

Isolation of 4-Chlorophenol-Degrading Bacteria, *Bacillus subtilis* OS1 and *Alcaligenes* sp. OS2 from Petroleum Oil-Contaminated Soil and Characterization of its Catabolic Pathway

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Abstract: Chlorophenols are carcinogenic and toxic environmental pollutants which massively discharged to the environment from uncontrolled industrial activities. Many industrial wastes were screened for chlorophenols-degrading bacteria. Two bacterial strains designated as OS1 and OS2, were isolated from petroleum oil-contaminated soil and characterized for their ability to degrade some chlorophenols. Strains OS1 and OS2 were able to catabolize only 4-chlorophenol (4-CP) and use it as a main carbon source. 4-chlorophenol was degraded up to 100 mg/L above which the degradation rate was reduced dramatically due to the cytotoxicity of chlorophenol. Strains were identified by the most recently used molecular typing technique by sequencing V3 variable region of their 16S-rRNA genes. According to their 16S-rDNA base sequence, strains OS1 and OS2 were identified as *Bacillus subtilis* OS1 and *Alcaligenes* sp. OS2. UV/VIS spectrophotometric analysis showed that *Alcaligenes* sp. OS2 is catabolizing 4-CP via extradiol *meta*-ring cleavage pathway revealed by the formation of 5-chloro-hydroxymuconate semialdehyde (5-CHMS) as a result of extradiol ring cleavage of 4-chlorocatechol (4-CC) primarily produced via hydroxylation of 4-CP by phenol hydroxylase (PH). GC-MS analysis showed that *Alcaligenes* sp. OS2 is catabolizing 4-CP via dehydrogenative branch of extradiol *meta*-ring cleavage pathway revealed by detection and identification of 4-CC and 5-chloro-hydroxymuconate (5-CHM) as intermediate metabolites in their cell free extract. However, 5-CHMS was not detected in culture filtrate of *B. subtilis* OS1, suggesting a possibility for a modified *meta*-pathway. Cell free extract of *B. subtilis* OS1 with 4-CP was found to contain 5-CHM as a major intermediate metabolite. Extradiol *meta*-ring cleavage pathway in these strains was assigned to the activity of catechol 2,3-dioxygenase (C23O).

Key words: chlorophenol, biodegradation, *meta*-pathway.

INTRODUCTION

Many chlorinated phenols are introduced to the environment as a contaminating compound from chemical manufacturing companies (Freiter, 1979). The natural pool of phenolic compounds is being increased as by-products of industrial origin. Chlorophenols are utilized in the production of detergents, and various biocides. Large quantities of higher chlorophenols are used in the wood preservation industry and as different fungicides (McAllister *et al.*, 1996). Significant amounts of chlorophenols can be formed and subsequently released into the environment as by-products of chlorine bleaching process in the pulp and paper industry (Annachhatre and Gheewala, 1996; Tartakovsky *et al.*, 2001), the chlorination of waste-water and drinking-water, and the incineration of municipal waste (Contrerasa *et al.*, 2003). Most of chlorinated phenolic compounds are very resistant to microbial degradation in sewage plants and accumulate in soil and water after they have fulfilled their useful function. Chlorophenols are toxic for a wide range of organisms (Herrera *et al.*, 2008), a property that accounts for many of their uses as antiseptics and disinfectants (Field and Sierra-Alvarez, 2008).

Chlorophenols due to their toxicity, carcinogenicity and recalcitrant nature are listed as priority pollutants by the Environmental Protection Agency (EPA, USA) and Agency for Toxic Substances and Disease Registry (ATSDR) and hence desire effective removal (ATSDR, 1999). Effective treatment of chlorophenolic waste requires simultaneous removal of other phenolics. However chlorophenols are much more environmentally stable than the parent unsubstituted phenol and desire more attention for effective removal (Ingols *et al.*, 1966).

