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Kinetic Analysis of Phenol Biodegradation by Isolated Bacteria and Mixed Culture by Cells Immobilized on Loofa (*Luffa Cylindrica*) Sponge in Airlift Bioreactor

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Various contaminated soil samples were examined for isolation of microorganisms responsible for phenol degradation. One type pure culture gram positive bacteria was isolated and identified as *Bacillus pulvifaciens*. Isolated bacteria and an activated sludge were acclimated for 75 days to a medium containing phenol in a separate 250 ml Erlenmeyer flasks. After this period of time, biomass was transferred into airlift bioreactor with loofa sponge for cells immobilization and acclimation which was operated by fill and draw mode for 70 days. The immobilized cells grew within the void space of the loofa sponge, reaching a high cell concentration of 21.4 g-cells/l-sponge volume and the free cells in the effluent became around of 0.7 g/l. The performance of airlift bioreactor for 27 hours operation after acclimation period, showed that decrease in phenol concentration after 24th hour culture with the isolated bacteria and the activated sludge culture were 99.8% and 84.2% removal, respectively. The phenol and COD degradation by the isolated bacteria was higher than that of the activated sludge culture. Based on the experimental data, kinetic model was discussed. Through the model the biokinetic parameters were evaluated, which represented the behavior of reactor very well.

Keywords: *Bacillus pulvifaciens*, Kinetic Parameters, Luffa Cylindrica, Immobilized cells

INTRODUCTION

Several types of industrial water contain various phenols. Many of these compound are extremely harmful as they are highly toxic both towards microorganisms and vertebrates. Enzymatic approaches to removing phenols have been tried for some years as they have several advantages compared with the conventional methods (solvent extraction, chemical oxidation, adsorption to activated carbons, etc.) (Luca *et.al.*, 1996). Conventional methods for removal of phenols from industrial wastewater may suffer from serious drawbacks as high cost, incomplete removal, formation of toxic byproduct and applicability to a limited concentration range (Yimin, *et. al.*, 1997).

A catalytic process which uses horseradish peroxidase (HRP), an enzyme isolated from the roots of horseradishes, has been shown to be applicable for the treatment of several industrial phenolic wastewater. HRP catalyzes 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. However, large amounts of enzyme required, the need to add H_2O_2, high catalyst cost, disposal of solid reaction product and possible formation of hazardous soluble byproduct may limit the application of the process (Cooper, *et.al.*, 1996; Luca *et. al.*, 1996).

Activated sludge process is one of the most widely accepted biological systems for the treatment of phenolic wastes. Bacteria, yeasts and fungi capable of utilizing phenolic compounds are found in soil and water environments (Kumaran, *et. al.*, 1997). The capacity of microorganism to degrade industrial effluent containing phenols is well known. However, in practice very few systems are highly efficient mainly because of poor control of microorganisms involved in degradation. Immobilization of cells in carrier is one way to maintain high cells concentration and prevent washout of slow growing microorganism. Immobilization of cells in support particles such as polyethylene (Lazarova, *et. al.*,1997), ceramic beads (Salter, *et. al.*, 1990) and polyurethane (Xing, *et. al.*, 1999) has been extensively investigated. Immobilization by cells entrapment in polymeric materials cause environmental problem after use.

Loofa *(luffa cylindrical)* sponge which produced abundantly in many developing countries within the tropical and sub-tropical zones is an excellent carrier for immobilizing flocculating cells and non flocculating cells (James, *et. al.*,1994; Ogbona, *et. al.*, 1977). Aerobic surface and anaerobic subsurface regions of the carriers are suitable for both aerobic and anaerobic microorganisms, respectively. Microbial degradation of phenol was examined by the culture incubated by the isolated microorganism (Bioreactor A) and the culture incubated by activated sludge (Bioreactor B) in comparison without microbes as a control (Bioreactor C).

EXPERIMENT

Preparation of microbial seeds

Various contaminated soil in pulp and paper mill (S), palm oil mill (IA), natural gas plant (IB), petroleum refining mill (TA) and urea formaldehyde plant (TB) were examined for selection of microbes for phenol degradation. The data on screening and characterization of microbial degradation of phenol from soil were presented elsewhere (Faisal, 2000). Enrichment cultures were prepared in 250 ml Erlenmeyer flasks. Five gram of various soil was enriched in the 50 ml medium containing phenol, yeast extract and microelement (Table 1).

