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A N I N T E R N A T I O N A L J O U R N A L

Physiological Changes of *Candida tropicalis* Population Degrading Phenol in Fed Batch Reactor

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ABSTRACT

Candida tropicalis *can use phenol as the sole carbon and energy source. Experiments regarding phenol degradations from the water phase were carried out. The fermentor was operated as a fed-batch system with oxistat control. Under conditions of nutrient limitation and an excess of oxygen the respiration activity of cells was suppressed and some color metabolites (black-brown) started to be formed. An accumulation of these products inhibited the cell growth under aerobic conditions. Another impact was a decrease of the phenol hydroxylase activity as the key enzyme of the phenol degradation pathway at the end of the cell respiration activity. This decrease is linked with the above mentioned product inhibition. The cell death studied by fluorescent probe proceeded very slowly after the loss of the respiration activity. The starvation stress induced an increase of the endogenous respiration rate at the expense of phenol oxidation.*

Key words: Phenol, biodegradation, *Candida tropicalis*, phenol hydroxylase, starvation stress, viability

INTRODUCTION

Phenol belongs to pollutants, which are present in a variety of environments, such as waste waters from oil refineries, cooking plant and coal conversion process. Environmental contamination problems can be solved by many technologies. Phenolic compounds can be removed from the water phase by adsorption using many sorbents, by oxidation using UV radiation (Scheck and Frimmel, 1995), H_2O_2, treated by ultrasound and/or with catalyzer (Drijvers et al., 1999). Also biooxidation belongs among these procedures. Stillage is industrial waste containing high amount of phenolic contaminants. Garcia et al. (1997) studied a possibility to reduce phenolic content by biodegradation. *Geotrichum candidum* and *Aspergillus tereus* were successfully used. Though both microorganisms behave in a similar way *Geotrichum candidum* seems to be slightly better for removing phenols from stillage (Garcia et al., 1997).
An impact of the phenol and phenolic compounds to the living organisms was also studied. Since 19th century, phenol has been used as a biocide. Phenolic compounds probably exert a toxic effect at the level of the mebrane, as it follows from the high correlation between the toxicity and hydrophobicity. This conclusion is supported by an observation that phenol changes membrane

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function and influences protein –to-lipid ratios in the membrane (Sikkema et al., 1995). A mechanism of the toxic effect of phenol is called polar narcosis or narcosis II. This effect can involve two or more toxic mechanisms (including physical interaction with membrane components) (Sixt et al., 1995). It can also reduce the methanogenic activity of biogranules in the waste water treatment under shock-loading conditions but some tolerance was observed too (Fang and Chan, 1997). However, phenol is easier degradable then other aromatics (Orupõld et al., 2001). Many studies have been focused on biodegradation of phenol and phenolic compounds with respect to degradation pathways (Dagley 1985, Katayama-Hyraiama et al., 1991). Catabolism of benzene compounds by ascomycetous and basidiomycetous yeast and yeast like fungi was describe by Middelhoven (1993). The metabolic pathway in *Candida* and *Trichosporon* goes from phenol to catechol and then by ortho fission of the benzene ring (Middelhoven, 1993).

A degradation of phenolic compounds by the yeast *Candida tropicalis* HP15 was studied by Krug et al (1985 a). They described a substrate inhibition and a degradation ability for many phenolic compounds including substituted compounds (Krug et al., 1985 a). Properties of the phenol hydroxylase and catechol 1,2-oxygenase were also studied (Krug et al., 1985 b). The phenol oxidation by *Candida tropicalis* was studied by Neujahr et al (1974). They observed that the range of degradable compounds is more restricted than in the case of *Trichosporon cutaneum*. *Candida tropicalis* could not oxidize tri-hydroxy-phenols, cresols, hydroxybenzoates, benzoate, xylene etc. (Neujahr et al., 1974). Chang et al (1998) determined the optimum pH at 7 and the optimum temperature of 32°C for *Candida tropicalis* and its fusant. Data demonstrated a better phenol degradation received with the fusant and also lower sensitivity to phenol inhibition as compared to the control strain. The fusant was more sensitive to pH and temperature variations than the control strain (Chang et al., 1998).

MATERIAL AND METHODS

Microorganism

Candida tropicalis Ct2 was isolated from activated sludge of an industrial waste water treatment plant.

