Cloning and Kinetic Properties of Catechol 2,3-dioxygenase from Novel Alkaliphilic BTEX-degrading *Pseudomonas* sp. HB01

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Abstract: Nowadays, there has been a special interest in isolation and characterization of alkaliphilic organisms for their ability to degrade aromatic compounds. Alkaliphilic strain, HB01, was isolated from soil in Kafr El Ziat Egypt around some chemical, insecticides, and pesticides producing factories. 16S rRNA gene sequence-based phylogeny suggested that strain HB01 is a member of the genus *Pseudomonas*. The focus of this study showed the capability of this strain for BTEX degradation at pH range from 9-11. Based on this analysis, it was hypothesized that BTEX is converted to phenol and then to catechol by phenol hydroxylase components. The resulting catechol undergoes ring cleavage via the *meta* pathway by Catechol 2,3-dioxygenase (C2,3O) to form 2-hydroxymuconic semialdehyde, which enters the tricarboxylic acid cycle. Catechol 2, 3-dioxygenase (C2,3O) was amplified and expressed in *E. coli* DH5α using pGEM®-T Easy Vector, Kinetic properties of catechol 2,3 dioxygenase (C2,3O) from alkaliphilic *Pseudomonas* sp. HB01 were analyzed catechol and 2,3-Dihydroxybiphenyl could be identified as the preferred substrates. C2,3O from *Pseudomonas* sp. HB01 had been severely affected and its activity rapidly inactivated by 3-Chlorocatechol. This finding may be necessary in order to estimate the true potential of this strain to be applied in the remediation of BTEX contaminants or natural attenuation of alkaline BTEX contaminated sites.

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1. Introduction

Among aerobic organisms, there is only a 1-10% occurrence of isolating alkaliphiles. While the two main physiological groups are general alkaliphiles and haloalkaliphiles, all alkaliphiles require some sodium ions for growth and maintenance, making all general alkaliphiles halotolerant to a certain degree (Hamdy *et al.,* 2012). Alkaliphiles have the potential advantage of being able to treatment of wastes or degrade aromatic and chlorinated hydrocarbons in high pH industrial wastewaters, oil-polluted salt marshes, and other contaminated environments (Margesin and Schinner, 2001), even locations with a bulk pH that is neutral or acidic (Grant *et al.,* 1990; Horikoshi, 1999). The alkaliphiles provide the industrial biotechnology with alkaline enzymes for use in detergent additives, wood-pulp treatment and dehairing of hides (Grant *et al.,* 1990; Horikoshi, 1999). Monocyclic aromatic compounds such as benzene, tolune, ethylebenzene, and xylene (BTEX) are common soil and groundwater contaminants and are classified as priority pollutants by the U.S Environmental Protection Agency. BTEX are category A carcinogen. They are highly water soluble, hence can contaminate a large volume of groundwater. Leakage from underground storage tanks, pipelines, spills, and seepage from surface contaminated sites can cause major BTEX contamination (Philip *et al.,* 2005). BTEX are included in the crude oil, which contains thousands of components which is separated into saturates, aromatics, resins and asphaltenes.

Studies have been carried out to understand the biochemical pathways of biodegradation of BTEX by bacteria at neutral pH, however very few reports are available on the complete degradation pathways under alkaline conditions, more so by using obligate alkaliphilic bacteria, few have been reported in alkaliphillic sites as *Alcanivorax* sp. HA03, which isolated on the basis of its ability to utilize benzene, and toluene from the extremely saline and alkaline lakes (Soda lakes) of the Wadi el Natrun (Hamdy *et al.,* 2012).

Two important enzymes involved in the aerobic degradation of BTEX by microorganisms, the ring hydroxylating oxygenase and the ring cleavage oxygenase resulting catecholic intermediates. Catechol 2,3 dioxygenase (C2,3O) is a key enzyme of many bacterial pathways for the degradation of BTEX. The majority of C2,3Os are phylogenetically closely related, belonging to the subfamily 1.2.A of the 1.2 extradiol dioxygenase family, and are of particular importance in the degradation of monocyclic aromatic compounds (Eltis and Bolin, 1996).