Table 1. The composition of medium and microelement solution

The composition of the medium		The composition of microelement solution	
Component	Amount	Component	Amount
K_2HPO_4	6 g/l	$FeSO_4.7\ H_2O$	2.8 g/l
KH_2PO_4	2 g/l	$MnSO_4.5H_2O$	2.4 g/l
$(NH_4)_2SO_4$	2 g/l	$CoCl_2.6H_2O$	2.4 g/l
$MgCl_2.6H_2O$ solution (34 g/l)	10 ml/l	$CaCl_2.2H_2O$	1.7 g/l
Microelement solution.	1 ml/l	$CuCl_2.2H_2O$	0.2 g/l
Yeast extract	1 g/l	$ZnSO_4.7H_2O$	0.3 g/l
Phenol	1 ml/l	$NaMoO_4$	0.25 g/l

Culture was kept on a rotary shaker for 7 days at 25 °C. On 8th day (second enrichment stage), 10 ml of culture solution in Erlenmeyer flasks was transferred into the 50 ml fresh medium (Table 1), in which yeast extract was decrease to 0.5 g/l. On 15th days (third enrichment stage) and on 23rd day (fourth enrichment stage) yeast extract was decreased to 0.2 g/L and to 0.004 g/L respectively.

After these enrichment culture (on 30th day), the culture was plated on agar to isolate the bacteria responsible for phenol biodegradation. The composition of agar plate was the same as the composition of the medium where agar concentration was 15 g/l. Culture in which growth took place in the liquid medium was then used further for isolation of phenol degrader by streaking on medium solidified with agar. By repeated picking-up and restreaking of the well isolated colony, a sufficiently pure culture, which was obtained was transferred to a bioreactor.

The stock culture was used as an original microbial seed which had been acclimated in the medium containing the microelement shown in Table 1. The microorganism from stock pure culture was cultivated at room temperature and activated sludge from the aerobic treatment pond of wastewater treatment plant in Mobil Oil Indonesia, Aceh, Indonesia was used as inoculums for seeding and acclimation of mixed culture. Seeding and acclimation of pure culture and mixed culture were performed in a 250 ml Erlenmeyer flask by fill and draw mode for 75 days at room temperature to increase the biomass concentration, then transferred into the airlift bioreactors.

Bioreactors and its operation

The three of bioreactors were made of glass as shown in Figure 1. The design for all bioreactors, i.e., bioreactor A (pure culture), bioreactor B (mixed culture) and bioreactor C (without microorganism) was 35 cm in height, 7 cm in width and 7 cm in length, total effective volume being 1500 cm^3. The riser and

