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Rate of Biodegradation of Phenol by *Klebsiella oxytoca* in Minimal Medium and Nutrient Broth Conditions

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ABSTRACT The rate of biodegradation of phenol by *Klebsiella oxytoca* strain was studied in the nutrient broth and M9 minimal medium. It was found that *K. oxytoca* degrade phenol at elevated phenol concentration where 75% of initial phenol concentration of 100 ppm will degrade within 72 h. This rate was increased with increasing the initial cell densities, increasing the aeration rate and increasing the time required for complete degradation. At phenol concentration above 400 ppm, the cells were unable to degrade the substrate efficiently due to the increasing concentration of phenol in the medium. The culture conditions were also showed a significant impact on the ability of these cells to remove phenol. The optimum solution pH and temperature were 6.8 and 37°C, respectively. The growth of these cells in the presence and absence of phenol was modeled and it was found that the Recatti equation best fit the growth in the absence of phenol whereas the Voltera equation accounted for the history of the cell population in the presence of phenol.

KEYWORDS *Klebsiella oxytoca*, phenol biodegradation, Reccati model, Voltera model

INTRODUCTION

Phenolic compounds are considered one of the major hazardous materials that have a hazardous impact on environment. They generally are found to contaminate streams, rivers, and ground water. Once these compounds enter into the food cycle, they can cause several organ problems to human. Exposure to high dose can cause liver damage, hemolytic anemia, and paralysis. The major source of phenolic compounds arises from industrial activities such as petroleum processing, plastic manufacturing, and the production of resins.

A variety of techniques have been used for the remediation of phenol. Conventional methods of treatment have been largely chemical or physical, but these processes have led to secondary effluent problems and are costly. Biological treatment is an effective method that is used where many organisms have been shown to grow on phenol as the sole source of carbon (Müller, 1992).

Among the research areas that focused on sensitivity of the microorganisms to phenol as sole carbon is the work of Stoilova et al. (2006) who used

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Aspergillus awamori cells to degrade high concentration of phenol, catechol, 2,4-dichlorophenol, and 2,6-dimethoxyphenol in aqueous solution. Khleifat (2006) studied the effect of carbon starvation and some growth conditions on phenol degradation of using *Ewingella americana*. Ehlers and Rose (2003) showed that the biodegradation efficiency of phenol and 2,4,6-trichlorophenol by immobilized white-rot fungal cultures was enhanced in pinewood chip and foam glass bead–packed trickling reactors. Dursum and Tepe (2005) studied the rate of biodegradation of phenol using a free and Ca-alginate–immobilized *Ralstonia eutropha*. They found that the phenol has a strong inhibitory effect on the microbial growth. Arutchelvan et al. (2006) used *Bacillus brevis* for reduction of phenol concentration in a batch reactor, were the *brevis* strain was optimized for various environmental conditions and the biodegradation of phenol was as high as 1750 mg/L obtained at pH 8.0.

Previous research has demonstrated that the biodegradation rate of phenol can be enhanced by immobilizing the cells on the surface of the solid-based material, by mixing phenol with another carbon source, or by selecting a cell line with the capability of degrading phenol. *K. oxytoca* has already been isolated from the chlorination tank of the effluent wastewater treatment plant located on our University campus. It is gram-negative, belongs to the family Enterobacteriaceae. Because of this bacterium is resistant to more than 1000 ppm phenol and was already isolated from wastewater-containing phenol, we encouraged to use *K. oxytoca* as an Enterobacteriaceae-representing model to degrade phenol and study the kinetic parameters that affect the phenol removal capacity.

