JASEM ISSN 1119-8362

Full-text Available Online at www.bioline.org.br/ja

J. Appl. Sci. Environ. Mgt. *September*, 2006
Vol. 10 (3) 75 - 81

# Detection of *meta*- and *ortho*-cleavage dioxygenases in bacterial phenol-degraders

***SAHAR ZAKI**

*Environmental biotechnology Department, Genetic Engineering and Biotechnology Research Institute, Mubarak City for Scientific Research and Technology Applications, Post code 21934, New Burg-Elarab, Alexandria, Egypt.*

**ABSTRACT:** In the last five years, in our lab, several bacterial genera capable of degrading phenol as sole carbon source were isolated from different Egyptian ecosystems. Phenol mineralization using these isolates was ranged from 55% to 0.4%. In the present work, randomly chosen representative strains; W-17, DF4 (*Acinetobacter*), Sea-8 (*Stenotrophomonas*), W-6 (*Klebsiella*), S-5 (*Bacillus*) and W-15 (*Ralstonia*) were analyzed for the type of ring-cleavage (*ortho* or *meta*) dioxygenase physiologically induced during growth in the presence of phenol as a sole carbon and energy source. The specific activities of the phenol-degrading enzymes phenol hydroxylase, catechol-1,2-dioxygenase and catechol-2,3-dioxygenase were investigated. In addition, whole-cell dioxygenases activities of the examined isolates were determined. Out of the results, only the *Acinetobacter* strains W-17 and DF-4 showed activity with the enzymes phenol hydroxylase and catechol-1,2-dioxygenase, which responsible for phenol degradation through *ortho*-cleavage pathway. In contrast, isolates S-5, Sea-8, W-6, W-15 and Pla-1 showed activity with the enzyme catalyzing the second step in the phenol degradation *meta*-cleavage pathway, catechol-2,3-dioxygenase. On the basis of our previous and present analysis, the investigated isolates are considered to have a good potential for application in remediation of phenol contaminated environments and industrial wastewater. @JASEM

Phenol and phenolic compounds are of widespread use in many industries such as polymeric resin production and oil refining. As a result, these compounds are commonly encountered in industrial effluents and surface water. These pollutants are usually treated in activated sludge processes because many aerobic bacteria and fungi are able to use phenol as a source of carbon and energy (Rebhun and Galil 1988; Watanabe et al., 1996). Biodegradation of phenol, therefore, has long been the subject of numerous investigations (Ruiz-Rrdaz et al., 2001; Chang et al., 1998; Fava et al., 1995; Abd-El-Haleem et al., 2003; Dean-Ross, 1989; Solomon et al., 1994; Ahmed et al., 1995; Alleman et al., 1995; Collins and daugulis, 1997; Fulthorpe and Allen, 1995; Lin et al., 1990; Morris and Lester, 1994; Ryu et al., 2000; Wang et al., 1996). A typical pathway for metabolizing an aromatic compound like phenol is to dihydroxylate the benzene ring to form a catechol derivative and then to open the ring through *ortho* or *meta* oxidation. Catechol is either oxidized in a reaction catalyzed by catechol-1,2-oxygenase which is described as an *ortho* pathway, or is oxidized in reaction catalyzed by 2,3-dioxygenase the *meta* pathway to 2-hydroxymuconic semialdehyde. The final products of both the pathways are molecules that can enter the tricarboxylic acid cycle (Powlowski and Shingler 1994; Harayama et al. 1992).

Previously in our lab twelve bacterial phenol-degraders which could grow aerobically and assimilate 500 mg/l phenol for a period of 15 h were isolated and Molecular characterized by Abd-El-Haleem et al. (2002 and 2003). Their comparative sequence analysis of the 16S ribosomal DNA, classified them to different affiliated groups. Four isolates of them W-17, DF-4, Sea-9 and Sea-12 were closely related to the genus of *Acinetobacter*. Two strains Sea-8 and Sea-11 were belonging to *Stenotrophomonas maltophilia*. Strains DM-5 and W-6 were affiliated to *Klebsiella.* Isolates S-5 and W-15 were closely related to *Bacillus* and *Ralstonia* respectively. Some of these isolates have been immobilized into organic and inorganic carriers and has shown good capacity for long-term degradation of high phenol concentration (Abd-El-Haleem et al. 2003; Beshy et al. 2003).

