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Effects of Pesticides and Respiratory Inhibitors on Phenol Degradation by *Acinetobacter* sp. strain AQ5NOL 1 Immobilized in Gellan Gum

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Phenol pollution often associated with agriculture industries involving pesticides and respiratory inhibitors. Microorganism used for biodegradation of phenols should also be resistant to the pesticides and respiratory inhibitors. In this study, 1 ppm of carbofuran, paraquat dichloride, atrazine, potassium cyanide (KCN), sodium azide (NaN_3) and rotenone were used to investigate the ability of *Acinetobacter* sp. strain AQ5NOL 1 freely suspended and immobilized in gellan gum beads to degrade phenol in the presence of pesticides and respiratory inhibitors. Results from this study showed that the degradation of phenol after 48 hours of incubation by free cells was inhibited by KCN at 47.48%. However, the degradation of phenol after 18 hours incubation by immobilized cells was inhibited by KCN at 52.68%. The lowest concentration of KCN that showed inhibition to phenol degradation was 0.8 ppm. Prolonging the incubation time from 18 hours to 20 hours for KCN has alleviated the inhibition. Other respiratory inhibitors such as carbofuran, paraquat dichloride, atrazine, NaN_3 and rotenone showed no effect on phenol-degrading activities and bacterial growth by both free and immobilised cells compared to control ($p>0.05$).

Key words: Immobilised cell, phenol degradation, pesticides, respiratory inhibitors, *Acinetobacter* sp.

Xenobiotics referred as chemical that are foreign to a biological system and may bring adverse effects to the biota. They often found as pollutants and present in several ecosystems, especially marine system, leading to environmental pollution and death of living organism as most of them are toxicants. Generally, phenolic compounds are pollutant that present most commonly in wastewater due to their extensive use in various industrial activities such as petrochemical, pulp, tanning, pharmaceutical and coal refining industries[1]. It has already being considered as a priority pollutant by the US EPA[2]. Phenol is a recalcitrant compound, which is toxic and carcinogenic to all forms of life, including microorganism and human[1]. Therefore, the removal of phenol and phenolic compounds from wastewater before discharging is crucial and need to be strictly regulated[3,4].

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Various methods have been surveyed to combat the xenobiotics since some of them are resistant to degradation. The methods involve both physicochemical and biological approaches, for example, ultra filtration, chemical dispersant, and bioremediation[5,6]. Among all methods available, bioremediation is the most preferable as it is affordable and causes less damage to the environment[7,8].

Bioremediation is an efficient way to remove pollutants by using microorganisms[9]. Phenol can be degraded by a wide variety of microorganisms, either single pure culture or bacterial consortia[2]. Moreover, phenol degradation can be carried out through free cell or Immobilised cell method. By comparing both methods, cell immobilisation showed better degradation as it can minimise cells loss and reduce the cost of regeneration[10]. Gellan gum is commonly used as encapsulating agent by aggregation of helical sequences to convert into polymer network of gellan gum in which it forms double helix network from disordered coil when it is cooled[11]. The advantages of gellan gum in cell immobilisation include high transparency, quick setting characteristics, high thermal and acid stability, good flavour release, adjustable gel elasticity and rigidity[12,13,14].

Nevertheless, there are some factors that constrain phenol degradation. Such factors include microbial acclimatisation to phenol, the presence of inhibitive metabolites, and environment conditions[7]. Pesticides and respiratory inhibitors such as carbofuran, paraquat dichloride, atrazine, potassium cyanide, sodium azide and rotenone are widely used in industrial activities[15,16,17,18,19.]

In a previous work, we reported that *Acinetobacter* sp. strain AQ5NOL 1 utilises phenol as a sole carbon source and showed the ability to degrade 100% phenol up to 1100 and 1900 mg/L by free and Immobilised cells, respectively[20,21]. Thus, a study was carried out to determine the effect of selected pesticides and respiratory inhibitors on phenoldegradation. This is due to both respiratory inhibitors and phenols that are harmful pollutants and often occur together. This study is important to favour the designs of effective bioremediation strategies.

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical grade and purchased either from Merck (Germany) or Sigma (USA).