MATERIAL AND METHODS

Medium and Culture Conditions

The bacterium used in these experiments was *Klebsiella oxytoca*, which was previously isolated from the wastewater treatment plant in Al-Karak Country, Jordan (Khleifat et al. 2006). Its biochemical identity was reverified using the REMEL kit (RapID ONE and RapID NF plus systems) procedure; also the morphological characteristics were always being microscopically checked. Nutrient broth (NB) was used in all experiments unless otherwise stated. This contains 1.0 g meat extract, 2.0 g yeast extract, 5.0 g peptone, and 5.0 g sodium chloride per liter of solution. The pH of the medium was adjusted to 7.4 ± 0.2 by addition few drops of sodium hydroxide. Minimal medium (M9) (Miller 1972) with slight modifications was used for the growth of the bacterial cells. This medium contains 3.0 g Na_2HPO_4, 1.5 g KH_2PO_4, 1.0 g NH_4Cl, and 0.5 g NaCl, dissolved in 500 ml distilled water and the pH was adjusted to 7.4 with NaOH. Then the following were added: 0.24 g $MgSO_4$, 0.05 g $CaCl_2 \cdot 6H_2O$, and 0.05 g $FeCl_3 \cdot 6H_2O$. After autoclaving, 0.6 g L-proline and 0.001 g thiamine-HCl were added to the mixture and the volume was adjusted to 1 L by addition of sterilized distilled water. Disodium succinate was omitted when using phenol as sole carbon and energy source.

The cells of *Klebsiella oxytoca* were grown in nutrient broth (NB) medium and NB-containing 300 ppm phenol in a batch culture. The growth conditions were a shaking rate of 200 rpm ($1.12 \times g$), incubation temperature of 37°C, and pH 7.0 (unless otherwise stated). The starting inocula were adjusted while 1.0 ml of bacterial suspension of *K. oxytoca*, $OD_{600} = 0.2$, was grown in NB and inoculated into 125-ml Erlenmyer flasks containing 50 ml of minimal medium (MM). After inculcation, the growth mass was measured as OD_{600} (Spectronic genesys 2) at 2-h intervals.

Effect of Cell Density on Phenol Degradation

Klebsiella oxytoca cell densities of 0.2, 0.4, and 0.6 OD_{600} were mixed with 100 ml each of M9 minimal medium in 250-ml Erlenmeyer flask. Then a sterilized stock solution of phenol was added to obtain initial phenol concentration of 300 ppm. The mixture temperature and acidity were adjusted to 37°C and pH 7.5, respectively. The solution was mixed continuously at 200 rpm ($1.12 \times g$) to attain fixed aeration rate. The phenol concentration was monitored spectrophotometrically by drawing 1 ml from each of these solutions at 12, 24, 48, 72, 96 h. The samples were centrifuged at 12000 rpm ($4032 \times g$) for 10 min and the supernatant was diluted 10 times with distilled water and the phenol concentration was measured at 270 nm using spectrophotometer model Spectronic Genesys 2 (Bastos et al., 2000).

Effect of pH on Phenol Degradation

The effect of variation in pH (pH 4.5, 5.5, 6.5, 6.8, 7.5, and 8.0) on *Klebsiella oxytoca* grown in nutrient broth on percentage degradation of phenol was conducted. Growth conditions and phenol measurement

were conducted as described above except that the different pH values were used by buffering the solution using potassium phosphate buffer. The phenol concentration used was 300 ppm. Similar procedure was carried out for control solution without having the cells in the solution.

Effect of Incubation Temperature

To check the effect of incubation temperature as a function of time on the percentage degradation of phenol by *Klebsiella oxytoca*, different incubation temperatures were used: 25°C, 30°C, 35°C, 37°C, 42°C. Growth conditions and phenol measurement were conducted as described above except that of incubation temperature was varied; the phenol concentration used was 300 ppm. Similar procedure was carried out for control solution without having the cells in the solution.

Effect of Aeration on Phenol Degradation

Different agitation rates using centrifuge with 25 mm radius of rotation (50, 75, 100, 150, 200, and 250 rpm) were used to assess the effect of aeration on the degradation ability of phenol by *Klebsiella oxytoca*. Growth conditions and phenol measurement were conducted as described above except that the agitation rates were varied. The initial phenol concentration used was 300 ppm. Similar procedure was carried out for control solution without having the cells in the solution.