The main aim of the present study was to isolate a new alkaliphilic BTEX-degrading bacterium from

soil in Kafr El Ziat Egypt around some chemical, insecticides, and pesticides producing factories, where the pH has been increased by industrial activities till pH 11, and the compare the capability of this isolates for the degradation of benzene, toluene, ethylbenzene, and xylene. Study in-depth the *meta* pathway catalysed by catechol 2,3-dioxygenase, the amplification, cloning and its kinetic properties, such information is important for understanding the rate-limiting of this important step that have to be overcome for the efficient removal of toxic compounds.

2. Material and Methods

Isolation of alkaliphilic BTEX utilizing Bacterium

BTEX-degrading bacteria were isolated from soil in Kafr El Ziat Egypt around some chemical, insecticides, and pesticides producing factories, where the pH was measured with rang from 9-11. Soil (1 g) was incubated in a 1-liter Erlenmeyer flask containing 100ml of mineral medium (Dorn, et. al. 1974) adjusted to various pH values: pH 7–9 (adjusted by adding $NaHCO_3$) and pH 10–11 (adjusted by adding Na_2CO_3) with BTEX each compound separately benzene, toluene, ethylbenzene 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 and cultured for a further month. 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 were sprayed with filter-sterilized catechol or 2,3 dihyroxybiphenyl (DHB) (10mM each). Yellow colonies due to extradiol cleavage of either catechol or DHB were purified on minimal medium agar plates with BTEX as the sole carbon source. One predominant colony morphotype, which grew rapidly at 30°C, was selected for further studies.

Phylogenetic analysis

Phylogenetic identification of the isolate was enabled by means of sequence analysis of the 16S rRNA gene. A 3 ml sample of the pure culture was centrifuged at 12,000 rpm 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 rDNA. These include: Bact 27f (5'-AGAGTTTGATC(A/C)-TGGCTCAG-3'), Bact 1492r (5'-TACGG(C/T)-ACCTTGTTACGACTT-3'), and Bact 1098r (5'-AAGGGTTGCGCTCGTTGCG-3') (Chang *et al.,* 2000). Theoretically, amplification with Bact 27f -1492r should yield 1505bp and amplification with Bact27f -1098r should yield 1108bp from the 16S rRNA. Amplifications with these two primer sets were used to obtain the nearly full-length sequence (1505 bp) 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, 0.6 µM of each primer, 1.75 mM MgCl2, 200µM of dNTPs, 1.25 U 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 94°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 53°C for 1 minute, and extension at 72°C for 2 minutes, and a final extension at 72°C for 8 minutes. PCR products were checked for expected size on 1.5% agarose gels. The PCR product was purified by Gene JET™ 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 Tree View version 1.6.6.

Growth condition and Growth curve

To quantify growth rate and substrate disappearance, *Pseudomonas* sp. HB01was grown as described above and cultures harvested during late exponential growth phase by centrifugation at 7000 rpm for 10 min. Cells were washed twice with 50mM phosphate buffer (pH 7) and resuspended in liquid mineral medium to give an OD600nm of 0.1. Degradation of BTEX were tested in sterilized glass tubes containing 2 ml cell suspension (OD600nm = 0.1) and 2 mM 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. 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 (with DMSO only) served as controls.

Optimum pH for BTEX degradation

The ability of the isolate to degrade BTEX compounds was assessed in flasks containing 60ml of MM-$NaHCO_3$ or Na_2CO_3 and inoculated with 3ml liquid culture from *Pseudomonas* sp. HB01and spiked with 1µl (~25µmole/Flask) of undiluted BTEX the

sole carbon and energy source. Flasks containing 60 ml MM- $NaHCO_3$ or Na_2CO_3 were incubated static at 30 °C in the dark in the presence of different pH ranging from 8 - 11.