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Microbial degradation of aromatic compounds has given much attention nowadays. It was reported that *Ralstonia eutropha* strains JMP134 and JMP222 can grow on trichlorophenol as the sole carbon source (Padilla *et al.*, 2000) while *Sphingomonas* strains K74 and MT1 and *Nocardioides* sp. K44 can degrade tetrachlorophenol as the sole carbon and energy sources (Minna *et al.*, 2001). Some aerobic pentachlorophenol (PCP)-degrading bacteria have also been isolated from contaminated soils (Crawford and Ederer, 1999). *Sphingobium chlorophenicum* (Takeuchi *et al.*, 2001) is one of the bacteria capable of completely mineralizing PCP. Mono- and dichlorophenols are usually degraded by aerobic bacteria via hydroxylation to corresponding chlorocatechols (Schwien and Schmidt, 1982). These chlorocatechols are further degraded through the modified *ortho*-pathway (Reineke and Knackmuss, 1988; Schlömann, 1994). Alternatively to the intradiol type of ring cleavage, the pathway may be initiated by extradiol ring cleavage in some microorganisms (Kaschabek *et al.*, 1998). *Meta*-cleavage by catechol 2,3-dioxygenase (C23O) is known to sometimes result in incomplete metabolism due to the production of dead-end or suicide-metabolites (Schmidt *et al.*, 1983). Successful degradation of chloroaromatics via chlorocatechols using the *meta*-cleavage pathway has been demonstrated (Arensdorf and Focht, 1995; Mars *et al.*, 1997), but is difficult. Generally, *meta*-cleavage of 3-chlorocatechol (3-CC) results in the inactivation of C23O either by 3-CC itself, acting as a chelating compounds (Klecka and Gibson, 1981), or by the production of a highly reactive acylchloride, the product of the cleavage of 3-CC, which binds irreversibly to the *meta*-cleavage enzyme (Bartels *et al.*, 1984). The *meta*-cleavage of other chlorocatechols results in the production of a chlorinated aliphatic compound that finally enters tricarboxylic acid cycle (TCA) (Reineke *et al.*, 1982; Westmeier and Rehm, 1987; Weiser *et al.*, 1994). Many reports have shown that further metabolism of chlorocatechols intermediates do occur, resulting in complete degradation of chlorophenols via a *meta*-cleavage pathway (Sung *et al.*, 1996; Hollender *et al.*, 1997). Chloroaromatic compounds containing more than two chlorine atoms are converted to hydroxyquinol or chlorohydroxyquinols and then cleaved by specific intradiol dioxygenases (Li *et al.*, 1991; Joshi and Gold, 1993; Travkin *et al.*, 1997).

MATERIALS AND METHODS

Sampling and Culture Conditions:

Samples were collected from different chlorophenols and/or petrochemical contaminated soils in Egypt, including; contaminated soil from Clorox company for chlorine production (El-Asher Men Ramadan city), Oil stations, and Racta company for paper production (El Tabia, Alexandria). Samples were collected in clean and sterile bottles, kept in 4°C for further analysis. Soil samples were enriched on basal mineral medium (Farrell and Quilty, 1999) with 100 mg/L chlorophenols and incubated at 30°C with daily examination for removal of chlorophenols added. Ten percent from each enrichment culture were transferred onto basal mineral media supplemented with chlorophenols and kept at 30°C with daily examination for removal of chlorophenols added. Subculturing in a fresh media was continued until a stable culture was obtained showing stable biodegradation of selected chlorinated phenols. Aliquots from each culture were transferred onto basal mineral agar plates and spreaded out aseptically. Chlorophenols were used in the medium as a main carbon source. Screening for potential chlorophenol degrading bacteria was made possible using a defined basal medium. Chlorophenol was added to culture medium after sterilization to a final desired concentration. Chlorophenols (2-CP, 4-CP, 2,4-DCP, 2,4,6-TCP, 2,3,4,5-TeCP and PCP, Loba Chemie, India) were added to the media in different concentrations ranging from 20 to 100 mg/L. Cultures were incubated at 30°C in shaking incubator, adjusted at 150 rpm and examined periodically for removal of chlorophenols.

Analysis of Chlorophenols:

Chlorophenols concentrations were measured using the 4-aminoantipyrine colorimetric method based on the procedure detailed in Standard Methods for the Examination of Water and Wastewater (Greenberg *et al.*, 1992). Briefly, One hundred microliter of chlorophenol-containing sample was placed in clean tube. One hundred microliter of distilled water was used instead of sample to prepare blank and a series of 100 µl chlorophenol standards containing 500, 250, 125, 62.5, 31.25, 15.6 and 7.8 mg/L chlorophenol were also prepared. Sample, blank and standards were treated by adding 100 µl 0.5 N NaHCO₃ and mixed well. One hundred microliter of 4-aminoantipyrine solution (0.6%) was added and mixed well. One hundred microliter of potassium ferricyanide solution (2.4%) was added and mixed well. After 15 min, the developed color was measured by reading absorbance of sample and standards against the blank at 500 nm.