In the present study randomly selected representative strains; W-17, DF4 (*Acinetobacter*), Sea-8 (*Stenotrophomonas*), W-6 (*Klebsiella*), S-5 (*Bacillus*), W-15 (*Ralstonia*) and Pla-1 (*Microbacterium*) were analyzed for the type of ring-cleavage dioxygenase physiologically induced during growth in the presence of phenol as a sole carbon and energy source. The specific activities of the phenol-degrading enzymes were investigated.

## MATERIALS AND METHODS

*Materials:* Unless specified otherwise, all reagents were of the highest purity available.

*Bacterial strains:* Isolation and molecular characterization of the all bacterial strains used in this study described previously by Abd-El-Haleem et al. (2002).

**Corresponding author: *Phone: (00203) 459-3420 Fax: (00203) 459-3423*
*E-mail: saharzaki@yahoo.com*

*Medium and culture conditions:* To achieve enough amounts of cells, isolates were grown in LB-medium, which contained per liter: 5 g yeast extract, 10 g casein peptone, 10 g NaCl, while the pH was adjusted to 7.0 before autoclaving. Subsequently, 10 % inocula from LB cultures were transferred to 50 ml freshly prepared minimal salts medium {(MSM) containing (per liter) 2.75 g of $K_2HPO_4$, 2.25 g of $KH_2PO_4$, 1.0 g of $(NH4)_2SO_4$, 0.2 g of $MgCl_2$.6H2O, 0.1 g of NaCl, 0.02 g of $FeCl_3.6H_2O$, and 0.01 g of $CaCl_2$ (pH 6.8 to 7.0)} supplemented with 5 mM phenol as sole carbon and energy source. All growth experiments were run in 250 ml Erlenmeyer flasks containing 50 ml of medium at 30 °C on a rotary shaker at 200 rpm.

*Enzyme assays:* All enzyme assays were routinely performed at 30°C using a Beckman DU7500 spectrophotometer with integral chart recorder and temperature control. All enzyme rates are the average of at least three determinations. The enzyme activity was defined as the initial rate of indigo formation or enzyme specific activity normalized to the protein content of the sample.

*Determination of normalized dioxygenases activity in whole cells***:** The optical density of a 1 ml sample of the culture was determined; then the sample was immediately transferred to a microcentrifuge tube. The cells were harvested and washed with phosphate buffer solution (pH=7.2) by centrifugation (1 min at 14 000 rpm at 4°C). The enzyme reaction was initiated by addition of indole (Jenkins and Dalton, 1985). Five microliters of 100 mM indole in *N*,*N*-dimethlylformamide was added to the cells and the formation of indigo was monitored spectrophotometrically at 600 nm over the reaction time against a control (resuspended cells without indole). The initial rate of indigo formation was determined by plotting the increase in indigo absorbance as a function of time.

*Phenol hydroxylase:* The oxidation of NADH in the presence of phenol by hydrogen peroxide-treated cell extracts was monitored at 340 nm. Reaction mixtures (3 ml) containing 50 mm $KH_2PO_4$: $K_2HPO_4$ buffer pH 7.2, 1.0 mmol NADH, and 100 nmol phenol were equilibrated at 55°C before the addition of the cell extract (100 ml). One unit of activity was defined as the amount of enzyme catalyzing the oxidation of 1 mmol NADH min (Gibson et al. 1990).

*Catechol-2,3-dioxygenase:* Reaction mixtures (3 ml) in 50 mm $KH_2PO_4$:$K_2HPO_4$ buffer pH 7.2 containing 1 mmol catechol were equilibrated at 55°C before adding the cell extract (100 ml). The increase in absorbance at 375 nm caused by the formations of the reaction product 2-hydroxymuconic semialdehyde was monitored (Masai**,** et al. 1995) One unit of enzyme activity was defined as the amount of enzyme catalyzing the production of 1 mmol 2-hydroxymuconic semialdehyde per minute at 55°C.