Microorganism and culture condition

The phenol-degrading *Acinetobacter* sp. strain AQ5NOL 1 used in this study described previously was isolated from pesticide-polluted site at Johor, Malaysia by Ahmad *et al.*, (2011)[20]. Bacterial strains were cultured in mineral salt medium (MSM) containing (g/L): K_2HPO_4, 0.4; KH_2PO_4, 0.2; NaCl, 0.1; $MgSO_4$, 0.1; $MnSO_4.H_20$, 0.01; $Fe_2(SO_4).H_2O$, 0.01; $NaMoO_4.2H_2O$, 0.01; $(NH_4)_2SO_4$, 0.4 at pH 7.5. The MSM were supplemented with 0.5 g/L phenol as carbon sources.

Immobilised cells

In this study, gellan gum gel was selected as cell immobilisation matrix following the method described by Ahmad et al., (2012)[21]. Combination of 7.5% of gellan gum concentration, bead size of 3 mm diameter and bead number of 300 per 100 ml medium was used as the entrapment matrix. In this study, degradation of 0.5 g/L phenol by free and Immobilised cells was tested. Phenol media without the presence of bacteria in gellan gum beads were used as control. Phenol degradations were monitored via colorimetric assay for phenol using 4-aminoantipyrene as the reagent, respectively[22].

The effect of pesticides and respiratory inhibitors

The effect of pesticides and respiratory inhibitors on phenol degradation was determined and compared between free and Immobilised cells. In this experiment, MSM containing 0.5 g/L phenol was separately supplied with 1 ppm respiratory inhibitors consisting of carbofuran, paraquat dichloride, atrazine, potassium cyanide (KCN), sodium azide (NaN_3) and rotenone. Bacterial cultures in free and Immobilised cells were inoculated in a rotary shaker in room temperature at 150 rpm. As shown in Fig 1, degradation of 0.5 g/L phenol was analysed after incubation of 18 hours for free and Immobilised cells and 48 hours for free cells. At the same time, phenol degradation and bacterial growth were verified using 4-aminoantipyrene and CFU methods.

Effect of different KCN concentration

Respiratory inhibitorsuch as KCN (Table 1) that have an effect on 0.5 g/L phenol degradation in Immobilised cells were further investigated with different concentrations from 0 to 1 ppm. The degradation of phenol was verified in 2 hours interval until it was fully degraded.

Statistical analysis

All experiments were carried out in triplicates. The data shown in the corresponding figures are the mean values of the experiment and expressed as mean± standard deviation (STDEV).

RESULTS AND DISCUSSION

To determine the effect of phenol degradation, free and Immobilised cells were grown in liquid MSM medium (pH 7.5) at 30ºC in the presence 0.5 g/L phenol. Fig. 1 shows phenol degradation by both free and Immobilised cells. Results from this study showed that complete degradation of phenols in free and Immobilised cells occurred after 48 and 18 hours of incubation, respectively. Meanwhile, in the control beads, no significant degradation has been observed. Cell immobilisation is a promising approach for phenol biodegradation compared to free cells. In this study, Immobilised *Acinetobacter* sp. strain AQ5NOL 1 in gellan gum beads showed faster phenol degradation than in free cells. The entrapment of cells in gellan gum had become a promising method for microbial degradation of toxic substances and is being used in recent years[20]. The gellan gum was also recommended in fermentation processes due to its mechanical and thermal stability[23,24,25].

Industrial facilities without proper means to control runoff have contributed toxic chemicals such as pesticides and respiratory inhibitors. In agriculture, the application of pesticides such as carbofuran, paraquat dichloride, and atrazine have become inevitable to protect crop plants from diseases and pests but at the same time, pesticides pollution had also caused the increase concerns among the public[15,16,17]. Phenol pollution is often associated with agriculture industries involving pesticides[26]. Wastewater containing phenols may also contain pesticides, giving multicomponent composition of wastewaters[27]. Respiratory inhibitors such as KCN, NaN_3 and rotenone act on cytochrome oxidase enzyme and inhibit cytochrome oxidase activities via respiratory chain[28]. They are widely used in industrial activities[18,19]. Therefore, the strains used for biodegradation of phenols should not only be highly active to phenols, but also resistant to the residue of pollutants or possess different biodegradation mechanism. To determine the effect of pesticides and respiratory inhibitors on phenol degradation, free and Immobilised cells were grown in liquid MSM medium (pH 7.5) at 30ºC in the presence of different types of pesticides respiratory

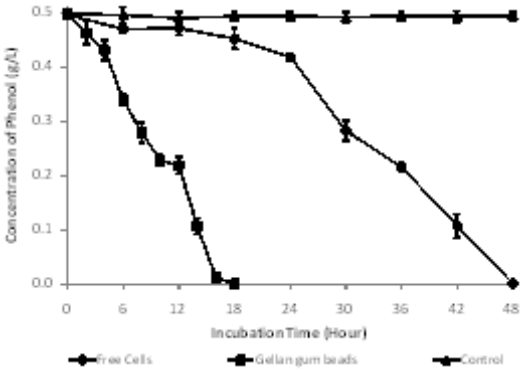

Fig. 1. Degradation of phenol at 0.5 g/L concentration by (●) free and (■) immobilized *Acinetobacter* sp. Strain AQ5NOL 1. Gellan gum beads without the presence of bacterial were used as (▲) control.