Uv/vis Spectra of Intermediate Compounds:

For detection of intermediates, samples were centrifuged to remove cells; the resulting supernatant was measured using UV-visible spectrophotometer (Unicam UV-300, UK). Spectra were recorded between 200 and 600 nm.

GC-MS Analysis of Intermediate Compounds:

Intermediate compounds were analyzed in culture filtrate after removing cells by filtration through 0.2 µm Millipore filters. Filtrates were extracted with dichloromethane (methylene chloride). Extracts were injected into a GC-MS (HP 5973/HP 6890) oven system equipped with a column (Aquatic 0.25 mm I.D. x 60 m, df 1.0 m, GL-Sciences, Japan). Temperature program was set as follows: column oven temperature, 40°C for 1 min, 40°C to 190°C at 10°C/min, 190°C for 10 min. Helium was used as a carrier gas. Main parameters related to the mass spectrometer were: EI energy 2000 eV; temperature, 220°C; scan rate, 1.0 scan/s; scan range, *m/z* 0 to 600.

Identification of Bacterial Isolates:

A- PCR Amplification of 16S-rRNA Genes:

Amplification of 16S-rDNA was performed by a modified method of Arturo *et al.* (1995). A loopful of overnight grown cells was transferred to 50 µl TE buffer and boiled for 5 min. Then, 1 µl of cell suspension was used as template for PCR reaction. PCR was performed using Premix Taq (Ex Taq Version, Takara, Japan) according to instruction manual. A pair of flanking sequences was used for primer binding sites to partially amplify target 16S-rRNA genes from the bacterial isolates 16S-1F (5'-AGAGTTTGATCCTGGCTCAG-3') and 16S-1500R (5'-ACGGCTACCTTGTTACGACT-3'). PCR was performed in MyCycler thermal cycler (Bio-Rad, UK). The PCR conditions were adjusted to 5 min for initial denaturation at 94°C and then 35 cycles of 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C, and finally 10 min at 72°C. PCR was terminated after the program was completed and amplified genes were electrophoresed out on a 1% agarose gel with size marker.

B- Cloning and Sequencing of 16S-rRNA Genes:

16S-rDNA-containing plasmid vectors were constructed using pGEM-T Easy vector system (Promega, USA) according to instruction manual. Constructed vectors were used to transform competent *E. coli* cells (JM 109, Takara, Japan). Recombinant plasmid DNA was isolated using standard plasmid miniprep procedure based on Wizard plus plasmid DNA purification system (Promega, USA) according to instruction manual and used for subsequent sequence base determination. The nucleotide sequence analysis of the selected clones were determined by automated fluorescent dye terminator sequencing method originally developed by Sanger *et al.* (1977) (DYEynamic ET Terminator Cycle Sequencing Kit, Amersham Pharmacia Biotech.) with a model ABI 310 genetic sequence analyzer (Applied Biosystems, CA, USA) according to user manual. Dye terminator-based sequencing was performed using PCR-amplified segments of about 517 bases covering up V3 region of 16S-rRNA genes with M13F vector specific primer with the following program (95°C, 20s; 50°C, 15s; 60°C, 60s for 25 cycles). Obtained sequences were analyzed by Genetyx-Win MFC application software version 4.0. Related sequences were identified using BLAST search program, National Center for Biotechnology Information (NCBI), National Library of Medicine, USA (Altschul *et al.*, 1997). Sequence alignments were performed by Clustal W1.83 XP software and phylogenetic trees were constructed using neighbor-joining method (Saitou and Nei, 1987) using MEGA3 software.

Nucleotide Sequence Accession Numbers:

Partial 16S-rDNA base sequences determined in this study have been deposited in NCBI GenBank under accession numbers AB474001 and AB473832 for strains OS1 and OS2 respectively.

