

3.3. Identification and Cloning catechol 2,3-dioxygenase Gene from *Pseudomonas* sp. HA10

Recently the BTEX degraders in halophilic and alkaliphilic conditions received great attention, specially genes, pathways, and mechanisms of their degradation. Also, it is not known whether haloalkaliphilic bacteria degrade BTEX using novel genes and pathways compared to those used by non-haloalkaliphiles, where under aerobic conditions, most of BTEX are primarily oxidized by dioxygenases into a catechol and then these intermediates undergo further ring cleavage at *meta* position by catechol 2,3-dioxygenase. These studies suggest that perhaps similar genes and pathways are present in our newly isolated organism that degrades bacterial cell biomass concomitant with a decrease in that code for catechol 2,3-dioxygenase was successfully amplified using degenerate primer set (C23O-ORF-F & C23O-ORF-R) from Genomic DNA from *Pseudomonas* sp. HA10 grown on BTEX at pH 10 and 7% salinity. The yield of PCR product and correct size was verified by the agarose gel electrophoresis (Fig. 4).

The obtained PCR product was ligated into pGEM®-T Easy Vector and transformed into *E.coli* DH5*α* using Transform Aid Bacterial Transformation kit (Thermo Scientific). After plating, the colonies were screened by catechol, the colonies showed a yellow coloration indicating these colonies to contain the vector harboring expressed catechol 2,3-dioxygenase, The positive colonies plasmids were isolated using The GeneJET Plasmid Miniprep Kit (Thermo Scientific) and the catechol 2,3-dioxygenase gene was amplified by PCR using the M13f and M13r primers. Analysis of the nucleotide sequence revealed that the C23O gene encodes an ORF of 912bp initiating at ATG and terminating at TGA. The C23O gene exhibited 58 % G + C content. Homology searches with C23O sequence suggested that it exhibited 100 % identity with that from *P. putida* (Miyakoshi *et al.*, 2012) and no less than 95 % identity with those from *P. oryzihabitans* (Jussila *et al.*, 2007) and *Pseudomonas* sp. S-47 (Noh *et al.*, 2000) But the identity was less than 85 % as compared to the corresponding enzymes of *Marinobacter adhaerens* HP15 (Gärdes *et al.*, 2010). To explore the phylogenetic relationship among various extradiol dioxygenases, phylogenetic trees were constructed. The C23Os from *Pseudomonas* sp. ZJF08 and *P. putida* belong to the same branch on phylogenetic trees (Fig. 5). The C23Os were divided into two groups the members of each group exhibit greater homology among one another than to members of the other group. Group 1 belonged to the catabolic pathway of polyaromatic hydrocarbon (PAH) and Group II belonged to the catabolic pathway of

monoaromatic hydrocarbon (MAH). Discovering novel genes and pathways from haloalkaliphilies is worthy and helpful in the development of the effective and the low cost bioremediation strategies. The reported enzymes in the metabolic pathways from halophiles or alkaiphilies were few and described for many nonhaloalkaliphiles as well.

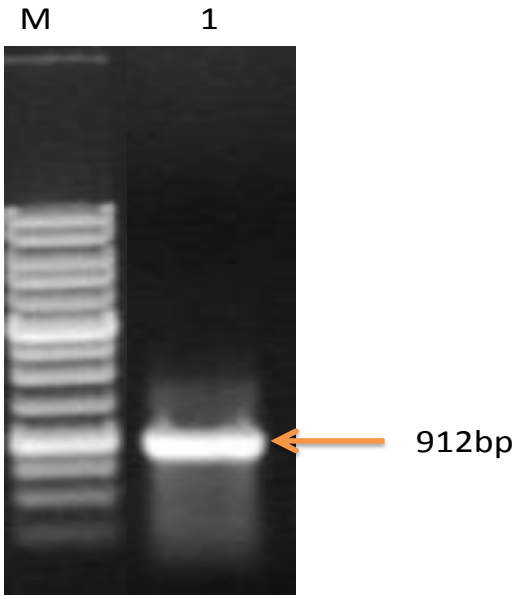

Fig. 4 Agarose gel electrophoresis of the ~912bp product obtained by using fw- C23O-ORF and rev-C23O-ORF primer set (lane1), Lan 1 PCR product for *Pseudomonas* sp. HA10, M, molecular weight marker O'Generuler TM 1 KB DNA ladder (Thermo Scientific).