Media

The inoculum was cultivated in yeast nitrogen base medium (YNB) 0.70 g/L yeast nitrogen base (Difco) and 5.00 g/L $(NH_4)_2SO_4$, 1.00 g/L KH_2PO_4, 0.50 g/L $MgSO_4.7H_2O$, 0.10 g/L NaCl, 0.10 g/L $CaCl_2.6H_2O$, and amino acids – 0.01 g/L L-histidin, 0.02 g/L L-methionin, 0.02 g/L L-tryptophan. The medium was supplemented with phenol (500 mg/L).

The cultivation media: The limited medium (25.00 mg/L $(NH_4)_2SO_4$ and 25.00 mg/L KH_2PO_4 in a distilled water). The base synthetic medium (BSM) (4.30 g/L KH_2PO_4, 3.40 g/L K_2HPO_4, 2.00 g/L$(NH_4)_2SO_4$, 0.34 g/L $MgCl_2.6\ H_2O$, 0.50 mg/L $FeSO_4.7\ H_2O$, 0.50 mg/L $ZnSO_4.7\ H_2O$, 0.10 mg/L $MnSO_4.7\ H_2O$, 0.10 mg/L $CuSO_4.5\ H_2O$, 0.10 mg/L $CoCl_2.6\ H_2O$, 0.10 mg/L $Na_2B_4O_7$, 0.10 mg/L $Na_6Mo_2O_7$).

Aparatus and Conditions

Experiments were carried out in a bench-scale bioreactor (B.Braun Biotech GmbH, Melsungen, Germany) with a 1.5 L working volume under aerobic conditions. pH was kept at the constant value of 5.2. The fed-batch cultivation under oxistat control were used for phenol feeding (Vojta et al., 2002). Each phenol solution addition resulted in the overall phenol concentration of 100 mg/L.

Analytical methods

The biomass concentration was determined using two methods. The cell dry weight concentration (X_{dw}) was determined gravimetrically. The samples were centrifuged, washed twice with distilled water and dried for 1h at 70°C and for 2h at 105°C. The biomas concentration (X) was determined photometrically. The optical density (OD) was measured at 500 nm and X was computed from the following calibration equation:

$$X = 0.492 \cdot OD - 0.004 \quad (g/L)$$

A metabolite accumulation was measured as an increase of the absorbance (A) of the medium at 305 nm and 360 nm.

Microscopy observation

The cell viability was determined using a fluorescent probe (FUN-1$^{(tm)}$ cell stain, Molecular Probes Inc.). The viable cells show light green with red spots coloring while the cells with damaged membrane structures (dead cells) show a radiated yellow color. The number of the dead cells (X^d) was calculated from the formula:

X^d = dead cell number / total cell number (%)

The specific phenol consumption rate was calculated from:

$$q_s = \Delta S / (X_{dw} \cdot \Delta t) \quad \text{(mmol/g . h)}$$

where the ΔS is the substrate consumption in mmol/L and the Δt is the time period (h).

RESULTS AND DISCUSSION

Fig. 1 shows a typical course of the phenol biodegradation under nutrient limitation. The cells were able to use phenol as a sole carbon and energy source during approximately the first 15 h under the starvation stress of all nutrients but carbon and energy source. The cells excreted some acidic metabolites during this period as it follows from NaOH consumption (Fig. 1). The end of the cell growth, phenol and NaOH consumptions resulted from a nutrient limitation (in the limited medium). Just before the cell respiration ceases an accumulation of color metabolites starts to occur as it follows from absorbance increase in Fig. 2. The color of the medium changed during elapsed time from white to yellow and brown. During the phenol degradation in excess of nutrients these colour changes were not observed.

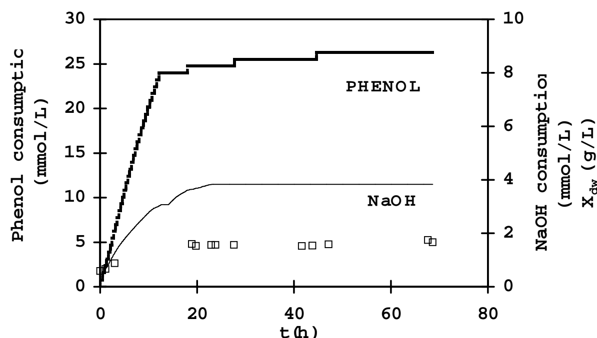

Figure 1 - Course of aerobic phenol degradation in the fed batch reactor
▬ phenol consumption; — NaOH consumption; □ biomass concentration (X_{dw})

A similar behavior was observed by Daratchkiev et al (1996) in a semifixed packing bioreactor but for *Pseudomonas putida*. Mörsen and Rehm (1990) using also *Pseudomonas putida* cells identified 2-hydroxymuconic acid semialdehyd as the only metabolite that accumulated in the medium.