RESULTS AND DISCUSSION

Isolation of Chlorophenol-Degrading Bacteria:

4-Chlorophenol-degrading bacteria were isolated from petroleum oil contaminated soil at oil stations, Egypt. Such soils were found to include sources of contamination by derivatives of petroleum compounds such as chlorophenols. Two bacterial strains designated as OS1 and OS2 have been isolated and characterized from such contaminated soil. Strains were identified by analysis of their 16S-rDNA base sequence. The 16S-rRNA gene (~1500 bp) was amplified, cloned and partially sequenced. About 517 bp was sequenced (1 - 517) as this

region, particularly from the bases 341 to 517 represents hypervariable region, where sequences have diverged over evolutionary time. Alignment of the obtained gene sequences with closest matches from the Blast GenBank database with the highest percentage of identity was performed. Strain OS1 shared 99.4% sequence similarity to *Bacillus subtilis* while strain OS2 shared 99.7% sequence similarity to *Alcaligenes* sp. Among all chlorophenols tested, 4-CP was effectively degraded by both strains. Both strains used 4-CP as a main carbon source, a property restricted to few types of bacteria and in contrast to known bacteria co-metabolizing chlorophenols in presence of utilizable carbon sources (Ziagova *et al.*, 2009).

Biodegradation of 4-Chlorophenol by *B. subtilis* OS1:

B. subtilis OS1 was able to degrade 4-CP up to 100 mg/L, above which no biodegradation was observed. *B. subtilis* OS1 was unable to degrade 2-CP, 2,4-DCP, 2,4,5-TCP, 2,3,4,5-TeCP and PCP. The rate of 4-CP degradation was efficient at low concentrations (20 – 60 mg/L) However; the rate of biodegradation was much decreased at elevated concentrations of 80 and 100 mg/L. At high 4-CP concentrations an inhibitory effect on the rate of biodegradation was observed resulting in incomplete degradation of 4-CP. Such inhibitory effect was attributed to the cytotoxicity of chlorophenols at elevated concentrations (Herrera *et al.*, 2008). Fig. (1,B) shows the time dependant change in 4-CP concentration by *B. subtilis* OS1 growing on different concentrations of 4-CP, (20, 40, 60, 80, and 100 mg/L). 4-CP was completely catabolized at concentrations ranging from 20 – 60 mg/L.

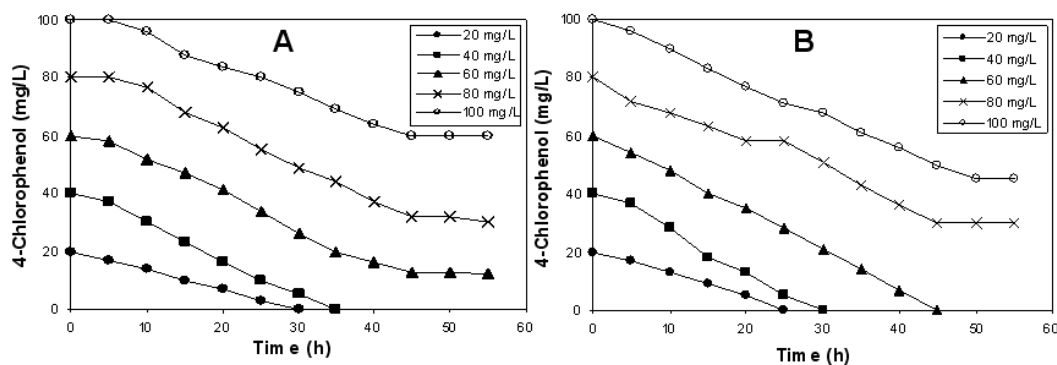


Fig. 1: Time dependent biodegradation of 4-CP by *Bacillus subtilis* OS1 (A) and *Alcaligenes* sp. OS2 (B) supplemented with different initial concentrations (20 – 100 mg/L).

Biodegradation of 4-Chlorophenols by *Alcaligenes* sp. OS2:

Alcaligenes sp. OS2 was able to degrade only 4-CP up to 100 mg/L, above which no biodegradation was observed. Biodegradation was efficient and complete for concentration of 20 and 40 mg/L (Fig. 1, A). *Alcaligenes* sp. OS2 was unable to degrade 2-CP, 2,4-DCP, 2,4,5-TCP, 2,3,4,5-TeCP and PCP. Fig. (17) shows the time dependant change in 4-CP by *Alcaligenes* sp. OS2 using different concentrations of 4-CP, (20, 40, 60, 80, and 100 mg/L). At high 4-CP concentrations an inhibitory effect on the rate of biodegradation was observed resulting in incomplete degradation of 4-CP. Again, cytotoxicity of chlorophenols at elevated concentrations was the cause for such an inhibitory effect.