*Catechol-1,2-oxygenase:* Reaction conditions were identical to those of the catechol-2,3-dioxygenase assay except the formation of *cis*, *cis*-muconic acid which was monitored at 260 nm (Ornston, et al. 1966)

*Protein concentration:* Protein concentrations were measured by the Bradford method (Bradford, 1976) using the Bio-Rad protein assay reagent. Bovine serum albumin (Sigma, Fraction V) was used as a standard.

## RESULTS AND DISCUSSION

A representative growth profile is shown in Fig 1, periodic monitoring of cell density revealed differences in growth characteristics between the cultures. All isolates reached their maximum growth after about ten hours of incubation. Thereafter, the growth rates of all isolates were approximately constant till 30 hours of incubation, then decreased dramatically until the end of the experiments (60 hours of incubation). The conversion of indole to indigo was used as an indicator of dioxygenase activity in the whole-cells. Therefore, all isolates were assayed for indigo production. As shown in Fig 2, whole-cell dioxygenase activity profiles were correlated with growth of the isolates. Dioxygenase activity was observed to vary from low to high between 0.3 (isolate DF-4) and 2.22 (isolate S-5) mg indigo/mg protein/min during the log growth phase (after incubation time for ~ 5 hours). During the stationary growth phase (from ~ 10-20 hours incubation), the enzyme activity increased to between 0.68 (DF-4) and 2.56 (S-5) mg indigo/mg protein/min. Hugouvieux-Cotte-Pattat et al. (1990) reported a similar growth-phase-dependent expression of the catabolic genes by *P. putida*.