Detection, identification and characterization of key ring-cleaving dioxygenase gene

Genomic DNA from *Pseudomonas* sp. HB01 grown on benzene in MM + Na_2CO_3 (pH 10). 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 SAT GAA MAA AGG -3'), and C23O-ORF-R (5'- TYA GGT 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* DH5α 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 and 2,3 dihydroxybiphenyl 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.

Analysis of kinetic data

V_{max}, k_{cat} and apparent K_m values of extradiol dioxygenases were determined using substrate concentrations of 0.2 - 5 times the determined K_m values in air-saturated buffer and kinetic data were calculated from the initial velocities using the Michaelis-Menten equation by non-linear regression (KaleidaGraph, Synergy Software) (Junca *et al.,* 2004).

Data deposition

The 16S rRNA sequence reported in this study has been deposited in the GenBank database under accession number KF010865, catechol 2,3 dioxygenase gene under accession number KC987073.

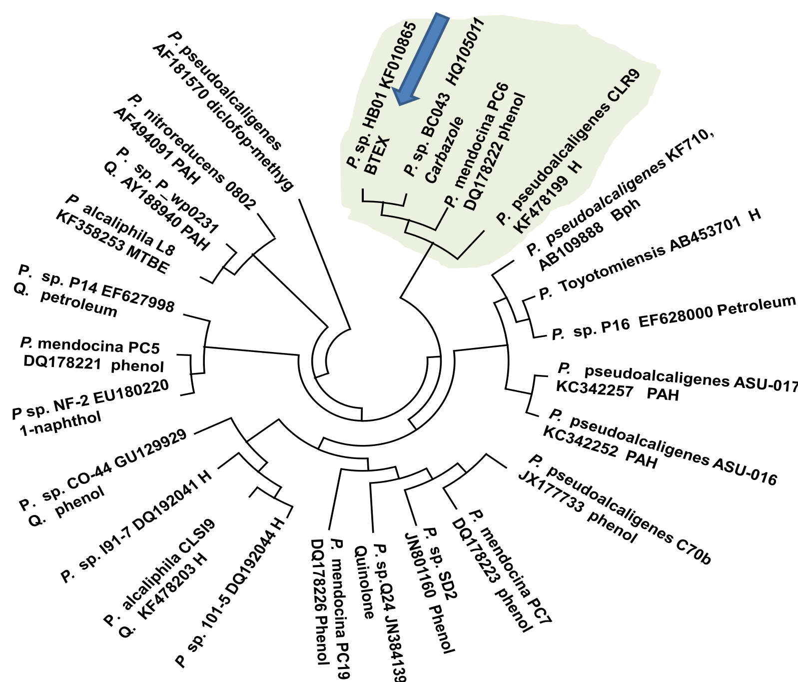

Figure 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of *Pseudomonas* sp. HB01 strain to other reported *Pseudomonas* strains. The strain followed by the accession numbers and degraded substrate. “A” aromatic degrader, “PAH” polyaromatic hydrocarbon degrader, “H” aliphatic hydrocarbon, and “Bph” biphenyl degrader.

3. Results

Identification of the alkaliphilic bacterium

Alkaliphilic bacterium that degrades benzene or toluene BTEX as the sole source of carbon was isolated from soil in Kafr El Ziat Egypt around some chemical, insecticides, and pesticides producing factories. The isolate is a gram-negative, rod-shape alkaliphilic aerobic bacterium. The isolate grew on minimal medium agar plates on BTEX vapors within 2-4 days. Comparison of the 16S rRNA gene sequence (>1400bp) of the isolate with the sequences in GenBank showed 99% sequence similarity with *Pseudomonas* sp. BC043 the most closely related organism which is carbazole-degrading isolated from activated sludge of a coking wastewater treatment plant (Zhao *et al.,* 2011), and *Pseudomonas mendocina* strain PC6, which is phenol and p-cresol-degrading and isolated from phenol-polluted water (Merimaa *et al.,* 2006) (Figure 1).