Effect of Carbon Starvation on Phenol Degradation

Klebsiella oxytoca cells were grown on nutrient broth (NB) at 37°C (150 rpm, $0.63 \times g$) to med-log phase ($OD_{600} \approx 0.50$) prior to starvation. The cells were harvested by centrifugation (5000 rpm, 25 mm radius of rotation, 10 min, 4°C), washed twice with equal volumes of sterile M9 minimal medium, and suspended in the same medium to an OD_{600} of 0.20 ($\approx 4 \times 10^8$ cells/ml). The cell suspension was immediately used as the nonstarved experimental control to assess phenol degradation by *Klebsiella oxytoca* cells. A subsample of the cell suspension was C-starved in the M9 minimal medium at 37°C with an agitation rate of 150 rpm ($0.63 \times g$) and left for 24 h. Then the carbon-starved cells containing M9 were supplied with 300 ppm phenol and tested for their phenol-degrading ability as usual. Growth curves also for the cells under two conditions (C-starved and nonstarved) were generated. The data for both phenol degradation experiments and growth curves were plotted together for carbon-starved and nonstarved cells.

Toxicity of to *Klebsiella oxytoca* on Different Phenol Concentration

To examine the toxicity of phenol on the growth of *Klebsiella oxytoca* in nutrient and M9 media, we varied the concentration of phenol from 100 to 1300 ppm. The evaluation of toxicity was based on the colony-forming units number measurements using NB plating methods.

Mathematical Methods

The biodegradation of phenol can be described by the nonelementary chemical reaction:

$$\text{Phenol} + \text{cells} \rightarrow \text{more cells} + \text{product}$$

The rate of cell production with time in a batch reactor can be expressed by the kinetic equation:

$$\frac{dCc}{dt} = kC_C(1 - \beta C_C) \tag{1}$$

where C_C is the concentration of the cells initially having the value of C_C^o, k is the rate constant, and β is constant related to the percentage of inhibition. This is *Riccati* equation, which can be integrated to give the logistic curve (Bailey and Ollis, 1986)

$$C_C = \frac{C_C^o e^{kt}}{1 - \beta C_C^o (1 - e^{kt})} \tag{2}$$

A drawback of the logistic equation is its failure to predict a phase of decline after the stationary population has exhausted all of a viable resource. This feature is found in one model developed by Volterra (Kumar et al., 2004):

$$\frac{dC_C}{dt} = kC_C(1 - \beta C_C) + K_o \left| \int_0^t C_C(r)dt \right| \tag{3}$$

where K_o is a constant account for the history or memory of population. The sign of K_o is taken as negative for an inhibitor and positive for a compound that promotes growth.

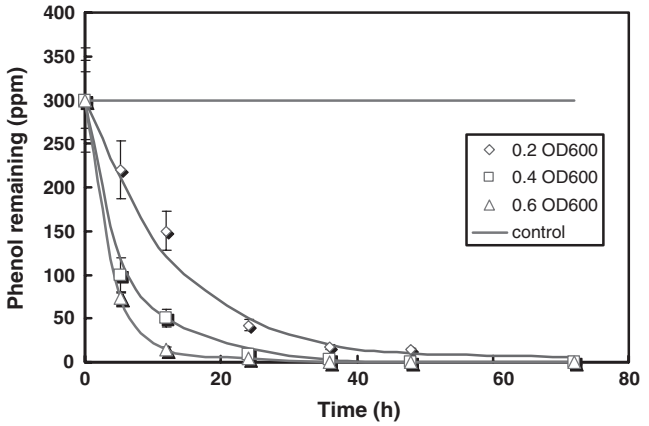

FIGURE 1 **Effect of different inoculation sizes on the biodegradation of 300 ppm of phenol by *Klebsiella oxytoca*. The temperature, agitation rates, and pH were 37°C, 200 rpm (1.12 × *g*), and 6.8, respectively.**

RESULTS AND DISCUSSION

In order to determine the effect of cell concentration on the rate of phenol degradation, freely suspended cell cultures with fixed phenol concentration (300 ppm) was carried out. Figure 1 shows the significant interdependence among the times needed for phenol degradation and the initial cell concentration. When initial cell concentration of 0.2 OD_{600} was used, partial degradation of phenol was achieved by 96 h; however, as the cell concentration increased, the time required for the complete degradation was decreased. The degradation rates for the three cell densities of 0.2, 0.4, and 0.6 OD_{600} nm were 10.5, 11.5, and 12 ppm/h, respectively. It has been shown that high initial cell concentration results in a high degradation rate of toxic compounds (Godjevargova et al., 2003; Lee et al., 1994; Marrot et al. 2006). These data suggest that high cell concentrations enhance the biodegradation of phenol.