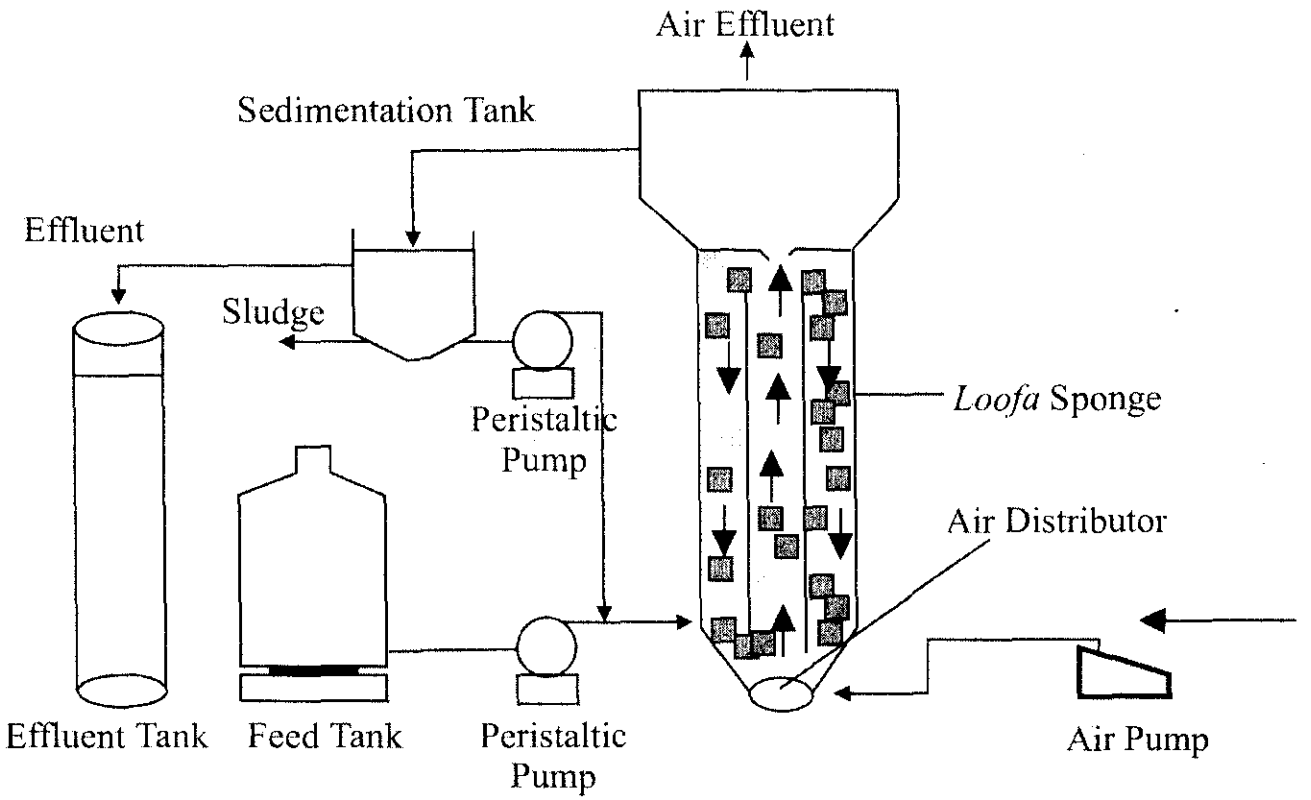


Figure 1. Air Lift Bioreactor

downcomer were 4 cm and 1.5 cm wide, respectively. The riser and the downcomer were interconnected near the top and the bottom of the reactor. Difference in gas holdup between the riser and the downcomer drives the circulation of fluid: up-flow in the riser and down-flow in the downcomer. Loofa sponges cut in to small rectangular blocks (average size of 10 x 10 x (3-5 mm)) were placed in the bioreactor at a volume ratio (sponge volume/medium volume = 0.1). The system were operated in batch process where the artificial sewage containing 30 mg phenol/L, 4000 mg COD/L, medium and microelement shown in Table 1 were fed by fill and draw mode with one cycle every three days. Supernatant in the bioreactors settled for 30 min were drawn before the fresh artificial sewage of the same volume was added. Operation progress was monitored by measurement of phenol, COD, MLSS, dissolved oxygen and pH.

Analytical methods

Identification of isolated bacteria was conducted using API 20 NE identification kit (bioMe'rieux) and Bergey's Manual of Determinative Bacteriology (Hold, *et. al.*, 1994). The microbial concentration was measured by optical density (OD) at wave length of 660 nm using a spectrophotometer (UV-1201, Shimadzu Corp.) after sonicating the sample. Nitrate-N concentration was measured by Brucine sulfanilic acid method at wave length of 410 nm and nitrite-N concentration using sulphanilamide and 1-naphtyl ethylene diamine dihydrochloride (NEDA) reagents at wave length of 540 nm. The COD and phenol concentration was measured by the standard dichromate and 4-aminoantipyrine method, respectively according to APHA, 1989. Concentration of dissolved oxygen and pH were monitored by DO and pH sensors.

RESULTS AND DISCUSSION

Identification of isolated bacteria

Identification of isolate conducted using API 20 NE identification kit (bioMe'rieux) and Bergey's Manual of Determinative Bacteriology (Hold, *et. al.* 1994), revealed the dominant strain was *Bacillus pulvifaciens*.

Performance of bioreactor

Figure 2 shows the time course of phenol degradation for 27 hours operation in bioreactor A and B. As observed from time course of phenol concentration, phenol in both reactor was degraded rapidly after the start of the culture.