3.4. Overexpression of catechol 2,3-dioxygenase protein

Catechol 2,3-dioxygenase (C23O) protein is a member of the extradiol dioxygenase family, and behaves as *meta*-cleavage enzyme. A Comparison of the amino acid sequence of C23O obtained from *Pseudomonas* sp. HA10 in this study with other extradiol-type dioxygenase whose structures have been characterized revealed that the amino acid residues were highly conserved as *meta*-cleavage enzyme for the mono aromatic hydrocarbons (Fig. 5). Where the dominant degradation pathway for BTEX compounds was via *meta*-cleavage (Sei and Fathepure, 2009). In this study BTEX compounds are degraded primarily via the *meta*-pathway using the C23O at haloalkalphilic conditions. These observations are important because C23O is a functional gene that has been shown to play an important role in the degradation of BTEX compounds. Therefore, this gene can be used as a suitable molecular marker for the *in situ* detection of BTEX degraders in haloalkalphilic. The recombinant C23O of *Pseudomonas* sp. HA10 was overexpressed in *E. coli* DH5*α*. Analysis of crude lysate prepared from *E. coli* DH5*α* harboring pGEM®-T Easy Vector with C23O by SDS-PAGE indicated an overexpressed protein band of 34.4kDa which was the expected size of ORF predicted a polypeptide of 303 amino acids with a calculated molecular weight of 34.429kDa of the C23O protein (Fig. 6). Among the conserved amino acid residues His199, His246 and Tyr253, may be important for catalytic activities and His153, His218 and Glu268 for metal binding ligands. One of the conserved histidine residues (His199) seems to have important roles in the catalytic cycle (Noh *et al.*, 2000; Takeo *et al.*, 2006; Shuang *et al.*, 2014). Presence of these amino acids residues may be suitable site directed mutagenesis, in addition the C23O has four subunits with four non-heme ferrous ion, which play as a sole cofactor in the active site and 303 amino acids (Dai *et. al* 2001; Takeo *et al.* 2007). The instability index (II) of C23O in this study was computed to be 24.46 this classifies the protein as stable.

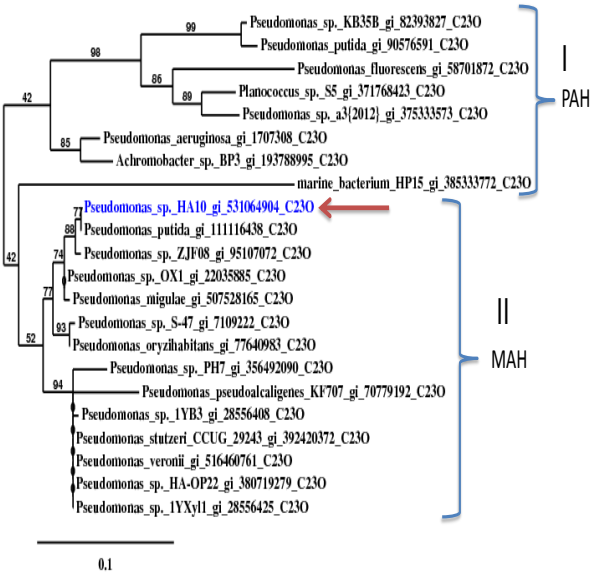

Fig. 5 Phylogenetic tree shows the relatedness of catechol 2,3dioxygenase (C23O). The dendrogram was calculated using Phylogeny.fr based on protein sequence alignments. The C23Os proteins from *Pseudomonas* sp. HA10 are shown by arrow. Group I C23Os of polycyclic aromatic hydrocarbons (PAH) and Group II C23Os of monocyclic aromatic hydrocarbons (MAH). The scale bar corresponds to an estimated evolutionary distance of 0.1 amino acid substitutions per site.

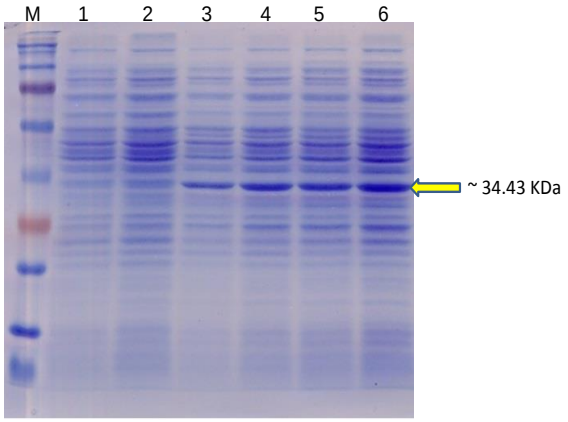

Fig. 6 SDS-PAGE analysis of cell extracts of *E.coli* DH5*α* with pGEM®-T Easy Vector but without insert Lane 1 and 2 (dil 1:4 and 1:1 respectively), *E.coli* DH5*α* with pGEM®-T Easy Vector with C2,3O insert from *pseudomonase* HA10, Lane 3, 4, 5 and 6 (1: 4, 1:3, 1:2 and 1:1 respectively) with 34.43Kda M PageRuler™ prestained Broad–way Dual ™ (INTRON).

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