To test the ability of the cells to tolerate the toxicity of the phenol, several initial concentrations were brought to the cells and the rate of degradation was monitored over time. It is shown (Figure 2) that the higher phenol concentration, the more time is required to be degraded by these cells. On the other hand, the effect of carbon starvation for a period of 24 h on the phenol degradation in M9 medium was investigated. As shown in Figure 3, when *Klebsiella oxytoca* cells were starved for carbon, the phenol degradation was almost advantageous for nonstarved cells during the first 24 h of incubation; however, after 24 h the degradation by C-starved cells become slightly better and complete degradation was achieved for both at the same time (72 h). In similar

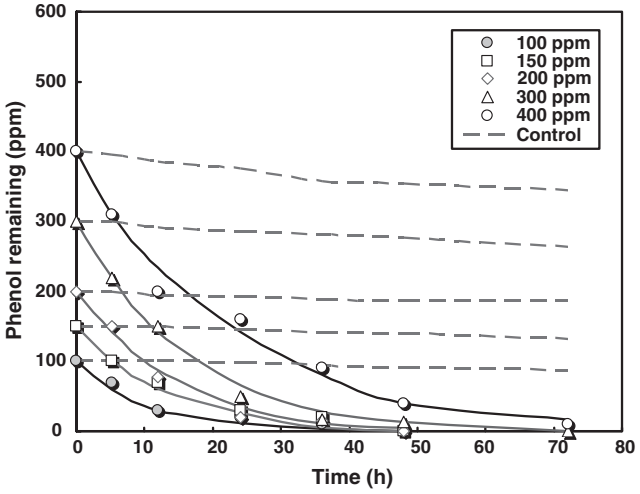

FIGURE 2 **Effect of substrate (phenol) concentrations on the biodegradation rate of phenol by *Klebsiella oxytoca* grown on M9 minimal medium. The figure represents the remaining phenol concentration of different initial phenol concentration as a function of time. Data are average of three independent experiments.**

manner, the growth of nonstarved cells in M9 medium (Figure 4) was better in the first 36 h, then the growth of starved cells started to be higher than the non-starved ones.

Leung et al. (2005) showed that the phenol degradation rate by *Moraxella* strains has increased significantly when these cells were starved for 24 h. This significant increase preceded by the decrease in the induction time. In our results, although the growth and degradation was better in sometimes, but the C-starvation did not reduce the induction time of phenol degradation of these cells, suggesting that C-starvation of the cells alone did not enhance the expression of the phenol-degrading genes. It was reported that carbon starvation could initiates an early expression of the *p*-nitrophenol catabolic genes in the bacterium *Moraxella* (Leung et al., 2005).

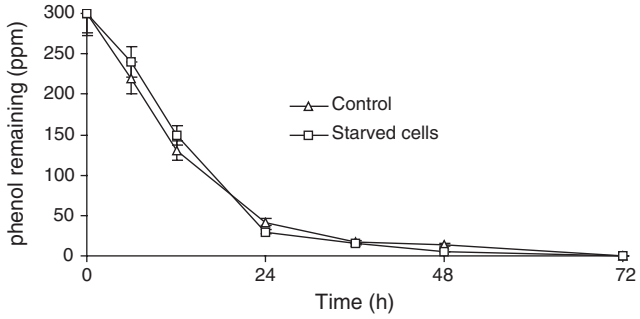

FIGURE 3 **Effect of carbon starvation on the phenol degradation percentage by *Klebsiella oxytoca*. The cells were grown on 300 ppm phenol–containing M9 minimal medium. The temperature and agitation rates were 37°C and 200 rpm (1.12 × *g*), respectively. Data are average of two independent experiments.**