Effect of pH on the degradation of BTEX by *Pseudomonas* sp. HB01

The optimal pH for biodegradation of BTEX by strain HB01 was 10.5 (Figure. 2), degradation was observed at pH 8 and11, but not at pH less than 8.0 or more than 11, indicating that strain HB01 is an alkaliphilic bacterium (Romano et al., 2005; Li et al., 2006). strain HB01 grew and degrade on toluene and xylene better than benzene and ethylbenzene.

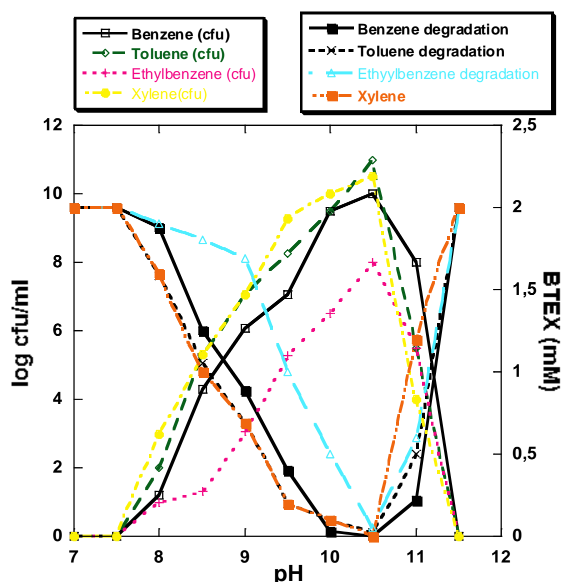

Figure 2. Biodegradation of BTEX by strain HB01 at different pHs (7- 12). The strain was grown in MM containing 2mM of BTEX with 5% concentrated cell suspension inoculum pregrown on BTEX, after 2 days incubation the CFUs were counted and BTEX degradation were measured.

Identification of extradiol dioxygenase in *Pseudomonas* sp. HB01

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 BTEX at high alkalinity. To ascertain our assumption, the key ring-cleaving gene that code for catechol 2,3-dioxygenase was amplified using degenerate primer set (C23O-ORF-F & C23O-ORF-R). The obtained PCR product was ligated into pGEM®-T Easy Vector and transformed into *E.coli* DH5*α* using transform aid bacterial transformation kit. After plating, the colonies were screened by catechol and also by DHB the colonies showed a yellow coloration indicating these colonies to contain the vector harboring expressed extradiol dioxygenase gene (catechol 2,3-dioxygenase), 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 and M13r primers. PCR products showing the presence of inserts of the expected size 1200bp (Figure 3) were purified. Sequence analysis found to be very similar to other catechol 2,3- dioxygenases from *Pseudomonas* species and showed the highest sequence identity 99% with Catechol 2,3-dioxygenase (C2,3O) from *Pseudomonas stutzeri* OX1, which is able to grow on various aromatic substrates as the sole source of carbon and energy (Viggiani *et al.,* 2004) with only four amino acids different and 95% with C2,30 from *Pseudomonas* sp. S-47 catalyzing the conversion of 4-chlorocatechol (4CC) as well as catechol to 5-chloro-2-hydroxymuconic semialdehyde

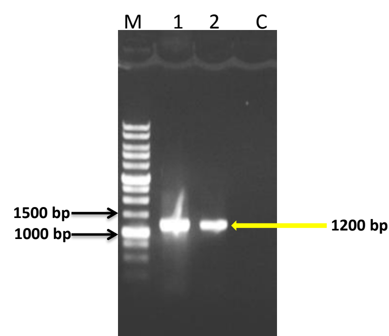

Figure 3. Agarose gel electrophoresis of PCR products obtained using M13 primers on *E.coli* DH5α colonies containing pGEM®-T Easy Vector with inserts obtained by amplification using the C23O-ORF-F / C23O-ORF-R primer set (lane 1-2). Lanes M GeneRuler 1 kb DNA Ladder (Thermo Scientific).

and 2-hydroxymuconic semialdehyde, respectively, through *meta*-ring cleavage (Noh *et al.,* 2000), 92% with C2,3O from *Pseudomonas stutzeri* AN10 (CCUG 29243) can be considered a model strain for aerobic naphthalene degradation that was isolated from polluted marine sediments of the West Mediterranean Sea (Brunet-Galmés *et al.,* 2012) (Figure 4).