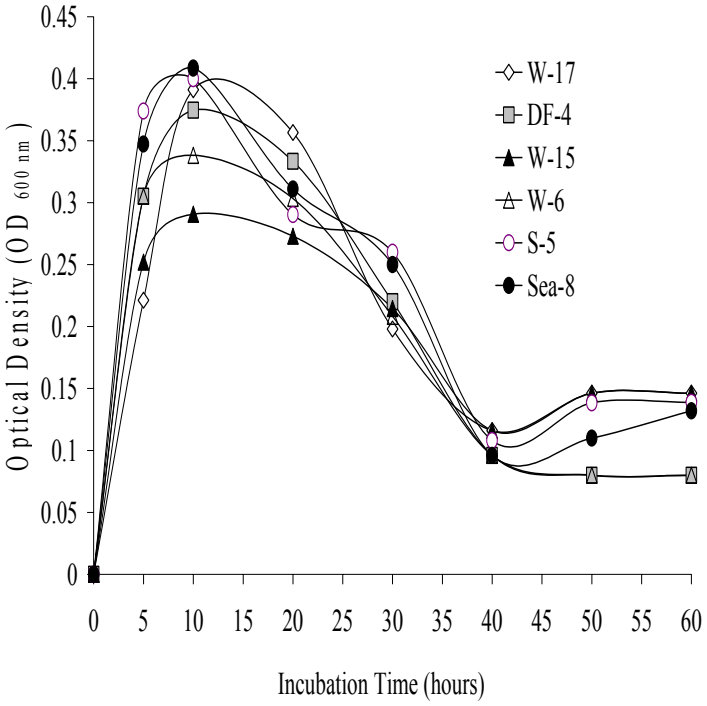


**Fig 1**: Cell growth of phenol degrading bacteria in MSM supplemented with 500 mg/l phenol

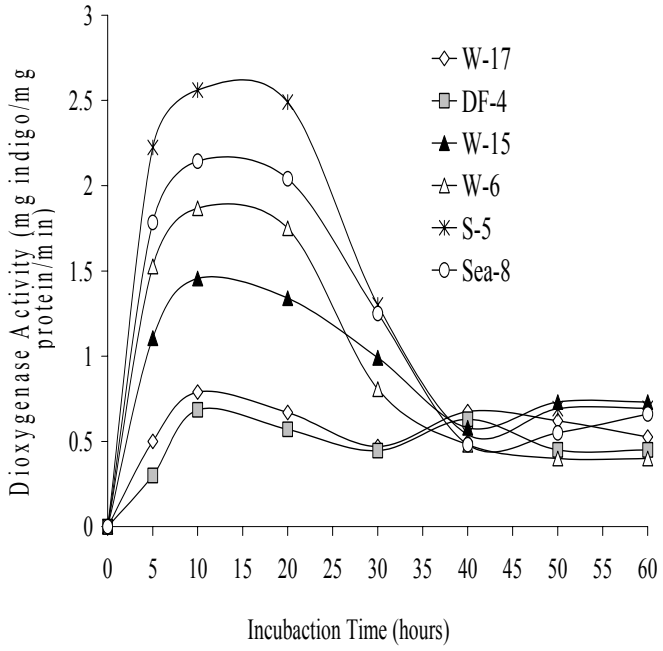


**Fig 2:** Dioxygenase activity profiles in whole-cells of the examined isolates in suspended batch culture as a function of time for indigo formation per mg protein.

Phenol-degrading aerobic bacteria are able to convert phenol into nontoxic intermediates of the tricarboxylic acid cycle via an *ortho* or *meta* pathway (Powlowski and Shingler, 1994). The first step of both routes is the monohydroxylation of the *ortho* position of the aromatic ring (Harayama et al. 1992). The enzyme responsible for the hydroxylations at the positions *ortho* is the monooxygenase phenol hydroxylase. It is also known that the efficiency of a certain catabolic pathway often depends on the properties of the involved key enzyme(s).Therefore, the specific activities of phenol hydroxylase and catechol 1,2-dioxygenase in cell-free extracts obtained by ultrasonication from investigated isolates were examined. As shown in Fig 3 A, B, the enzymes phenol hydroxylase and catechol 1,2-dioxygenase of exhibited certain similarities and differences between examined microorganisms. The data showed also that the highest activities of both enzymes were in the *Acinetobacter* isolates DF-4 and W-17, while no phenol hydroxylase activates were detected under similar conditions in the rest of the examined isolates. These results suggest phenol metabolism throughout occurred via *ortho* fission of catechol in the *Acinetobacter* isolates. In addition, isolate W-17 that presented the highest phenol mineralization rate (55%) as reported previously by Abd-El-Haleem et al. (2002), presented higher activities of both enzymes phenol hydroxylase and catechol 1,2-dioxygenase, respectively.