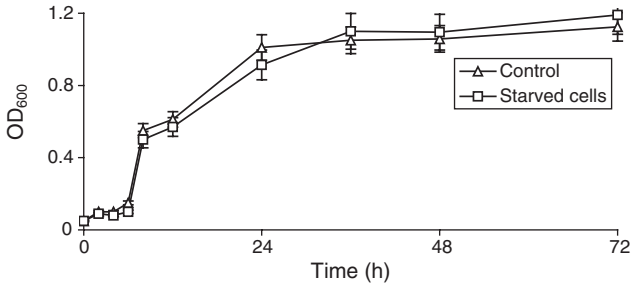

FIGURE 4 **Growth curve of *Klebsiella oxytoca* on M9 minimal medium containing 300 ppm phenol. The curve represents the absorbance at 600 nm as a function of time. The temperature and agitation rates were 37°C and 200 rpm (1.12 × *g*), respectively.**

The growth rate of cells in NB medium in the presence and absence of phenol is illustrated in Figures 5 and 6, respectively. It is clear that the cell growth in both cases is increased exponentially in the first 25 h, then started to decrease thereafter. The cell growth was analyzed using the Reccati and Voltera models. With NB medium in the absence of phenol, a logistic curve followed Reccati's equation was obtained. The parameters of this model were estimated using Mathematica (version 10): $\beta = 0.8$ and $k = 1.6$, with correlation coefficient of 0.98 and sum of the square errors of 0.07. On the other hand, the growth of the cells in the NB medium containing phenol was best fitted using the Voltera model, which predicts the phase of decline after the stationary population has exhausted all available resources. The Voltera parameters obtained were: $k = 0.95$, $\beta = 0.64$, and $K_o = -0.01$. The correlation coefficient is 0.88 and the sum of the square errors is 2.54.

Increasing the phenol concentration in both NB and M9 resulted in a decrease in the number of the cells required to degrade the phenol as a result of increasing the

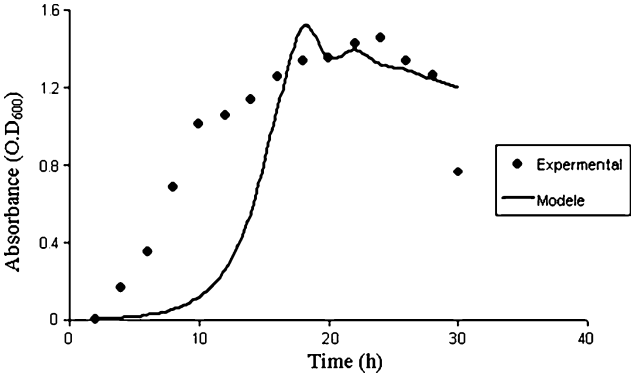

FIGURE 5 **Growth curve of *Klebsiella oxytoca* on nutrient broth medium plus 300 ppm phenol. The curve represents the absorbance at 600 nm as a function of time. Data were fitted by Volterra models. The temperature, agitation rates, and pH were 37°C, 200 rpm (1.12 × *g*), and 6.8, respectively.**

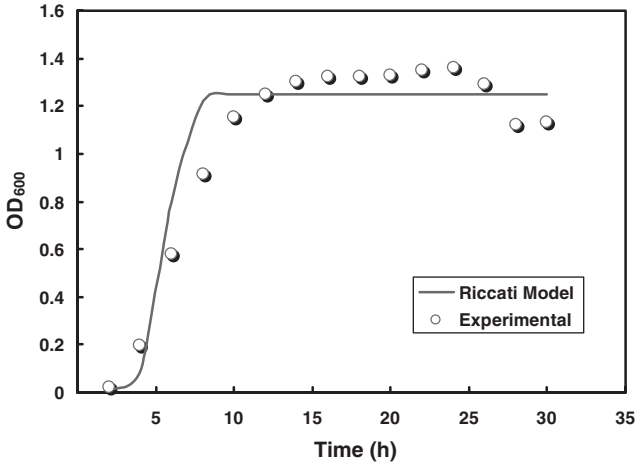

FIGURE 6 **Growth curve of *Klebsiella oxytoca.* The curve represents the absorbance at 600 nm as a function of time. Data were fitted by logistic models. The temperature, agitation rates, and pH were 37°C, 200 rpm (1.12 × *g*), and 6.8, respectively.**