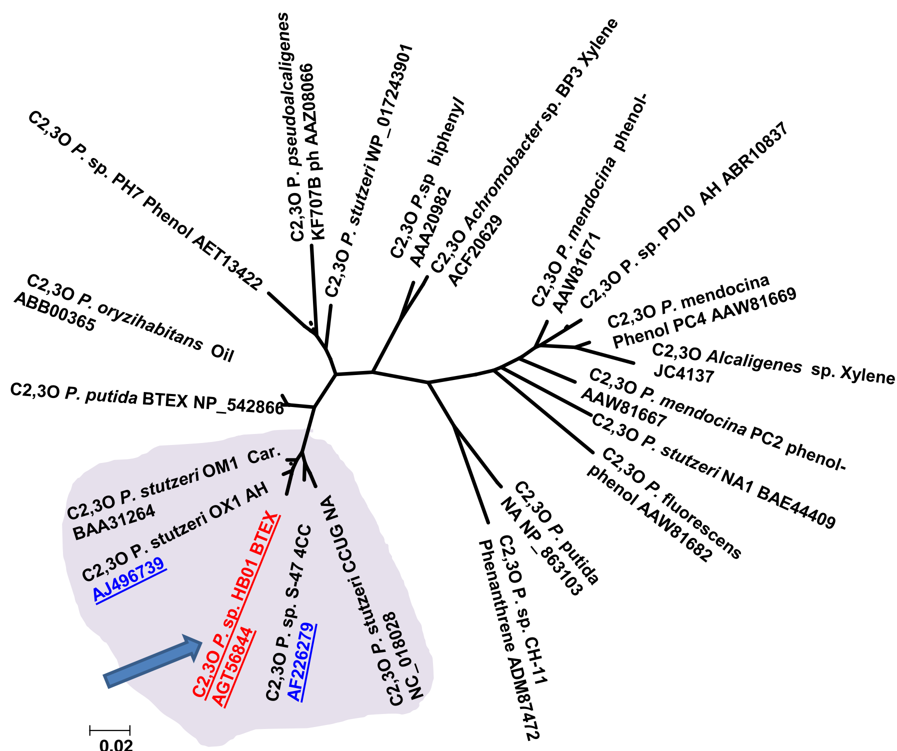

Figure 4. Dendrogram showing the relatedness of Catechol 2,3-dioxygenase (C2,3O). The dendrogram was calculated using MEGA tree version 3.1 based on protein sequence alignments calculated by ClustalX 1.81. The C2,3O proteins of *Pseudomonas* sp. HB01 are shown underlined, after the strain the substrate and the accession number. NA is the naphthalene, Car is the carbazole, AH is the aromatic hydrocarbon, 4CC is 4-chlorocatechol. The scale bar correspond to an estimated evolutionary distance of 0.02 amino acid substitutions per site

Kinetic properties of catechol 2,3 dioxygenase (C2,3O) from alkaliphilic *Pseudomonas* sp. HB01

The C2,3O was expressed in DH5α and its activity was tested in cell extracts of DH5α with 200 ± 50 U/g protein, where the cell extract comprising 30-40 g protein/l was using to determine the catalytic constants directly. Catechol and 2,3-Dihydroxybiphenyl could be identified as the preferred substrates with a K_m of 300 ± 30 μM and 1.2 ± 0.2 μM respectively(Figure 5 A, B). The K_m value with THB was even lower (0.3± 0.1μM, however, V_{max} was only 25% that with DHB as substrate. C2,3O exhibited reasonable activities with catechol derivatives and V_{max} values with catechol were of the same order of magnitude as that with DHB (140% and 90% with catechol and 3-methylcatechol, respectively), however, relatively high K_m values were observed (300 ± 30 μM and 50 ± 6 μM with catechol and 3-methylcatechol respectively (Table 1).