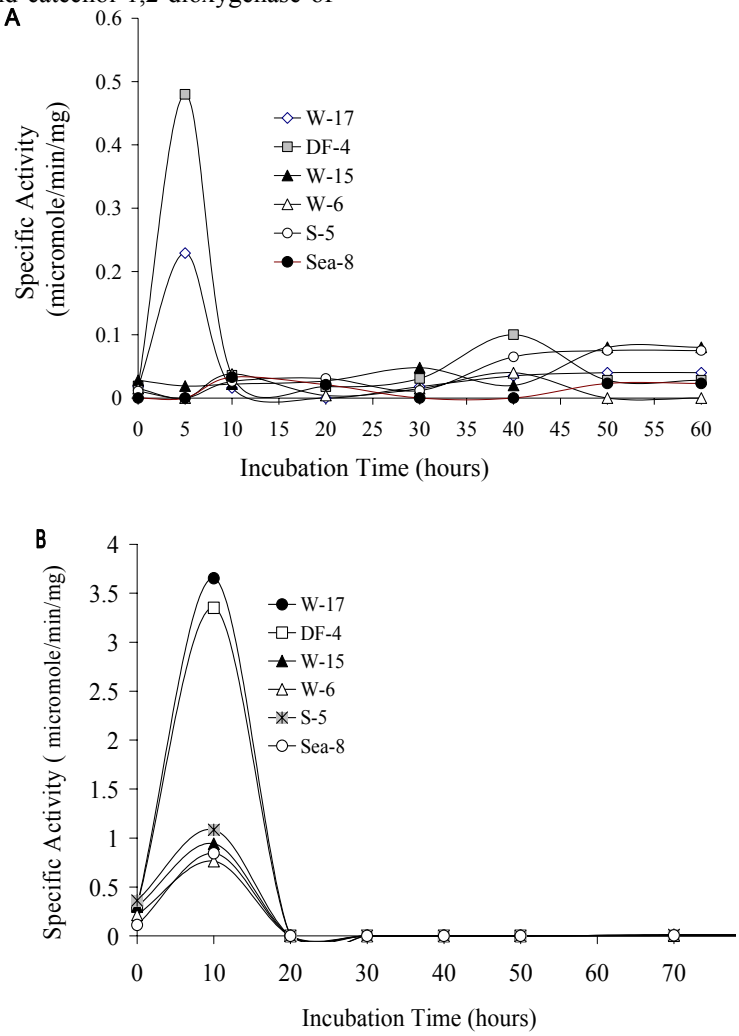


**Fig 3:** Specific enzyme activity as a function of time and the measured enzyme activity, micromole enzyme/ mg protein/ min.; A) phenol hydroxylase, B) 1,2 catechol dioxygenase.

In contrast to the above finding, a large amount of *meta*-cleavage dioxygenase (catechol 2,3-dioxygenase) activity was detected in the cells of isolates S-5 (*Bacillus*), Sea-8 (*Stenotrophomonas*), W-6 (*Klebsiella*) and W-15 (*Ralstonia*) respectively in order of decreasing activity; while no *meta*-

cleavage dioxygenase activity was detected under similar conditions in the *Acinetobacter* isolates W-17 and DF-4 (Figure 4). Previously, extradiol catechol 2,3 dioxygenases have been purified from a variety of organisms comprising *Pseudomonas*, *Alcaligenes* and *Bacillus* (Ornston and Stanier 1966; Kataeva and Golovleva 1990; Kang et al. 1998; Chen et al. 1998).

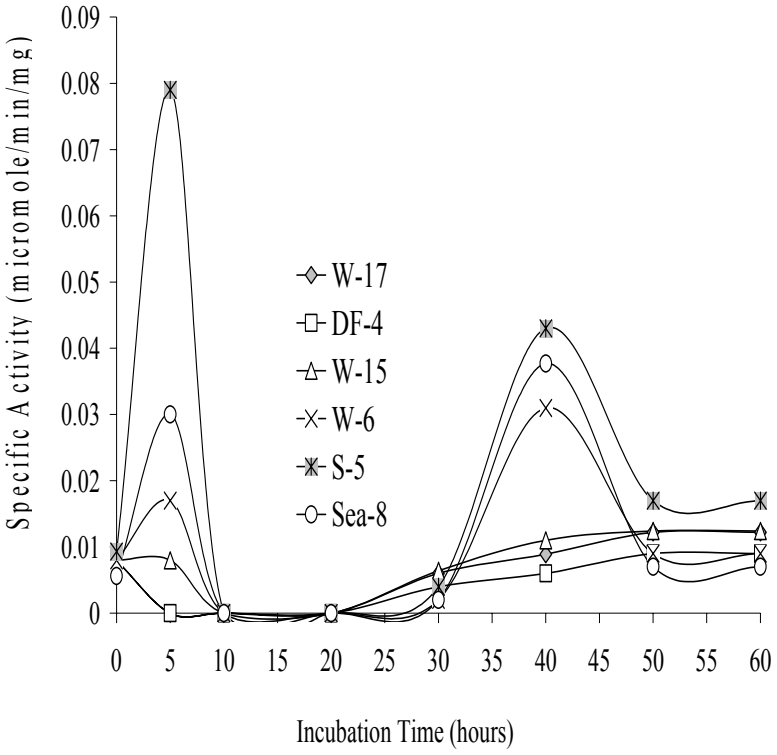


**Fig 4:** Specific activity of enzyme catechol 2,3-dioxygenase as a function of time and the measured enzyme activity micromole enzyme/ mg protein/ min.

