DEGRADATION PATHWAY OF PHENOL THROUGH *ortho*-CLEAVAGE BY *Candida tropicalis* RETL-Cr1

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ABSTRACT. *Phenols and its derivatives are environmental pollutant commonly found in many industrial effluents. Despite being toxic, phenol can be utilized by microbes as carbon and energy sources. The strain utilized up to 3mM phenol as a sole source of carbon and energy. Phenol catabolism was confirmed through the detection of the intermediary products namely catechol and cis,cis-muconic acid. Catechol was formed at the earlier stage of the reaction mixture while cis,cis-muconic acid was formed at the later stage of the biodegradation process. The maximum concentration of catechol was 20.4 mg L^{-1} after 7 h incubation. The HPLC chromatography detected* cis,cis-*muconic acid and enzymatic assays performed were found to be negative for catechol 2,3 dioxygenase activity. Hence, these results showed that this indigenous phenol-degrading yeast, Candida. tropicalis RETL-Cr1 (AY725426) seemed to metabolize phenol via ortho-cleavage pathway.*

KEYWORDS. Batch system, *cis-cis*-muconic acid, Intermediates, Phenol biodegradation

INTRODUCTION

Phenols and its derivatives are troublesome environmental pollutant commonly found in many industrial effluents of industrial processes. Despite being toxic, phenol can be utilized by microbes as carbon and energy sources (Gibson, 1968). A number of studies on phenol degradation with prokaryotic microorganisms have been carried out (Hinteregger *et al*., 1992; Collins and Daugulis, 1997; Leonard and Lindley, 1999). Only some members of yeast genera *Rhodotorula*, *Trichosporon*, and *Candida* can metabolize phenolic compounds as a sole carbon and energy source (Katayama-Hirayama *et al*.,1994; Alexieva *et al*., 2002; Chen *et al*., 2002; Santos and Linardi, 2004).

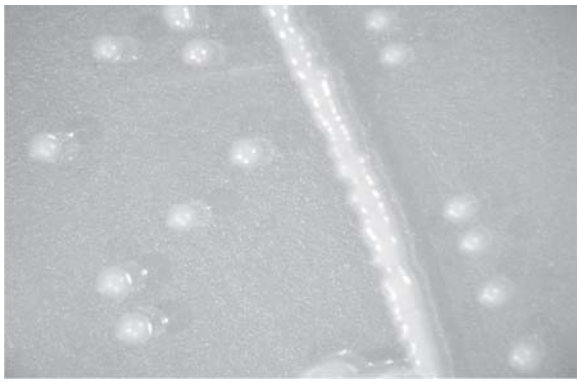
Aerobic organisms degrade phenol to catechol followed by oxidative cleavage of the ring. This oxidative ring cleavage of catechol can occur in one of two ways. The *ortho* cleavage to produce *cis,cis*-muconic acid (ccMA) or the *meta* cleavage to produce 2-hydroxymuconic semialdehyde (2-HMSA). The production and accumulation of these intermediates during phenol degradation has been commonly observed (Li and Humphrey, 1989; Mörsen and Rehm, 1990; Allsop *et al*., 1993). The identification of products formed during the biodegradation process of phenol is essential for a better understanding of the degradation mechanism.

The objective of this study was to determine the intermediates produced during phenol degradation by *C. tropicalis* RETL-Cr1 and to postulate possible phenol metabolism pathway.

MATERIALS AND METHODS

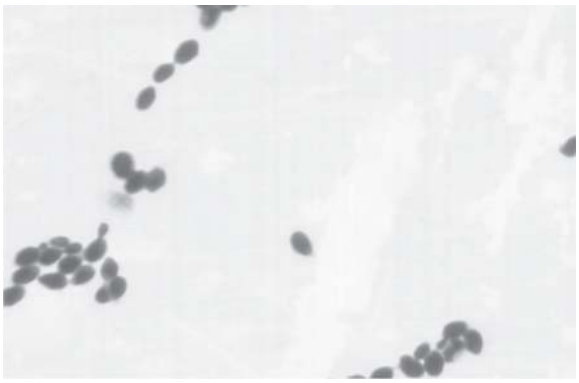
Microorganism

The yeast C. tropicalis RETL-Cr1 (AY725426) was used in this work (Figure 1a, b). This locally isolated yeast strain is capable of utilizing phenol as sole carbon source (Piakong, 2006).