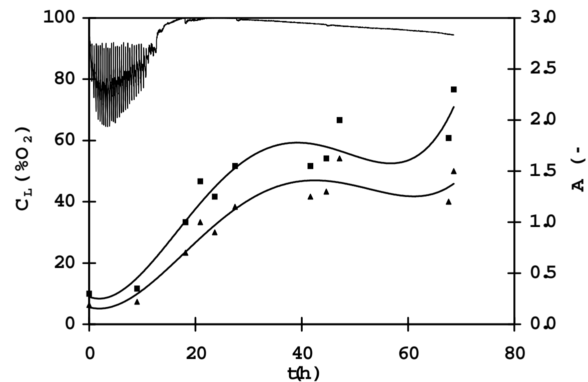

Figure 2 - Courses of the dissolved oxygen concentration (C_L) and absorbance (A) of the limited medium during phenol degradation.
---- C_L ■ A 305 ▲ A360

They observed intensive not fluorescing green-yellow color of the medium. Peroxidases catalyze the oxidation of phenols with hydrogen peroxide to form phenoxy radicals. The free radical products spontaneously form insoluble polymers which can be removed from solution by coagulation and sedimentation. A study with horseradish peroxidase for a foundry wastewater treatment was performed by Cooper and Nicell (1995) and the metabolites had an inhibitory effect to the enzyme. The ozonization of a phenolic water also resulted in a formation of phenoxy radicals and brown metabolites (Wu et al., 2000). This characteristic liquid coloring with the subsequent polymer formation were also reported in phenol oxidation by horseradish peroxidase (Wu et al., 1993). Masuda et al., (2001) described phenol polymerization by *Coprinus cinnereus* peroxidase as an alternative enzyme to horseradish peroxidase and *Arthromyces ramosus* peroxidase. A soil catalyzed polymerization of catechol also produces brown color changing to deep black but the spontaneous oxidation is negligible (Colarieti et al., 2002). These examples indicate two possible mechanisms of the metabolite formations but no one of them solve the problem what to do with polymerized products. To distinguish between the effect of the product inhibition and that of the intracellular reserve exhaustion the following experiments were carried out.

Inhibitory Effect of Metabolites

The metabolites produced during the nutrient limited aerobic phenol biodegradation process were

tested for their inhibitory effect. The respiratory characteristics are shown in Fig. 3. After a short cell cultivation in the base synthetic medium (BSM) the cells actively grew as it can be seen from changes of the dissolved oxygen concentration profile resulting from phenol additions using oxistat control (Fig. 3a left). At time A the cells were harvested and separated from the medium. At time B the cells were resuspended in the exhausted colored medium supplied with all the BSM salts. The presence of metabolites inhibited the cell respiration as it follows from the dissolve oxygen profile in Fig. 3a (right) and the cell growth (data not shown).

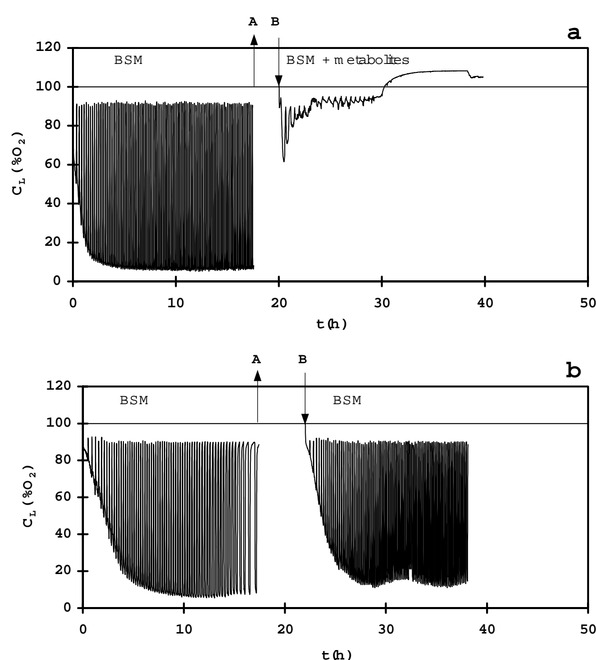

Figure 3 - Changes of the dissolved oxygen concentration resulting from repeatedly added phenolic water to the BSM medium (left part, Fig. 3a), to the exhausted medium supplied with the BSM nutrients (right part, Fig. 3a), and to the fresh BSM medium (Fig. 3b).