Table1. Catalytic properties of C2,3O from *Pseudomonas* sp. HB01 The kinetic parameters were determined using cell extracts of *E. coli* DH5*α* expressing C2,3O, Km values are expressed in μM for all substrates. Maximal turnover rates are expressed relative to those determined with 2,3-dihydroxybiphenyl as substrate.

Substrate	C2,3O from *Pseudomonas* sp. HB01			
	K_m (μM)	Vmax (%)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)
2,3-dihydroxybiphenyl (DHB)	1.2 ± 0.1	100	200 ± 5	166.6
2,2´,3-trihydroxybiphenylether (THB)	0.3 ± 0.1	25	60 ± 10	200
Catechol	300 ± 30	140	360 ± 6	1.2
3-Methylcatechol	30 ± 5	90	150 ± 5	5

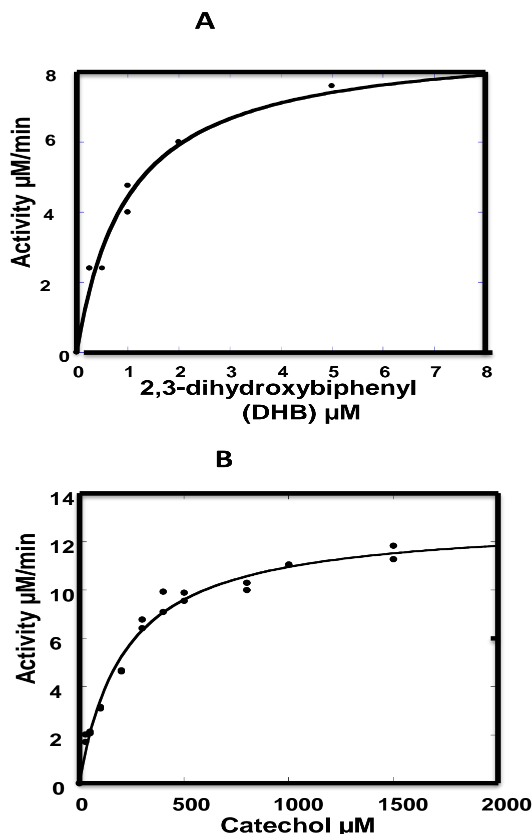

Figure 5. Michaelis-Menten plot of C2,3O from *Pseudomonas* sp. HB01 activity with 2,3-dihydroxybiphenyl (DHB, A), and catechol (B). The fitted parameters for catechol are Km = 300 ± 30 µM, and Vmax = 10.55 ± 0.48 µM/min and for 2,3-dihydroxybiphenyl Km = 1.2 ± 0.12 µM, and Vmax = 8.51 ± 0.45 µM/min.

As a result of severe effect of 3-Chlorocatechol on most of extradiol dioxygenase and its efficiently inactivity, C2,3O from *Pseudomonas* sp. HB01 had been severely affected and its activity rapidly inactivated by 3-Chlorocatechol. The determination of kinetic parameters of C2,3O directly by the inactivation 3-chlorocatechol was too efficiently (Figure 6). The K_m for this substrate was determined using DHB and catechol as reporter substrates. Similar values were obtained in both tests, indicating a low K_m value for this substrate (0.53 ± 0.13 µM when measured with catechol and 0.40 ± 0.11 when measured with DHB as reporter substrate). The apparent rate constant of inactivation was J= 230 ± 20 X $10^{-3}s^{-1}$ when measured with catechol as reporter substrate and J = 230 ± 32 X $10^{-3}s^{-1}$ when measured with DHB as reporter substrate and the J/K_m value of 380 mM^{-1} s^{-1} shows 3-chlorocatechol to be a potent inactivator for C2,3O (Table 2).