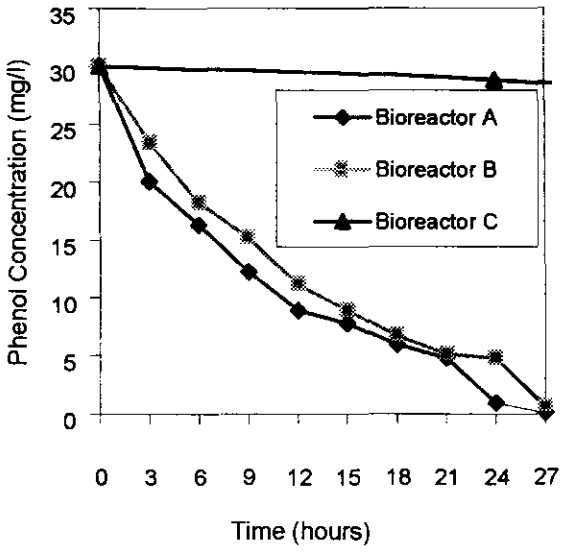


Figure 2. The profile of phenol concentration for 27 hours operation after 70 days acclimation period

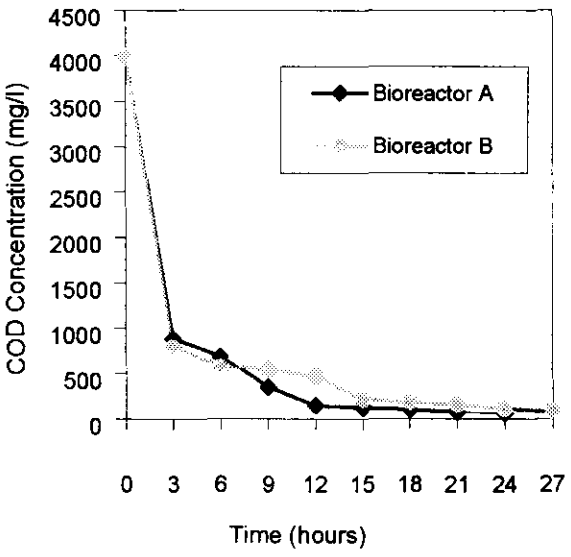


Figure 3. The profile of COD concentration for 27 hours operation after 70 days acclimation period

The phenol degradation by pure culture (bioreactor A) was faster than by mixed culture (bioreactor B). The degradation of phenol in bioreactor A was completed after around 24 hours while in bioreactor B the degradation of phenol was fully consumed after 27 hours. The observed removal efficiency of phenol in bioreactor A higher than bioreactor B mainly implied that the screening and multiplication of isolated microorganism would enhance the phenol degradation capacities.

Figure 3 shows that COD concentration in bioreactor decrease rapidly at thebeginning until 3rd hour from around 4000 mg/l to around 800 mg/l which corresponded to around 80% COD removal efficiency for both pure and mixed culture. Afterwards COD removal efficiency was getting higher and finally reach at level 98% after 27th hour operation.

Figure 4 shows during the acclimation in bioreactors, the suspended microbial concentration in both bioreactor A and bioreactor B showed a gradual decreased and reached stable levels after around 48 day.

The decrease in suspended microbial concentration in both reactor were indicative of microbial selection for individuals capable of phenol degradation, wherein phenol non-degraders would have died out and the cells were entrapped in the carriers particles. After acclimation period, the suspended microbial concentration in both bioreactor reached the constant level was about 500 mg/l in bioreactor A and 800 mg/l in bioreactor B, the retained cells concentration within the lattice structure of the sponge in bioreactor A was about 19,000 mg MLSS/l of sponge volume and 21,600 mg MLSS/l of sponge volume in bioreactor B.

The results of both culture A and B indicate that microbes not only reduce the phenol and COD but also removed ammonia normally present in the wastewater. The pH in the bioreactors were in the range of 5.21 to 6.45 in bioreactor A and 6.02 to 6.45 in bioreactor B, these acidic pHs were due to the formation of nitrite and nitrate during the degradation of ammonia. Fig. 5 shows the concentration of NO_3-N gradually increased with time course and reached a constant level of 16 mg/l in bioreactor A and 21 mg/l in bioreactor B. Only a small amount of NO_2-N was detected after 27 hours cultivation in both Bioreactors. This research demonstrates that using microbes