(a)

Figure 1. (a) Colony morphology of C. tropicalis. RETL-Cr1 under stereo- microscope (x12)



(b)

(b) Cellular morphology of C. tropicalis. RETL-Cr1 magnified x 1000

Culture Medium

A 10 ml of inoculum was transferred into a 250 ml conical flask containing 90 ml of medium described by Ramsay *et al*., (1983) containing (g/L): 2.0g NH_4NO_3, 0.5g KH_2PO_4, 1.0g K_2HPO_4, 0.5g $MgSO_4.7H_2O$, 0.01g $CaCl_2.2H_20$, 0.1g KCl and 0.06g yeast extract. The pH was adjusted between 6.5-6.8. Phenol was added as a sole carbon source. Duplicate yeast cultures in duplicates were incubated at 30^{o}C with shaking at 200 rpm. Samples were withdrawn at a regular intervals and analyzed for cell growth and phenol concentration

Determination of biomass concentration

During the period of batch growth, samples were periodically taken for optical density determination. Cell density was monitored spectrophotometrically by measuring absorbance at 600nm using Jenway 6300 spectrophotometer, U.K.

Determination of phenol

Phenol was determined by isocratic elution high performance liquid chromatography (HPLC) (W600 2487) using a Waters Hypersil C18 5µm (4.6 mm x 250 mm) column with UV detector at 280 nm. The mobile phases used were acetic acid (1% v/v) in water and acetic acid (1% v/v) in acetonitrile, at a flow rate of 1 mL min^{-1}. Solvents were of HPLC grade. 1 mL sample was centrifuged at 15,000 rpm for 10 minutes in a microfuge (Hettich centrifuge, Germany). The supernatant was filtered through a 0.25 µm nylon filter to remove cell debris. The filtrate was cooled and stored at -20^{o}C for subsequent analysis. Aliquots of 40 µL of filtered samples were injected into the HPLC for phenol determination and analyzed in duplicates.

Determination of cis,cis-muconic acid

Cis,cis-muconic acid as indicator of *ortho*-cleavage of phenol was determined using HPLC. The HPLC-analytical parameters and procedures used in determination of phenol.

Meta-cleavage dioxygenase assays

To determine whether *meta*-cleavage of phenol was involved, spray plate method and a test tube assay as described by Kim and Zylstra (1995) were performed. A spray plate method was used to screen for colonies showing *meta*-cleavage dioxygenase activity on plates. An ether solution of catechol (0.1% w/v) was sprayed on to colonies of *C. tropicalis* RETL-Cr1 and observed for yellow colour formation as a result of a *meta*-cleavage of catechol by *meta*-cleavage dioxygenase.

A test tube assay was also employed for detection of low levels of *meta*-cleavage dioxygenase activity. One loopful of cells grown overnight on plates was suspended in 1 mL of 50 mM phosphate buffer (pH 7.5 and pH 6.5). 20µL Catechol (20 mM stock solution in methanol) was added, and the formation of a yellow colour was monitored visually over time.

RESULTS AND DISCUSSION

Determination of intermediates of *C. tropicalis* RETL-Cr1

The first step taken to determine the possible phenol metabolic pathway of *C. tropicalis* RETL-Cr1 was to perform the enzymatic assays to detect catechol 2,3 dioxygenase activity for *meta*-pathway. The enzymatic assays performed were found to be negative for this enzyme's activity. On the *meta*-cleavage pathway, catechol would be converted to 2-hydroxymuconic semialdehyde (2-HMSA) catalyzed by catechol 2,3-dioxygenase (C2,3D). Colonies of *C. tropicalis* RETL-Cr1 cultured on plates or suspended in phosphate buffer did not develop a yellow colour when sprayed or added with catechol. This indicates that there was no formation of 2-hydroxymuconic semialdehyde of *meta*-pathway (Bushwell, 1975; Kim and Zylstra (1995).

It was then assumed that *C. tropicalis* RETL-Cr1 probably may metabolize phenol via the *ortho*-pathway. Then the next step was to perform the HPLC chromatography method to detect the presence of *cis,cis*-muconic acid, the intermediate indicator for *ortho*-cleavage pathway.