toxicity of the media for these cells. Figure 7 showed the colony forming units (CFU) of the cells as a function of phenol concentration. It is clear that increasing this concentration from 100 to 400 ppm resulted in a gradual decrease in CFU; however, further increase in phenol concentration resulted in steep decrease in CFU till 1000 and 1300 ppm for M9 minimal medium and NB, respectively, beyond which no viable cells are seen.

Effect of pH, Temperature, and Aeration of Solution

Environmental factors such as pH, temperature, and other conditions in the environment may affect the

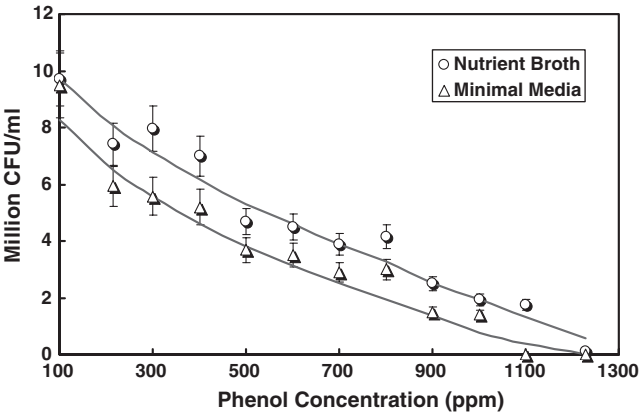

FIGURE 7 **Toxicity of *Klebsiella oxytoca* cells toward phenol. The data represent the CFU/ml as a function of phenol concentration for both bacterial cells that were grown in nutrient broth (NB) and M9 minimal media. The cells were incubated for 24 h. The temperature, agitation rates, and pH were 37°C, 200 rpm (1.12 × *g*), and 6.8, respectively.**

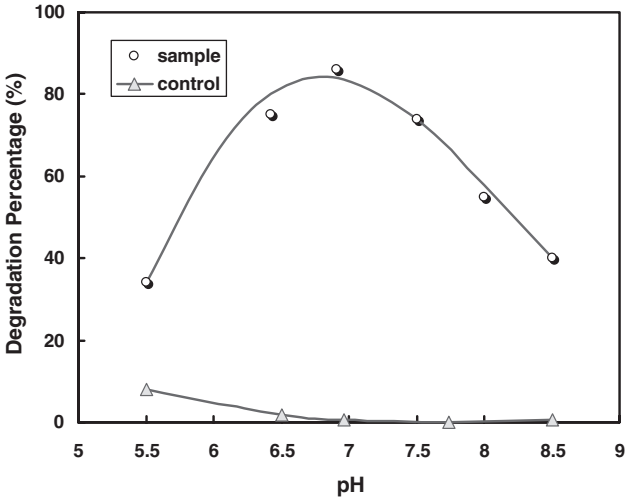

FIGURE 8 **Effect of pH on the biodegradation percentage of phenol by *Klebsiella oxytoca*. Cells were grown in nutrient broth (NB) plus 300 ppm phenol. The temperature and agitation rates were 37°C and 200 rpm (1.12 × *g*), respectively.**

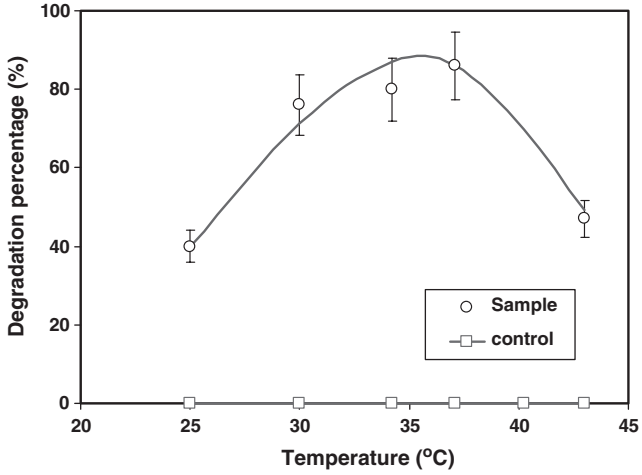

FIGURE 9 **Effect of Temperature on the biodegradation percentage of phenol by *Klebsiella oxytoca*. Cells were grown in nutrient broth (NB) plus 300 ppm phenol. The agitation rates and pH were 200 rpm (1.12 × *g*) and 6.8, respectively.**