Determination of Chlorophenol Biodegradation Pathway:

GC-MS and UV/VIS spectrophotometric analysis showed that *Alcaligenes* sp. OS2 degrade 4-CP via extradiol *meta*-pathway. Firstly 4-CP was hydroxylated to 4-CC, which then cleaved via extradiol *meta*-pathway to give a typical yellow 5-chloro-2-hydroxy muconic semialdehyde (5-CHMS). Fig. (2) Shows the absorption spectra of supernatant of cell suspension of *Alcaligenes* sp. OS2 showing the production of 5-CHMS, λ_{max} at 380nm. Production of hydroxy muconate semialdehyde as a result of *meta* ring cleavage of many aromatic hydrocarbons have been reported (Kaschabek *et al.*, 1998; Sung *et al.*, 1996; Hollender *et al.*, 1997). hydroxy muconate semialdehyde is a precursor for down stream compounds that fed eventually to the tricarboxylic acid cycle.

To follow up the biodegradation pathway for 4-CP in both bacterial strains, a GC-MS analysis was performed with resting cell conditions. Fig. (3) shows the mass spectra of obtained intermediates and the identification of each selected metabolite in extracted culture filtrate of resting cells of *Alcaligenes* sp. OS2 with 4-CP as a starting compound. Beside 4-CP as a starting compound, two main metabolites have been identified as, 4-chlorochatechol (4-CC) and 5-chloro-2-hydroxy muconate (5-CHM).

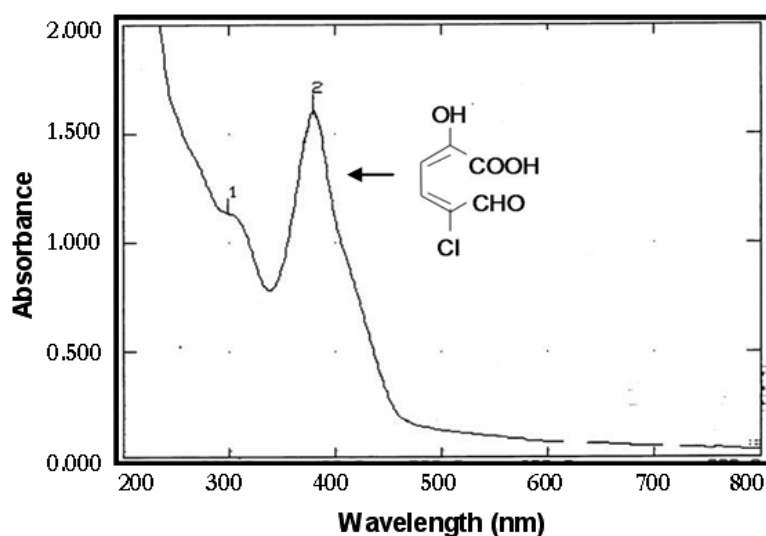


Fig. 2: UV/VIS absorption spectra of supernatant of cell suspension of *Alcaligenes* sp. OS2 with 4-CP showing the production of 5-CHMS, λ_{max} at 380 nm.

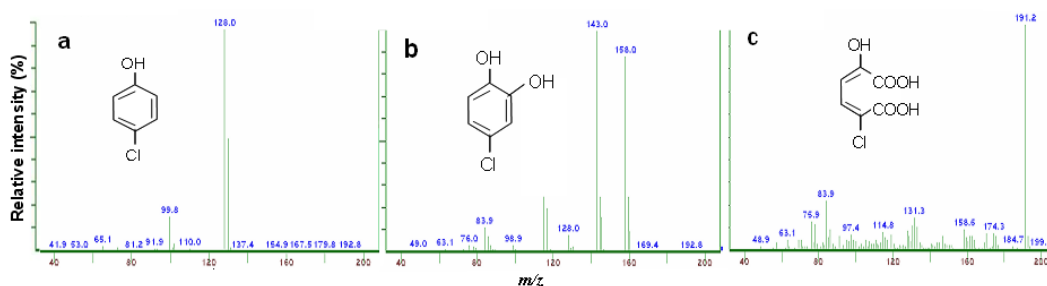


Fig. 3: Mass spectra of intermediate metabolites produced as a result of 4-CP biodegradation by *Alcaligenes* sp. OS2 showing the presence of 4-CP as a starting compound (a), 4-CC (b), and 5-CHM (c).