During degradation of phenol using free cells of *C. tropicalis* RETL-Cr1, HPLC analysis of the samples taken from batch system containing varying initial phenol concentration (IPC) from 282- to 940 mg L^{-1} (3 mM-10 mM) revealed the presence of catechol and *cis,cis*-muconic acid as intermediates. Figure 2 illustrates a typical HPLC chromatogram recorded for samples taken after 7 hours for phenol degradation by *C. tropicalis* RETL-Cr1 in medium (pH 6.5) under aerobic condition at 30^{o}C, at an initial phenol concentration of 3 mM.

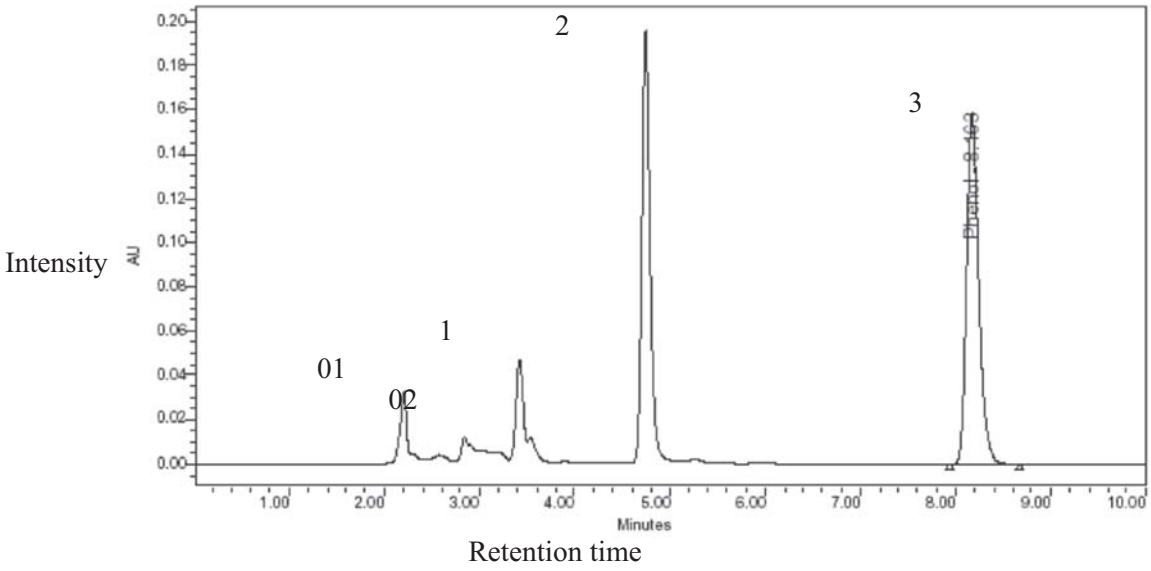


Figure 2. Typical HPLC chromatogram recorded for an aerated suspension: (01 & 02) unidentified products; (1) *cis,cis*-muconic acid ; (2) catechol ; and (3) phenol during phenol degradation in batch culture of *C. tropicalis* RETL-Cr1 at initial phenol concentration of 3 mM after 7h incubation.

The formation of other products (unidentified) was also observed. These products; 01 and 02 are assumed to be any of the other intermediates produced of the *ortho*-cleavage pathway such as muconalactone, 3-oxioadipic acid (ß-ketoadipate), or succinic acid (Bugg and Winfield, 1998).