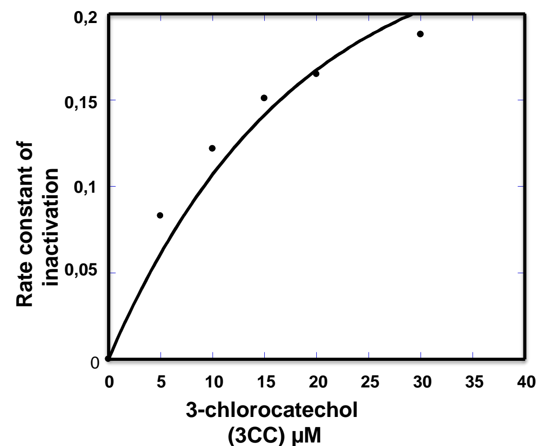

Figure 6. The inactivation C2,3O from *Pseudomonas* sp. HB01 by 3-chlorocatechol (3CC). C2,3O was incubated with DHB (100 µM) and 3CC in concentrations of 10 – 40 µM. The appearance of ring-cleavage product was monitored at 434 nm. The rate constant of inactivation J and the Km value for 3CC were calculated by fitting using KaleidaGraph. The fitted parameters are J = 0.20 ± 0.03 s^{-1} and Km = 0.53 ± 0.15 µM.

Table 2. Kinetic parameters and inactivation parameters of C2,3O from *Pseudomonas* sp. HB01 for 3-chlorocatechol (3CC).

Substrate	Km µM	J 10^{-3} s^{-1}	J/ Km 103 M^{-1} s^{-1}
3CC	0.64 ± 0.15	240 ± 30	375

4. Discussion

Understanding microbial diversity and identifying microorganisms that play a key role in the degradation of pollutants is important for defining new strategies for bioremediation. In order to gain insight into the genetic diversity and metabolic potential of microorganisms involved in the biodegradation of pollutants, analyzing for genes that code for key catabolic steps in the degradation pathways is important. Aerobic degradation pathway and genes involved in the metabolisms of BTEX have been fairly well characterized for many non-halophiles (Gross *et al.,* 1956; Jindrova *et al.,* 2002; Mampel *et al.,* 2005) this information is lacking in Alkaliphilic bacteria and there is no reports at molecular level are available on the characterization of aromatic-degrading genes in Alkaliphilies and only few Alkaliphilic bacteria reported for its capability of degrading BTEX have been reported (Yumoto *et al.,* 2002; Yamahira *et al.,* 2008, Hirota *et al.,* 2011), where in non-Alkaliphilic bacteria, a number of aerobic biodegradation pathways of aromatic compounds is catalyzed by dioxygenases that is followed by further steps of the catabolic

pathway, In the present study, alkaliphilic bacterium, strain HB01, that can degrade BTEX was isolated from soil in Kafr El Ziat Egypt around some chemical, insecticides, and pesticides producing factories. Phylogenetic analyses based on 16S rRNA sequences showed that the isolate can be considered as representing a novel species belonging to the genus *Pseudomonas.* Our results show that the strains isolated are better suited to benzene degradation than alkylebenzene and xylene. Although *Pseudomonads* are widely distributed in nature and have been reported as a metabolically and genetically diverse bacterial group (Mulet *et al.,* 2010). Few reports associated with the degradation of BTEX compounds by alkaliphilic *Pseudomonads* (Hirota *et al.,* 2011). In the present study one strain was highly similar with *Pseudomonas* sp. BC043 and *Pseudomonas mendocina* strain PC6 which carbazole and phenol degrading respectively, (Merimaa *et al.,* 2006, Zhao *et al.,* 2011). this indicate that the strain may has the capability to degrade the monocyclic and bicyclic compounds.