The inability of cells to oxidize phenol proves the inhibition effect of these metabolites (Fig. 3a). The effect of centrifugation (3500 g) and cell washing is negligible as it follows from the same cell inoculation material used in the case shown in Fig. 3b (right).

Effect of products elimination and intracellular reserve material exhaustion

To prove our hypothesis that the yeast population ceases its respiration ability due to the product inhibition and also change the cell physiology the experiment shown in Fig. 4 has been carried out. First the phenol degradation was performed with "fresh cells" received from an inoculum cultivation in the YNB medium. These cells, cultivated in a complex medium, were considered to be rich in a content of intracellular reserve materials. The courses of C_L and q_s received with this population can be seen from Fig. 4. After approximately 24 h of phenol degradation in the nutrient limited medium (time C) the cells were harvested, separated from the exhausted medium with accumulated metabolites, and inoculated again into the same nutrient limited medium. During the second (repeated) phenol degradation period the cell respiration rate achieved almost the same values as it follows from the dissolved oxygen concentration profiles shown in Fig. 4 a.

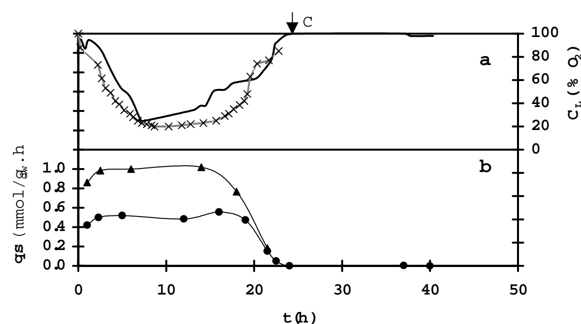

Figure 4 - Changes of the minimum dissolved oxygen concentration achieved after a phenol addition (a) and the specific phenol consumption rate (b) in the nutrient limited medium with:
-x- ; ▲ fresh cells from the YNB medium,
▬ ; ● exhausted cells from the limited medium

However, the maximum specific phenol consumption rate was only 50% of the value reached with the "fresh cells" (Fig. 4b). From this follows that the "exhausted cells" were able to respire with the same activity as the "fresh cells" however, at the expense of an increase of the endogenous respiration due to a partially changed enzymatic system of the cells. Then the overall physiological changes resulted from a gradual exhaustion of the intracellular reserve material.

Changes of Phenol Hydroxylase Activity
The phenol hydroxylase is the key enzyme for biodegradation of phenol. In ascomycetous and basidiomycetous yeasts and yeast like fungi phenol is assimilated via 3-oxoadipate pathway by ortho fission of catechol (Middelhoven, 1993). The phenol hydroxylase activity in *Candida tropicalis* strictly depends on the presence of NADPH (Neujahr et al., 1974). As found in our experiments the average course of the phenol hydroxylase activity during phenol oxidation under nutrition stress conditions can be seen from Fig. 5 (an average value from three repeated experiments).
At the start of phenol oxidation (approximately first 6 h) there is evident an induction effect of phenol to phenol hydroxylase.

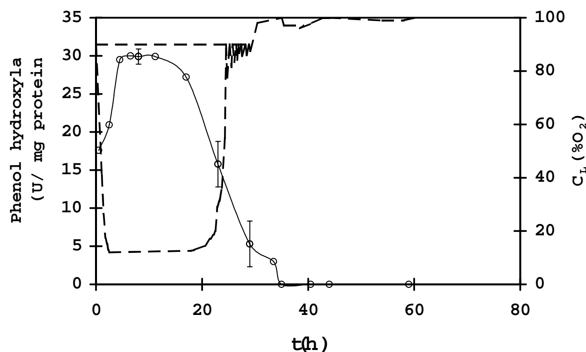

Figure 5 - Courses of the minimum dissolved oxygen concentration achieved after a phenol addition (--- C_L) and the activity of phenol hydroxylase (○) in the nutrient limited medium

The enzyme activity remains at a high value as long as the cells can utilize their reserve materials and completely oxidize phenol to CO_2 and H_2O (cf. the low C_L values resulting from the high respiration rates of the cells). Just at the start of nutrient starvation accompanied by a drop of the respiration rate a simultaneous decrease of the phenol hydroxylase activity occurs. Such a time plot of the phenol hydroxylase activity had not yet been published in the literature. However, Krug et al (1985 b) has already described the induction effect of various phenolic compounds, the temperature effect and the pH optimum for phenol hydroxylase. The zero activity, was observed at about 35th hour (Fig. 5), results from the reserve material exhaustion. This is a similar finding to that of Harder and Dijkhuizen (1983) who proved the metabolic changes coupled with adaptation to the starvation stress (Harder and Dijkhuizen, 1983).