By combining the data obtained from UV/VIS spectrophotometry and GC-MS analysis, it was possible to propose the biodegradation pathway for 4-CP in both bacterial strains (Fig. 4). *Alcaligenes* sp. OS2 initiates the degradation pathway by hydroxylation of 4-CP to the corresponding 4-CC. The produced 4-CC then undergoes a *meta*-ring cleavage by C23O to produce 5-CHMS which in turn is converted to 5-CHM. The downstream pathway was also found to proceed via the dehydrogenative branch revealed by the detection of 5-CHM, oxidation product of 5-CHMS, as an intermediate compounds in culture filtrate of *Alcaligenes* sp. OS2. The pathway then proceeds via *meta*-pathway and intermediates were introduced to the central metabolism (Arnesdorf and Focht, 1995; McCullar *et al.*, 1994; Hollender *et al.*, 1997).

Mass spectra of intermediate metabolites produced as a result of 4-CP biodegradation by *B. subtilis* OS1 showed the presence of 5-CHM indicating a *meta*-cleavage pathway. However, UV/VIS spectrophotometric analysis showed absence of 5-CHMS, a key metabolite in the *meta*-pathway. So, it was suggested that 4-CP biodegradation by *B. subtilis* OS1 proceeds via a modified *meta*-pathway. Such assumption was suggested due to the lack of 5-CHMS as a precursor to 5-CHM. Activated aromatic compounds undergo ring cleavage reactions via lower pathway and are further processed to give molecules that can eventually enter the tricarboxylic acid cycle (Cafaro *et al.*, 2004). Downstream pathway is documented to proceed by catabolizing 5-CHM to TCA. 5-CHM is converted to 5-chloro-2-oxopent-4-enoate by 4-oxalocrotonate isomerase and 4-oxalocrotonate decarboxylase (dehydrogenative pathway). 5-CHMS could be converted directly to 5-chloro-2-oxopent-4-enoate by Hydroxymuconate semialdehyde hydratase (HMSH) through what is known as hydrolytic pathway (Arnesdorf and Focht, 1995). 5-chloro-2-oxopent-4-enoate is converted by 5-chloro-2-oxopent-4-enoate hydratase to 5-chloro-4-hydroxy-2-oxopentate which is then converted to chloroactate and pyruvate by 5-chloro-4-hydroxy-2-oxopentate aldolase. Pyruvate enters TCA directly, while chloroactate enters it after conversion to glycolate (McCullar *et al.*, 1994). Beside determination of the 4-CP biodegradation pathway in our strains as a *meta*-pathway, it is also concluded that catabolism of 5-CHMS to 5-chloro-2-oxopent-4-enoic acid proceeds via dehydrogenative branch of the *meta*-pathway.

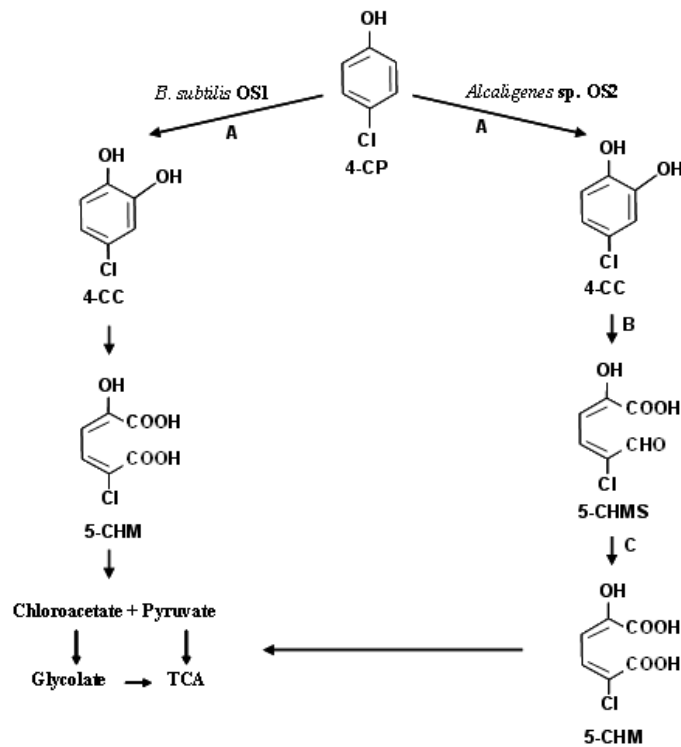


Fig. 4: Proposed pathway for the biodegradation of 4-CP by *Bacillus subtilis* OS1 and *Alcaligenes* sp. OS2. Enzymes involved in the pathway includes, phenol hydroxylase (A), catechol 2,3-dioxygenase (B), and 2-hydroxymuconic semialdehyde dehydrogenase (C).