growth of *Klebsiella oxytoca* and their ability to degrade the phenol. In this study *K. oxytoca* could degrade phenol at a wide range of pH (Figure 8), from 4 to 8, with an optimum value of 6.8. It is possible that the enzymes for phenol degradation have their optimum enzymatic activity at pH 6.8 and 72 h. On the other hand, the control solution showed a decrease in phenol concentration with decreasing pH. This could be attributed to the fact that phenols act as weak acids in aqueous solution and the dissociation of hydrogen ion from phenols strongly depends on the pH of the solution. In acidic solutions the molecular form dominates and in alkaline medium the anionic form is the predominant species (Abburi, 2003).

The dependence of the rate of phenol degradation on temperature is illustrated in Figure 9. Increasing the incubation temperature from 25°C to 37°C resulted in an increase in the degradation ability. However, further increase in the incubation temperature did not result in any further improvement; instead it caused a reduction in the percentage of degradation. In general, these bacterial cells were able to utilize phenol as the sole carbon source over a wide range of incubation temperatures, which is related to the metabolism of these microorganisms for the degradation process.

To assess the effect of aeration/agitation rate on the phenol degradation ability of *K. oxytoca*, different agitation rates were used for control and sample containing cells (Figure 10). It is clear that the difference in percent of degradation rate for phenol between the control and sample containing cells was achieved from 70% to 90% when the agitation speed is increased from 50 to 250 rpm (0.07 to 1.75 × *g*). On the other hand, when the culture was incubated without shaking there was no degradation. This phenomenon is probably due to the possibility of the phenol degradation process being aerobically occurred even under hypoxic conditions. Thus, this slight difference in the degradation ability probably may be attributed to the increase in cell mass with further increasing the agitation rate.

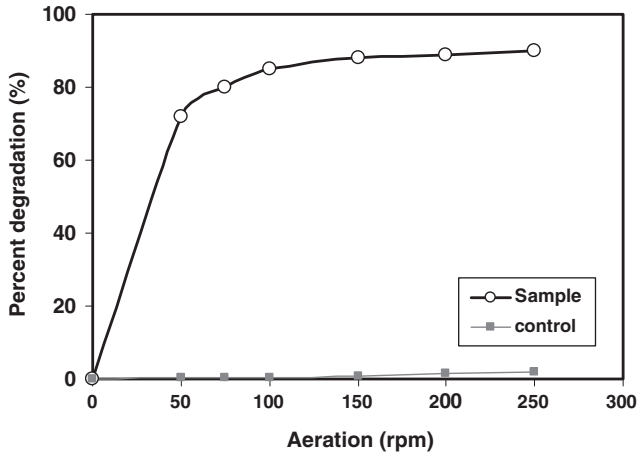

FIGURE 10 **Effect of agitation rates (rpm) on the biodegradation percentage of phenol by *Klebsiella oxytoca*. Cells were grown in nutreint broth (NB) plus 300 ppm phenol. The temperature and pH were 37°C and 6.8, respectively.**

CONCLUSION

Klebsiella oxytoca could be used for wastewater treatment for reduction of phenols from water. This degradation efficiency is affected by the condition of the medium such as the availability of oxygen, pH, and temperature as well as other substrates. It can tolerate the toxicity of phenol up to 400 ppm whereas starving these cells could promote slightly the phenol degradation efficiency. Two mathematical models were used to describe the rate of cell population increase with time; the growth of cells in NB medium containing phenol was best fitted using the Voltera model. It is recommended to extrapolate the research on these cells in the presence of multiple substrates under a variety of growth conditions.

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