A decisive step in the degradation of aromatic compounds is the cleavage of benzene ring. Under aerobic conditions, microorganisms use mono- and dioxygenase enzymes to hydroxylate a variety of ring compounds to a few key intermediates including catechol and protocatechuate that are subsequently broken down by ortho- or *meta*-cleavage dioxygenases leading to the formation of Kreb cycle intermediates (Powlowski and Shingler, 1994; Harwood and Parales, 1996; Andreoni and Gianfreda, 2007). Studies have used genes that code for the ring cleavage enzymes as molecular markers to detect aromatic compounds-degrading microorganisms in a wide variety of contaminated environments including extreme habitats (Garcia *et al.,* 2005a; Kasuga *et al.,* 2007). Halomonas species have been shown to express both *ortho*- and *meta*-cleavage pathways when degrading phenol at pH 10 and 10% (w/v) NaCl. At high carbon-to-nitrogen (C/N) ratios, only *meta*-cleavage was observed, while at low C/N ratios, phenol metabolized via both ortho- and *meta*-cleavages (Maskow and Kleinsteuber 2004) The assessment of BTEX degradation pathway using degenerate primers for the detection of key catabolic gene in the *Pseudomonas* sp. HB01 DNA showed that the strain degrades benzene via catechol and *meta*-cleavage pathway. These observations are important since they help develop specific probes for the detection of similar phylotypes in variety of Alkaline habitats. To analyze the cloned catechol 2,3 dioxygenase gene and its enzyme activity the resting cells grown on BTEX were incubated with 2,3DHB and catechol for detection of the *meta*- cleavage activity for monocyclic or bicyclic aromatic catabolic pathway of PCBs degradation, the activities were detected colorimetric by the production of yellow metabolites. The yellow color was observed in both substrates as an indication of *meta*-cleavage product of hydroxyl 6-oxo-6-penta 2,4-dienoic acid (HOPDA) in the case of 2,3DHB and 2-hydroxymuconic semialdehyde for catechol, this results emphasis that this strain may have the capability to grow on mono and bicyclic aromatic compounds. The amino acid sequence of C2,3O from our strain showed highly similarity with the C2,3O from *Pseudomonas stutzeri* isolated from soil and marine sediment for a population genetic study (Sikorski *et al.,* 2005).

The extradiol dioxygenases analyzed here C2,3O from *Pseudomonas* sp. HB01, showed a high affinity towards 3-chlorocatechol as indicated by low K_m values < 1 μM, similar to the observed Ki values of DHB12Os analyzed by Ohnishi (Ohnishi *et al.,* 2004) and relatively similar to the reported K_m of 4.8 μM for 3-chlorocatechol of BphCLB400 (Vaillancourt *et al.,* 2002) However, the important feature exerted by 3-chlorocatechol is not the simple inhibition, but its capability of mechanism based inactivation, the efficiency of which is reflected by the J/Km value. It is well documented, that BphC 2,3-dihydroxybiphenyl 1,2-dioxygenases are adapted to the transformation of bicyclic compounds and are only poorly active with catechol, and exhibit also only faint activity with 3-methylcatechol, which differentiate them from catechol 1,2-dioxygenases (Eltis & Bolin, 1996), which are active mainly with catechol. C2,3O from *Pseudomonas* sp. HB01 obviously is less restricted in their substrate preference, as indicated by a comparison of the kcat/Km values, which differed by only 1-2 orders of magnitude between the preferred bicyclic substrates THB and DHB and the less preferred substrate catechol/3-methylcatechol. Recently, some structural determinants of the substrate selectivity of bicyclic and monocyclic extradiol dioxygenases were investigated based on mutants in BphCLB400 (Vaillancourt *et al.,* 2005). Among the mutants a V148L derivative had increased specificity for catechol. V148 usually lines the distal ring of 2,3-dihydroxybiphenyl in 2,3-dihydroxybiphenyl 1,2-dioxygenases, whereas in catechol 2,3-dioxygenases, a larger leucine is located at the respective position (Vaillancourt *et al.,* 2005).

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1/20/2014