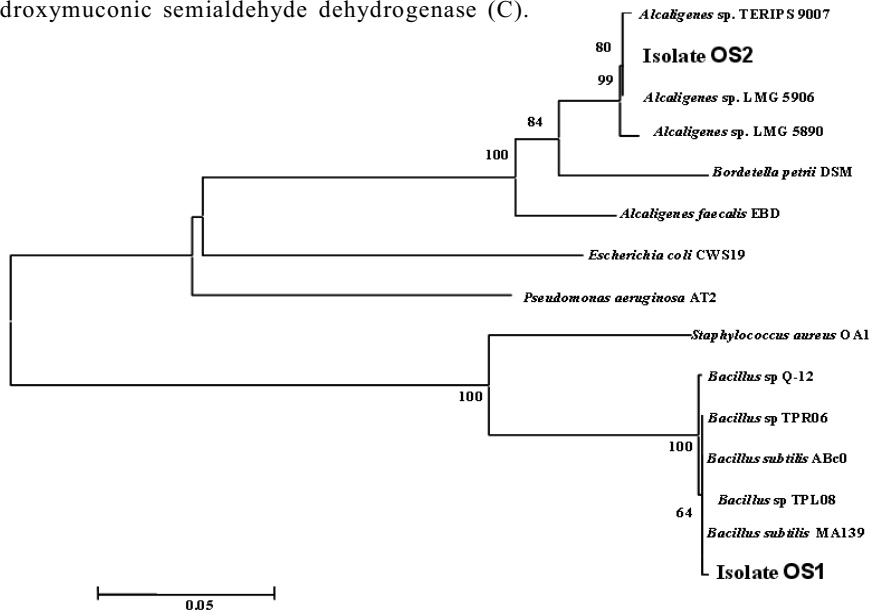


Fig.5: Neighbor-joining phylogenetic tree based on 16S-rDNA sequences analysis showing the relationship between isolates OS1 and OS2 and representative species of the genera *Bacillus* and *Alcaligenes* together with other related genera. NCBI GenBank accession numbers for all sequences are given as follows: *Alcaligenes* sp. TERIPS 9007 (AY499114); *Alcaligenes* sp. LMG 5906 (AY131213); *Alcaligenes* sp. LMG 5890 (AY131212); *Bordetella petrii* DSM (AM902716); *A. faecalis* EBD (EF011115); *E. coli* CWS19 (FM20752); *P. aeruginosa* AT2 (AB091760); *S. aureus* OA1 (D83356); *Bacillus* sp. Q-12 (AB199317); *Bacillus* sp. TPR06 (EU373402); *B. subtilis* ABc0 (EU862566); *Bacillus* sp. TPL08 (EU373378) and *B. subtilis* MA139 (DQ415893). The bar represents 0.05 nucleotide substitution per 100 nucleotides.

Phylogenetic Relationships:

A phylogenetic tree was constructed based on 16S-rDNA sequences obtained from both strains and comparison with analogues sequences from GenBank (Fig. 5). Such phylogenetic analysis showed the relationship between both strains and representative species of the genera *Bacillus* and *Alcaligenes* and other related genera. Strain OS1 was clustered at the same phylogenetic branch with *Bacillus* sp. while strain OS2 was clustered with *Alcaligenes* ones. *Pseudomonas aeruginosa* AT2 was included in this phylogenetic study as a known phenol and chlorophenol degrading bacterium (El-Sayed *et al.*, 2003). *Alcaligenes* sp. was more related to *P. aeruginosa* AT2. That was convenient with the fact that both strains catabolize chlorophenols via typical *meta*-cleavage pathway. On the other hand, strain OS1 was clustered away from strain AT2 confirming a modified *meta*- pathway.

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