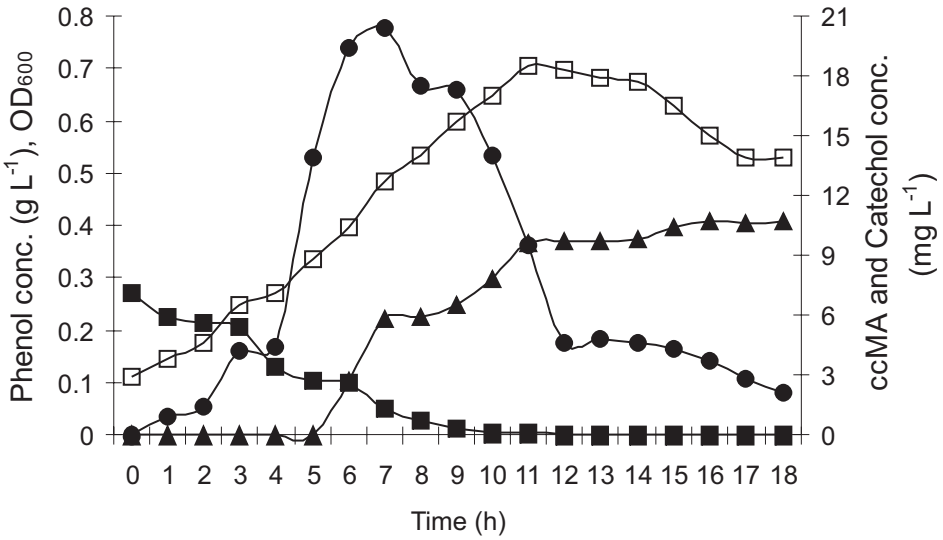


Figure 3. Time course of phenol (■) degradation in batch system (shake-flask) using *C. tropicalis* RETL-Cr1 at IPC of 3 mM, pH 6.5, and detection of intermediates; catechol (●) and *cis,cis*-muconic acid (ccMA) (▲). Biomass concentration (□) of *C. tropicalis* RETL-Cr1.

As clearly seen in Figure 3, catechol and *cis,cis*-muconic acid (ccMA) were not formed simultaneously. Catechol was formed at the earlier stage of the reaction mixture while *cis,cis*-muconic acid was formed at the later stage of the biodegradation process. The maximum concentration of catechol was 20.4 mg L^{-1} after 7 h incubation. This initial phenol concentration of 3mM was similar to that reported by Chung *et al*., (2003) whereby the concentration of catechol produced by *P. putida* CCRC14365 was always less than 21 mg L^{-1} at 30^{o}C and pH 6.8. In contrast, *cis,cis*-muconic acid was only formed at later stage of the incubation period. The onset of formation of ccMA was after 6 hours of the degradation process and increased exponentially as phenol concentration decreased and finally leveled off after 11 h incubation period and appeared to accumulate in the medium.

Phenol metabolic pathway of *C. tropicalis* RETL-Cr1

Many studies have focused on biodegradation of phenol and phenolic compounds with respect to degradation pathways (Dagley, 1985; Katayama-Hirayama *et al*., 1991). The general principle of pathways for aerobic aromatic catabolism is best described by Dagley, 1986 and Harayama and Timmis, 1992). This aerobic aromatic catabolic pathway generally consists of three stages namely the conversion of the growth substrate to catechol, ring-cleavage and metabolism of the ring-cleavage product to central metabolites by either the *ortho* or *meta*-pathways.

The first reaction in phenol degradation is catalyzed by phenol hydroxylase (PH) (EC 1.14.13.7) whereby one oxygen atom of molecular oxygen into the aromatic ring to form catechol as the central intermediate. This catechol is then converted to *cis, cis*-muconic acid through *ortho*-cleavage pathway. This pathway is also known as ß-ketoadipate pathway which is catalayzed by catechol 1,2-dioxygenase (C1,2D) (EC 1.13.11.1) or converted to 2-hydroxymuconic semialdehyde (2-HMSA) through the *meta*-pathway which is catalyzed by catechol 2,3-dioxygenase (C2,3D) (EC 1.13.11.2 (Feist and Hegeman, 1969;Ornston and Stainer,1966). After several subsequent reactions, *ortho* pathway will lead to succinyl-CoA and acetyl-CoA. On the other hand, *meta* pathway will lead to pyruvate and acetyldehyde and finally both pathways will incorporated into the tricarboxylic acid cycle (TCA) or Krebs cycle (Shingler, 1996). Therefore, from this phenol metabolic pathway, *cis,cis*-muconic acid (*cc*MA) is considered the indicator for *ortho*-cleavage pathway and 2-hydroxymuconic semialdehyde (2-HMSA) is the indicator for *meta*-cleavage.