Changes of Cell Viability
Fig. 6 shows courses of the biomass concentration and the dead cell quantity during a phenol oxidation in the nutrient limited medium. During a phase of the maximum respiration rate (total phenol mineralization) only 2% of the dead cells has been identified. After the loss of the respiration ability the dead cell number increased to about 9%. Therefore, at the end of the respiratory phase the cell population still remains viable and without any damage of the membranes that were supposed to occur at higher phenol concentrations (Ruiz-Ordaz et al., 1998, Mörsen and Rehm, 1990).

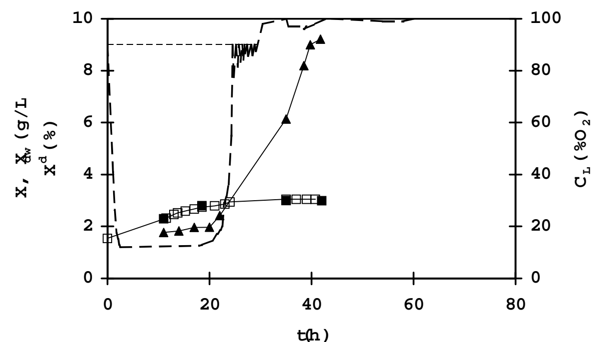

Figure 6 - Courses of the minimum dissolved oxygen concentration achieved after a phenol addition (--- C_L), the biomass concentration (□ X; ■ X_{dw}) and the dead cell quantity (▲ X^d) in the nutrient limited medium

CONCLUSIONS

- The cells produce extracellular metabolites under the starvation conditions.
- The starvation stress induces an increase of the endogenous respiration rate at the expense of phenol oxidation.
- The respiration inhibition is linked with metabolite accumulation in the reactor.
- It was proved that the respiration inhibition can be released by metabolite removal.
- The activity of phenol hydroxylase drops together with the loss of respiration ability.
- After the loss of the respiration ability the dead cell number slightly increases.

- The ratio of damaged cells remains below 10% as long as 15h after the loss of the respiration activity.

RESUMO

Candida tropicalis pode utilizar fenol como única fonte de carbono e de energia. O fermentador foi operado em um sistema "batelada-alimentada" e controle oxidativo. Em condições limitantes de nutrientes e excesso de oxigênio a atividade respiratória das células foi suprimida e o calor do metabolismo pode ser formado. Uma acumulação desses produtos inibiu o crescimento das células em condições aeróbicas. Outro impacto foi um decréscimo da atividade fenol hidroxilase como enzima chave da degradação do fenol no final da atividade respirométrica. Essa redução está relacionada com os fatos acima mencionados.
A morte da célula estudada por sonda de fluorescência ocorreu lentamente após a perda da atividade respiratória. O "stress" celular induziu um aumento na taxa de respiração endógena devido à oxidação fenólica.

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REFERENCES

Chang, Y. H.; Li, C. T.; Chang, M. C. and Shoieh, W. K. (1998), Batch phenol degradation by Candida tropicalis and its fusant. *Biotechnol. Bioeng*., **60** : (3), 391-395.

Colarieti, M. L.; Toscano, G. and Greco Jr., G. (2002), Soil catalyzed polymerization of phenolics in polluted waters. *Wat. Res.*, **36**, 3015-3022.

Cooper, V. A. and Nicell, J. A. (1996), Removal of phenols from a foundry wastewater using horseradish peroxidase. *Wat. Res,* **30** : (4), 954-964.

Dagley, S. (1985), Microbial metabolism of aromatic compounds. In: Moo-Young M. (Ed.): *Comprehensive Biotechnology*. Oxford : Pergamon Press. **v. 1**. pp. 483-505.

Daratchkiev, R.; Kolev, N.; Beschkov, V. and Aleksandrova, T. (1996), A new bioreactor with semi-fixed packing: investigation of degradation of phenol. *Bioprocess Engineering,* **16**, 5-7.