On the basis of our previous and present results, the investigated isolates are considered to have good prospects for its application for microbial detoxification of phenol pollutes industrial wastewater. A general comparison of the major pathways for catabolism of aromatic compounds in bacteria has revealed that the initial conversion steps are carried out by different enzymes but that the compounds are transformed into a limited number of central intermediates, such as protocatechuate and (substituted) catechols (Van Der Meer et al. 1992; Chaudhry and Chapalamadugu 1991). These dihydroxylated intermediates are channeled into the *ortho* cleavage pathway or *meta* cleavage pathway (Powlowski and Shingler 1994; Harayama et al. 1992). Both types of pathways lead to intermediates of central metabolic routes, such as the tricarboxylic acid cycle. Finally, it may possible to conclude that some bacteria employ more than one pathway to degrade hydrocarbons. These can easily allow the formation of new mixed metabolic pathways and might explain why bacterial strains capable of growing on contaminants emerge so quickly.

## REFERENCES

Abd El-Haleem, D., Beshay, U., Abdelhamid, A., Moawad, H., and Zaki S. (2003), Effects of mixed nitrogen sources on biodegradation of phenol by immobilized *Acinetobacter* sp. Strain W-17. African Journal of Biotechnology, 2. 8-12.

Abd-El-Haleem, D., Moawad, H., Zaki, E. and Zaki, S.: (2002), Molecular characterization of phenol-degrading bacteria isolated from different Egyptian ecosystems. Microbial Ecology, 43. 217-224.

Ahmed, A.M., Nakhla, G.F., and Farooq, S. (1995), Phenol degradation by *Pseudomonas aeruginosa.* Journal of environmental science and health, 30, 99-107.

Alleman, B.C., Logan, B.E., and Gilbertson, R.L. (1995), Degradation of pentachlorophenol by fixed films of white rot fungi in rotating tube bioreators. Water Research, 29. 61-67.

Beshay, U., Abd-El-Haleem, D., Moawad, H., Zaki, S. (2002) Phenol biodegradation by free and immobilized *Acinetobacter*. Biotechnology Letters, 24.1295-1297.

Bradford, M. M. (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of proteindye binding. Analytical Biochemistry, 72**.** 248–254.

Chang, Y.H., Li, C.T., Chang, M.C., and Shieh, W.K. (1998), Batch phenol degradation by *Candida tropicalis* and its fusant. Biotechnology and bioengineering, 60. 391-395.

Chaudhry, G.R. (1991), Chapalamadugu S. Biodegradation of halogenated organic compounds. Microbiology Reviews, 55.59–79.

Chen M.Q., Yin C.C., Zhang W., Mao Y.M., and Zhang Z.H. (1998), Purification, crystallization and preliminary X-ray diffraction studies on the thermostable catechol 2,3-dioxygenase of Bacillus stearothermophilus expressed in Escherichia coli. *Acta Crystallogr., D Biol. Crystallogr.* **54** Pt. 3, pp. 446–447

Collins, L.D., and Daugulis, A.J. (1997), Biodegradation of Phenol at High Initial Concentration in Two-Phase Partitioning Batch and Fed-batch Bioreactors. Biotechnology and bioengineering, 55. 155-162.

Dean-Ross, D. (1989), Bacterial abundance and activity in hazardous waste-contaminated soil. Bulletin of environmental contamination and toxicology, 43. 511-517.

Fava, F., Armenante, P., and Kafkweitz, D. (1995), Aerobic degradation and dechlorination of 2-chlorophenol, 3-chlorophenol and 4-chlorophenol by *Pseudomonas pickettii* strain. Applied microbiology and biotechnology, 43. 171-177.

Fulthorpe, R.R., Allen, D.G. (1995), A comparison of organochlorine removal from bleached Kraft pulp and paper-mill effluents by dehalogenating Pseudomonas, Ancylobacter and *Methylobacterium* strains. Applied microbiology and biotechnology, 42. 782-787.

Gibson, D.T., Zylstra, G.J., Chauhan, S., (1990), Biotransformations catalyzed by toluene dioxygenase from *Pseudomonas putida* F1. In: Silver, S., Chakrabarty, A.M., Iglewski, B., Kaplan, S. (Eds.), Pseudomonas: Biotransformations, Pathogenesis, and Emerging Biotechnology, American Society for Microbiology Press, Washington, pp. 121–132.