The HPLC chromatography and enzymatic results revealed that *C. tropicalis* RETL-Cr1 seem to metabolize phenol via *ortho* cleavage pathway (Figure 4). 2-hydroxymuconic semialdehyde (2-HMSA) has been reported responsible for colour change in culture medium (greenish to yellow) during phenol degradation (Li and Humphrey, 1989; Mörsen and Rehm, 1990). However, in this present study no colour changes of media was observed, which further suggested that *Candida tropicalis* RETL-Cr1 probably did not metabolize phenol through *meta*-pathway. This postulated *ortho*-pathway for phenol degradation of C. *tropicalis* RETL-Cr1 could be similar to one reported on *Candida tropicalis* by Hashimoto, (1970, 1973), Neujahr *et al*., (1974); Middelhoven, (1993), Krug and Straube, (1986) and Bastos *et al*., (2000).

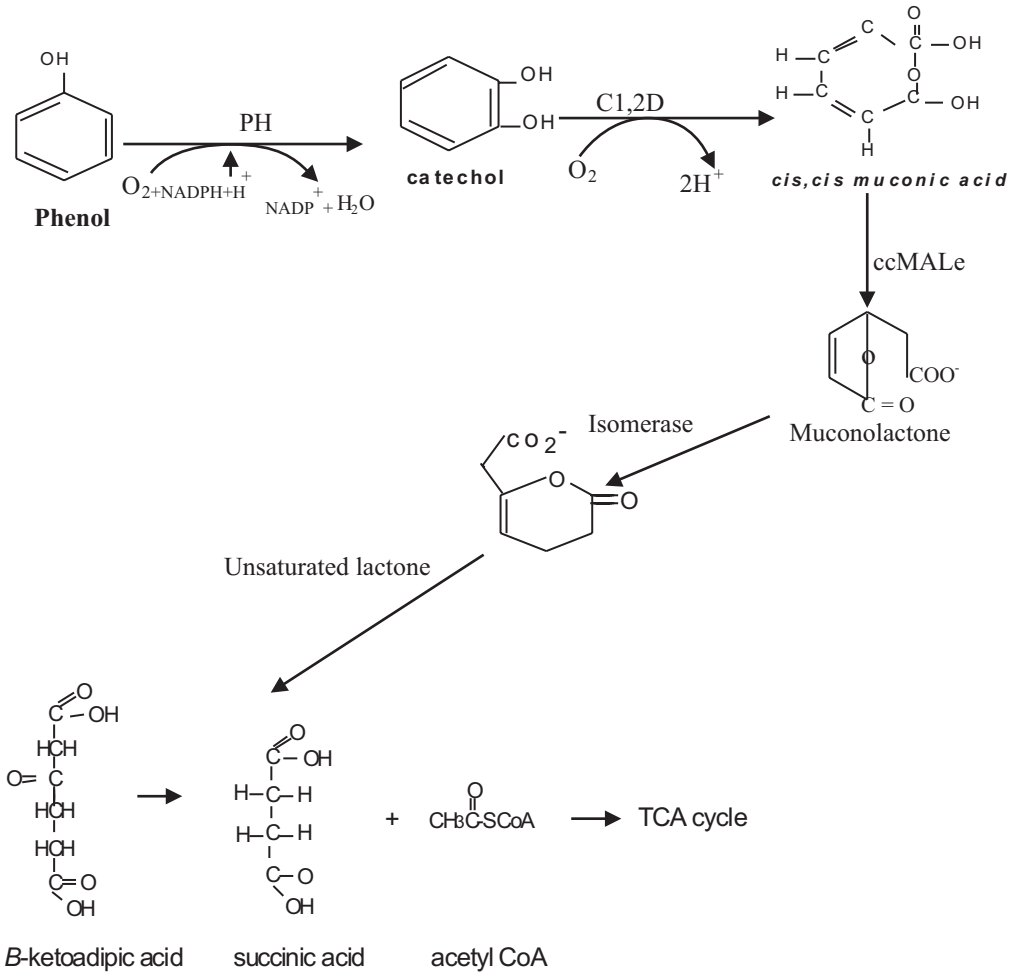


Figure 4. Postulated *ortho*-pathway for degradation of phenol by *C. tropicalis* RETL-Cr1. [Metabolic sequences for phenol catabolism: PH = phenol hydroxylase, C1,2D = catechol 1,2-dioxygenase, ccMALe = *cis,cis* muconic acid lactonizing enzyme (Gaal and Neujahr, 1979; Bugg and Winfield, 1998), and Reaction: phenol + O_2 + NADPH +H^+ $NADP^+$ + H_2O + catechol (Mörtberg and Neujahr, 1987), catechol + O_2 ccMA + $2H^+$ (Ngai *et al*., 1990)], NADP = Nicotinamide adenine dinucleotide phosphate.