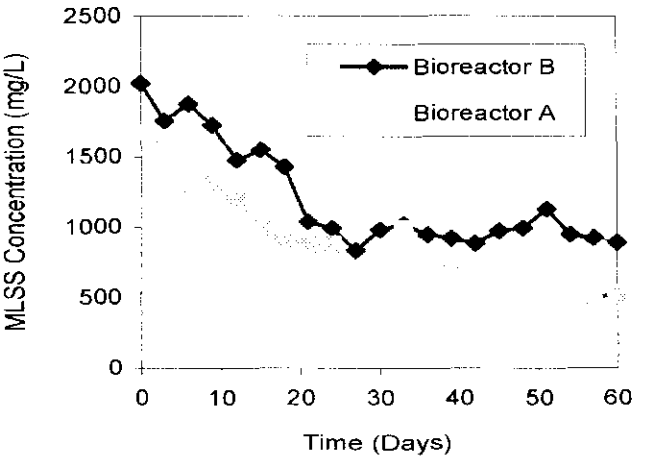


Figure 4. MLSS concentration during the acclimation

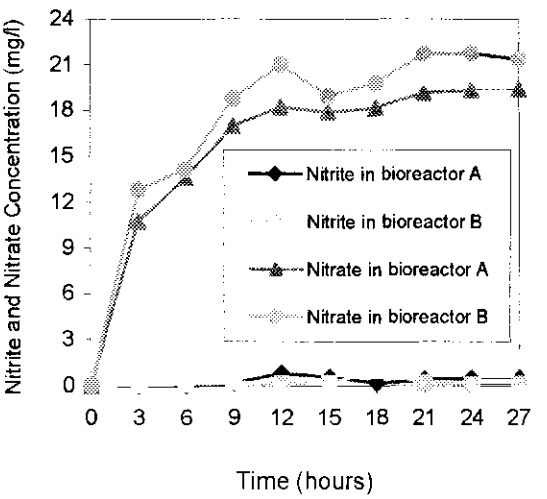


Figure 5. Nitrite and Nitrate concentration during the 27 hours operation after 70 days acclimation period

retained in loofa sponge as carrier is not only effective in degradation of phenol and COD but also enables transformation of NH_4-N to NO_3-N *(nitrification)*. Ammonia-N uptake for cell growth and *denitrification*, which was not measured, might have affected the reaction.

Kinetic of phenol biodegradation

Since the phenol degradation by immobilized *Bacillus pulvifaciens* and mixed culture proceeded with cell mass growth, the kinetic parameters were evaluated on the basis of Monod growth model.

$$\mu = \frac{I}{X}\frac{dX}{dt} = \frac{\mu_{max} S}{Ks + S} \qquad (1)$$

The above equation is rearranged in on order to find out the kinetic parameters:

$$\frac{1}{\mu} = \frac{Ks}{\mu_{max}}\frac{1}{S} + \frac{1}{\mu_{max}}$$

Introducing the yield coefficient

$$Y_{x/s} = \frac{-dX}{dS}$$

and assuming its volume to be constant throughout the fermentation, the rate of phenol degradation is presented as:

$$-\frac{dS}{dt} = \frac{1}{Y_{x/s}}\frac{dX}{dt} = \frac{\mu_{max} SX}{Y_{x/s}(Ks+S)} \quad (3)$$

The equation (3) is integrated in term of variable S by expressing by the following relation:

$$X = X_0 + Y_{X/S}(S_0 - S) \quad (4)$$

into equation (5).

$$t = -\int_{So}^{S} \frac{Y_{x/s}(Ks+S)dS}{\mu_{max} S[X_o + Y_{x/s}(S_o - S)]}$$

$$= \frac{Y_{x/s}K_s}{a}\ln\frac{S_o}{S} + \frac{Y_{x/s}}{b}\left(1 + \frac{bK_s}{a}\right)\ln\frac{a-bS}{a-bS_o} \quad (5)$$

where

$$a = m_{max}X_o + bS_o$$
$$b = m_{max}Y_{x/s}$$

The values of K_s and m_{max} can be determined using equation 2, by plotting 1/m against 1/S having slope K_s/m_{max}. The kinetic parameters m_{max} and K_s for culture A and B were calculated from Figure 6 and the values of $Y_{X/S}$ was calculated according to equation 4, as summarized in Table 2.