Drijvers, D.; Van Langenhove, H. and Beckers, M. (1999), Decomposition of phenol and trichlorethylene by the ultrasound/H_2O_2/CuO process. *Wat. Res.,* **33** : (5), 1187-1194.

Fang, H. H. P. and Chan, O. (1997), Toxicity of phenol towards anaerobic biogranules. *Wat. Res.,* **31** : (9), 2229-2242.

Garcia Garcia, I.; Bonilla Venceslada, J. L.; Jiménez Pena, P. R. and Ramoz Gómez, E. (1997), Biodegradation of phenol compounds in vinasse using *Aspergillus tereus* and *Geotrichum candidum. Wat. Res.*, **31** : (8), 2005-2011.

Harder, W. and Dijkhuizen, L. (1983), Physiological responses to nutrient limitation. *Ann. Rev. Microbiol.,* **37**, 1-23.

Katayama-Hyraiama, K.; Tobita, S. and Hirayama, K. (1991), Metabolic pathway of phenol in Rhodotorula rubra. *J. Gen. Appl. Microbiol.*, **37**, 379-388.

Krug, M.; Ziegler, H. and Straube, G. (1985a), Degradation of phenolic compounds by the yeast *Candida tropicalis* HP 15. I. Physiology of growth and substrate utilization. *J. Basic. Microbiol.,* **25** : (2), 103-110.

Krug, M.; Ziegler, H. and Straube, G. (1985b), Degradation of phenolic compounds by the yeast *Candida tropicalis* HP 15. II. Some properties of the first two enzymes of the degradation pathway. *J. Basic. Microbiol*., **25** : (2), 271-281.

Masuda, M.; Sakurai, A. and Sakakibara, M. (2001), Effect of reaction conditions on phenol removal by polymerization and precipitation using *Coprinus cinereus* peroxidase. *Enzyme and Microbial Technology,* **28**, 295-300.

Middelhoven, W. J. (1993), Catabolism of benzene compounds by ascomycetous and basidiomycetous yeast and yeast like fungi. *Antonie van Leeuwenhoek,* **63**, 125-144.

Mörsen, A. and Rehm, H. J. (1990), Degradation of phenol by a defined mixed culture immobilized by absorption on activated carbon and sintered glass. *Appl. Microbiol. Biotechnol.*, **33**, 209-212.

Neujahr, H. Y.; Lindslo, S. and Varga, J. M. (1974), Oxidation of phenols by cells and cell-free enzymes from *Candida tropicalis. Antonie van Leeuwenhoek*, **40**, 209-216.

Orupõld, K.; Maširin, A. and Tenno, T. (2001), Estimation of biodegradation parameters of phenolic compounds on activated sludge by respirometry. *Chemosphere*, **44**, 1273-1280.

Ruiz-Ordaz, N.; Hernández-Manzano, E.; Ruiz-Lagúnez, J.; Cristiani-Urbina, E. and Galíndez-Mayer, J. (1998), Growth kinetic model that desribes the inhibitory and lytic effects of phenol on *Candida tropicalis* yeast. *Biotechnol. Prog.,* **14**, 966-969.

Scheck, C. H. K. and Frimmel, F. H. (1995), Degradation of phenol and salycilic acid by ultravioloet radiation/hydrogen peroxide/oxygen. *Wat. Res*., **29** : (10), 2346-2352.

Sikkema, J.; Bont, J. A. M. and Poolman B. (1995), Mechanisms of membrane toxicity of hydrocarbons. *Microbiological Reviews*, **59** : (2), 201-222.

Sixt, S.; Altschuh, J. and Brüggerman, R. (1995), Quantitative structure-toxicity relationships for 80 chlorinated compounds using quantum chemical descriptions. *Chemosphere* , **30** : (12), 2397-2414.

Vojta, V.; Nahlik, J.; Paca, J. and Komarkova E. (2002), Development and verification of the control system for fed-batch phenol degradation processes. *Chem. Biochem. Eng. Q.*, **16** : (2), 59-67.

Wu, J.; Rudy, K. and Spark, J. (2000), Oxidation of aqueous phenol by ozone and peroxidase. *Advances in Environmental Research*, **5**, 339-346.

Wu, J.; Taylor, K. E.; Bewtra, J. K. and Biswas, N. (1993), Optimization of the reaction conditions for enzymatic removal of phenol from wastewater in the presence of polyethylene glycol. *Wat. Res.,* **27** : (12), 1701-1706.

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