CONCLUSION

Phenol catabolism was confirmed through the detection of the intermediary products namely catechol which was formed in the early stage and *cis,cis*-muconic acid in the later stage of incubation. HPLC chromatography and enzymatic results showed that this indigenous phenol-degrading yeast, *C. tropicalis* RETL-Cr1 seemed to metabolize phenol via *ortho*-cleavage pathway.

REFERENCES

Alexieva, Z., Ivanova, D., Godjevargova, T. & Atanasov, B. 2002. Degradation of Some Phenolderivatives by *Trichosporon cutaneum* R57. *Proc. Biochem*., **37**: 1215-1219.

Allsop, P.J., Chisit, Y., Moo-Young, M. & Sullivan, G.R. 1993. Dynamics of Phenol Degradation by *Pseudomonas putida*. *Biotechnol. Bioeng*. **41**: 572-580.

Bastos, A.E..R., Tornisielo, V.L., Nozawa, S.R., Trevors, J.T. & Rossi, A. 2000. Phenol Metabolism by Two Microorganisms Isolated from Amazonian Forest Soil Amples. *Ind. Microbiol. Biotechnol*., **24**(6): 403-409.

Bugg, T.D.H. & Winfield, C.J. 1998. *Enzymatic Cleavage of Aromatic Rings: Mechanistic Aspects of Catechol dioxygenases and Later Enzymes of Bacterial Oxidative Cleavage Pathways.* Nat. Prod. Report.: 513-530.

Buswell, J.A. 1975. Metabolism of Phenol and Cresols by *Bacillus stearothermophilus*. *Bacteriol*., **72**: 248-254.

Chen, K-C., Lin, Y-H., Chen, W-H. & Liu, Y-C. 2002. Degradation of Phenol by PAA-Imobilized *Candida tropicalis*. *Enzym. Microb. Technol.,* **31**: 490-497.

Chung, T-P., Tseng, H-Y., & Jung, R-S. 2003. Mass Transfer Effect and Intermediate Detection for Phenol Degradation in Immobilized *Pseudomonas putida* Systems. *Proc. Biochem*., **38**: 1497-1507.

Collins, L.D. & Daugulis, A.J. 1997. Biodegradation of Phenol at High Concentrations in Two Phase Partitioning Batch and Fed-batch Bioreactors. *Biotechnol. Bioeng*., **55**:155-162.

Dagley, S. 1985. *Microbial Metabolism of Xenobiotic Compounds. In: Comprehensive Biotechnology*. Moo-Young, M. (ed.): Vol.1: Pergamon Press, Oxford, U.K.: 483-505.

Dagley, S. 1986. Biochemistry of Aromatic Hydrocarbon Degradation in *Pseudomonads*: In: Gonsalus, I.C., Sokatch, J.R. & Ornston, L.N. (Eds.) *The bacteria*, **10**:527-556.

Feist, C.F. & Hegeman, G.D. 1969. Phenol and Benzoate Metabolism by *Pseudomonas putida*: Regulation of Tangential Pathways. *J. Bacteriol*., **100**: 869.

Gaal, A. & Neujahr, H.Y. 1979. Metabolism of Phenol and Resorcinol in *Trichosporon cutaneum*. *Bacteriol*., **137**(1): 13-21.

Harayama, S. & Timmis, K.N. 1992. Aerobic Biodegradation of Aromatic Hydrocarbons by Bacteria. In: Sigel, H. & Sigel, A. (Eds.) *Metal Ions in Biological Systems. Vol. **28**. Degradation of Environmental Pollutants by Microorganisms and Their Metalloenzymes*. Marcel Dekker Inc. New York: 99-165.

Hashimoto, K. 1970. Oxidation of Phenols by Yeast. I. A New Oxidation Product from *p*-cresol by an Isolated Strain of Yeast. *Gen. Appl. Microbiol*., **16**: 1-13.

Hashimoto, K. 1973. Oxidation of Phenols by Yeast. II. Oxidation of Cresol by *Candida tropicalis*. *Gen. Appl. Microbiol*., **19**: 171-187.