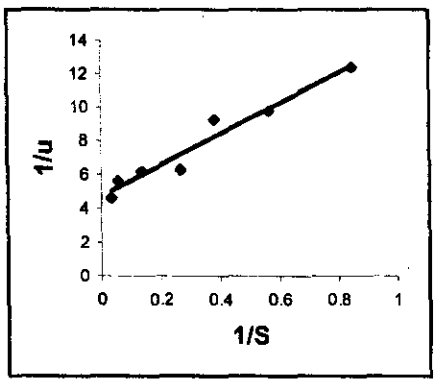
After the determination of kinetic parameters for culture A and B and the yield coefficient $Y_{x/s}$, simulation studies have been made for phenol degradation. The simulated and the experimental data in the plots of (S/S_0) vs fermentation time t, are shown in Figure 7. The data on phenol biodegradation for culture A and culture B were correlated very well as evidence from the plot of Figure 7.

CONCLUSION

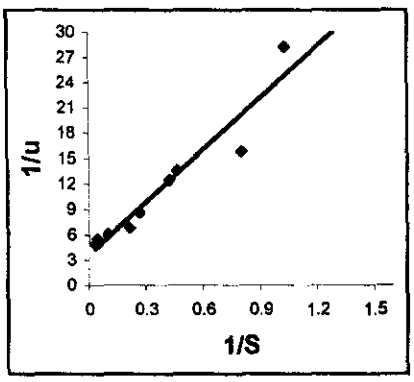
Loofa sponge is a good carrier for immobilizing of phenol degrading microbes. The cells grew very well within the lattice of the loofa sponge, reaching high cell concentration up to 21,000 mg MLSS/l of sponge volume. Isolated bacteria from contaminated soil (*Bacillus Pulvifaciens)* and acclimated activated sludge were inoculated in the airlift bioreactor together with the cell immobilizing loofa sponge applicable to degrade phenol and organic compound. Phenol and COD degradation by the culture inoculated by the isolated strain was faster than the culture inoculated by the activated sludge. Aerobic surface and anaerobic subsurface region of the

Table 2. Kinetic parameter for culture A and culture B

No	Culture	μ_{max} [1/h]	Ks [mg/l]	$Y_{x/s}$ [g/g]
1.	culture A	0 .21	1.92	262
2.	Culture B	0.28	5.68	403



(a) Culture A



(b) Culture B

Figure 6. Kinetic constants by application of Monod's Model (a) for culture A and (b) culture B

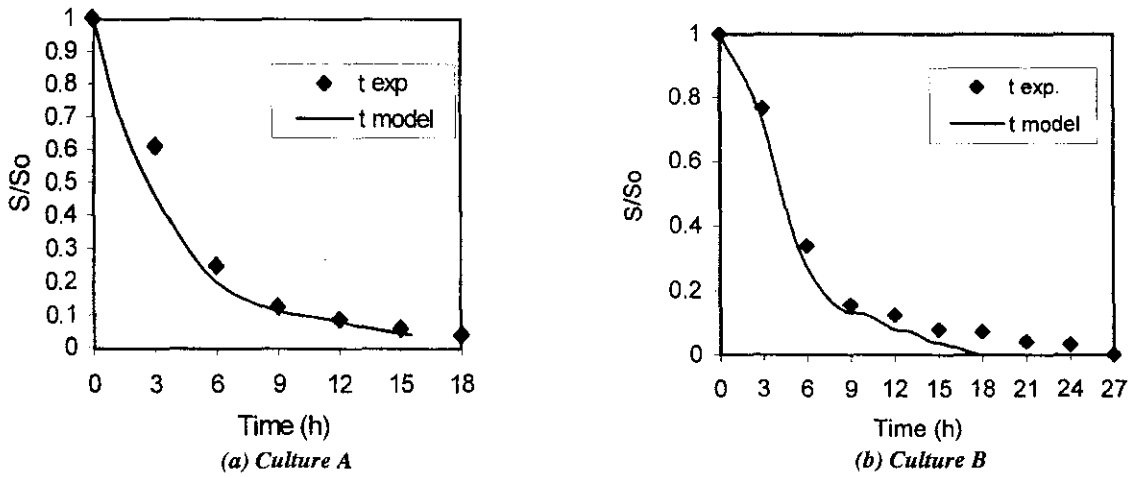


Figure 7. Phenol degradation experimental and predicted values from model using immobilized cells (a) Culture A and (b) Culture B for an initial phenol concentration of 30 mg/l

sponge was considered suitable for both aerobic nitrification and anaerobic denitrification simultaneously in Bioreactor. The microorganism not only reduced phenol and organic compound but also was able to removed ammonium in the wastewater. Through the developed biokinetic model, the behavior of the reactor in term of phenol concentration was represented well.

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