Harayama, S., Kok, M., and Neidle, E.L. (1992), Functional and evolutionary relationships among diverse oxygenases. Annual Review of Microbiology, 46. 565–601.

Hugouvieux-Cotte-Pattat, N., Kohler, T., Rekik, M., and Harayama, S. (1990), Growth-phase-dependent expression of the Pseudoputida TOL plasmid pWW0 catabolic genes. Journal of Bacteriology, 172. 6651–6660.

Jenkins, R.O., and Dalton, H. (1985), The use of indole as a spectrophotometric assay substrate for toluene dioxygenase. FEMS Microbiology Letters, 30. 227–231.

Kang, S. Ha J.Y., Lim J.C., Lee J., Kim C.K., Min K.R. and Kim Y. (1998), Structure of catechol 2,3-dioxygenase gene from Alcaligenes eutrophus 335. Biochemical and Biophysical Research Communications, 245**.** 791–796.

Kataeva, I.A., and Golovleva L.A. (1990), Catechol 2,3-dioxygenases from *Pseudomonas aeruginosa* 2x. Methods of Enzymology, 188. 115–121.

Lin, J.E., Wang, H.Y., and Hickey, R.F. (1990), Degradation kinetics of pentachlorophenol by *Phanerochaete chrysosporium.* Biotechnology and bioengineering, 35. 1125-1134.

Morris, S., and Lester, J.N. (1994), Behaviour and fate of polychlorinated biphenyls in a pilot wastewater treatment plant. Water Research, 28. 1553-1561.

Ornston L.N. and Stanier R.Y. (1966), The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. Journal of biological chemistry**,** 241. 3776–3786.

Ornston, L. N. and Stanier, R. Y. (1966), The conversion of catechol and pyrocatechuate to b ketoadipate by *Pseudomonas putida*. Journal of biological chemistry, 241**.** 3776–3786.

Powlowski, J., and Shingler, V. (1994), Genetics and biochemistry of phenol degradation by *Pseudomonas* sp. CF 600. Biodegradation, **5**. 219–236.

Rebhun, M., and Galil, N. (1988), Inhibition by hazardous compounds in an integrated oil refinery. Journal - Water Pollution Control Federation, 60. 1953-1959.

Ruiz-Ordaz, N., Ruiz-Lagunez, J.C., Castanon-Gonzalez, J.H., Hernandez-Manzano, E., Cristiani-Urbina, E., and Galindez-Mayer, J. (2001), Phenol Biodegradation Using a Repeated Batch Culture of *Candida tropicalis* in a Multistage Bubble Column. Revista Latinoamericana de Microbiologia, 43. 19-25.

Ryu, W.R., Shim, S.H., Jang, M.Y., Jeon, Y.J., Oh, K.K., and Cho, M.H. (2000). Biodegradation of Pentachlorophenol by White Rot Fungi under ligninolytic and Nonligninolytic Conditions. Bioprocess and biosystems engineering, 5. 211-214.

Solomon, B.O., Clemens, P., Michael, P.F., Harder, V.H., and Wolf-Dieter, D. (1994), Energetics of *Pseudomonas cepacia* G4 growth in a Chemostat with Phenol Limitation. Journal of chemical technology and biotechnology, 60. 275-282.

Van –Der-Meer, J.R., De-Vos, W.M., Harayama, S., and Zehnder, A.J.B. (1992), Molecular mechanisms of genetics adaptation to xenobiotic compounds. Microbiology Reviews, 56. 677–94.

Wang, K.W., Baltzis, B.C., and Lewandoski, G.A. (1996), Kinetics of Phenol Biodegradation in the presence of Glucose. Biotechnology and Bioengineering, 51. 87-94.

Watanabe, K., Hino, S., and Takahashi, N. (1996), Responses of activated sludge to an increase in phenol loading. Journal of fermentation and bioengineering, 82. 522-524.