Hinteregger, C., Leitner, R., Loidl, M., Ferschl., A. & Streichsbier, F. 1992. Degradation of Phenol and Phenolic Compounds by *Pseudomonas putida* EKII. *Appl. Microbiol. Biotechnol*., **37**:252-259.

Katayama-Hirayama, K., Tobita, S. & Hirayama, K. 1991. Metabolic Pathway of Phenol in *Rhodotorula rubra*. *Gen. Appl. Microbiol*., **37**: 379-388.

Katayama-Hirayama, K., Tobita, S. & Hirayama, K. 1994. Biodegradation of Phenol and Monochlorophenols by Yeast *Rodotorula glutinis*. *Wat. Sci. Technol*., **30**: 59-66.

Kim, E., & Zylstra, G.J. 1995. Molecular and Biochemical Characterization of Two *meta*- cleavage Dioxygenases Involved in Biphenyl and *m*-xylene Degradation by *Beijerinckia* sp. Strain B1. *Bacteriol.,* **177**(11): 3095-3103.

Krug, M. & Straube, G. 1986. Degradation of Phenolic Compounds by the Yeast *Candida tropicalis* HP-15: II. Some Properties of the First Two Enzymes of the Degradation Pathway. *Basic Microbiol*., **26**(5): 271-281.

Léonard, D. & Lindley, N.D. 1999. Growth of *Ralstonia eutrophia* on Inhibitory Concentrations of Phenol: Diminished Growth Can Be Attributed to Hydrophobic Perturbation of Phenol Hydroxylase Activity. *Enzym. Microb. Technol*., **25**:271-277.

Li, J.K. & Humphrey, A.E. 1989. Kinetic and Fluorometric Behavior of a Phenol Fermentation. *Biotechnol. Lett.,* **11**:177-182.

Middelhoven, W.J. 1993. Catabolism of Benzene Compounds by Ascomycetous and Basidomycetous Yeasts and Yeast-like Fungi. *Antonie van Leewenhoek,* **63**: 125-144.

Mörsen, A. & Rehm, H.J. 1990. Degradation of Phenol by a Defined Mixed Culture Immobilized by Adsorption on Activated Carbon and Sintered Glass. *Appl.Microbiol Biotechnol*., **33**:206-212.

Mörtberg, M. & Neujahr, H.Y. 1987. *In situ* and *In vitro* Kinetics of Phenol Hydroxylase. *Biochem.Biophys. Res. Commun*., **146**: 41-46.

Neujahr, H.Y., Lindsjo, S. & Varga, J.M. 1974. Oxidation of Phenol by Cells and Cells-Free Enzymes from *Candida tropicalis*. *Antonie van Leeuwenhoek*, **40**: 209-216.

Ngai, K-L, Neidle, E.L. & Ornston, L.N. 1990. Catechol and Chlorocatechol 1,2-Dioxygenase. In: Lidstrom, M.E. (ed). Hydrocarbon and Methylotrophy. *Meth. Enzymol*., **188**: 122-126.

Ornston, L.N. & Stainer, R.Y. (1966). The Conversion of Catechol and Protocatechuate to ß-ketoadipate by *Pseudomonas putida*. IV. Regulation, *J. Biol. Chem.*, **241**: 3800.

Piakong, M.T. 2006. The Performance of Phenol Biodegradation by *Candida tropicalis* RETL-Cr1 Using Batch and Fed-batch Fermentation Techniques. Ph.D Thesis. Universiti Teknologi Malaysia, Skudai.

Ramsay, B.A., Cooper, D.G., Margaritis, A. & Zajic, J.E. (1983). *Rhodochorous* Bacteria: Biosurfactant Production and Demulsifying Ability. *Microb. Enh. Oil Recov.* 61-65. Santos, V.L. & Linardi, V.R. (2004). Biodegradation of Phenol by a Filamentous Fungi Isolated from Industrial Effluents-Identification and Degradation Potential. *Proc. Biochem*., **39**: 1001-1006.

Shingler, V. (1996). Molecular and Regulatory Checkpoints in Phenol Degradation by *Pseudomonas* sp. CP600. In: Nakazawa, T., Furukawa, K., Haas, D. & Silver, S. (eds.,) *Molecular Biology of Pseudomonads*. *Am. Soc. Microbiol*., Washington, D.C.: 153-164.