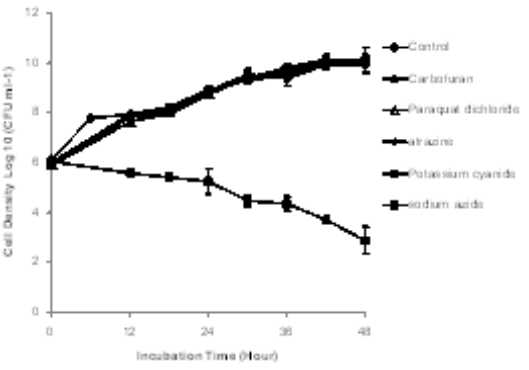

Fig. 2. Effect of different respiratory inhibitors and pesticides on bacterial growth by freely-suspended *Acinetobacter* sp. strain AQ5NOL 1 was determined using the colony count method in 48 hours incubation time.

Table 1. Comparison of the kinetic parameters of phenol degradation by free and immobilised *Acinetobacter* strain AQ5NOL 1 on different types of 1 ppm respiratory inhibitors and pesticides. Value are mean ±standard deviation (n=3).

Kinetics Parameters/Performance	Control	carbofuran,	paraquat dichloride	atrazine	KCN	NaN_3	rotenone
Free Cells (18 hrs)							
Maximum cell (Log_{10}(CFU))	8.221±0.147				5.395±0.199	8.108±0.115	8.222±0.092
Residual phenol (g/L)	0.453±0.019	0.472±0.015	0.476±0.015	0.466±0.030	0.446±0.009	0.466±0.009	0.425±0.033
Phenol degraded (g/L)	0.047±0.019	0.028±0.015	0.024±0.015	0.034±0.030	0.034±0.11	0.054±0.009	0.075±0.003
Phenol degradation rate (g/L/hr)	0.003±0.001	0.002±0.000	0.001±0.000	0.002±0.002	0.002±0.000	0.003±0.000	0.004±0.001
Percentage of phenol degradation (%)	9.360±5.107	5.600±3.005	4.733±3.036	6.827±6.007	6.774±2.27	10.715±1.884	14.905±6.630
Free Cells (48 hrs)							
Maximum cell (Log_{10}(CFU))	9.950±0.514				2.869±0.736	10.109±0.722	10.107±0.604
Residual phenol (g/L)	0.001±0.000	0.001±0.001	0.001±0.000	0.001±0.001	0.356±0.022	0.001±0.000	0.001±0.000
Phenol degraded (g/L)	0.499±0.000	0.499±0.001	0.499±0.000	0.499±0.001	0.144±0.021	0.499±0.000	0.499±0.003
Phenol degradation rate (g/L/hr)	0.010±0.000	0.010±0.000	0.010±0.000	0.010±0.000	0.003±0.000	0.010±0.000	0.010±0.001
Percentage of phenol degradation (%)	99.72±0.061	99.78±0.122	99.85±0.085	99.85±0.147	28.887±4.318	99.728±0.053	99.711±0.070
Gellan Gum (18 hrs)							
Residual phenol (g/L)	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000	0.263±0.044	0.002±0.000	0.001±0.001
Phenol degraded (g/L)	0.500±0.000	0.500±0.000	0.500±0.000	0.500±0.000	0.237±0.043	0.498±0.000	0.499±0.001
Phenol degradation rate (g/L/hr)	0.028±0.000	0.028±0.000	0.028±0.000	0.028±0.000	0.013±0.004	0.028±0.000	0.028±0.000
Percentage of phenol degradation (%)	99.99±0.043	99.99±0.122	99.99±0.085	99.99±0.140	47.310±8.718	99.643±0.095	99.842±0.171

* Phenol media without the presence of pesticides and respiratory inhibitors were used as control.

inhibitors. In this study, 1 ppm of pesticides such as carbofuran, paraquat dichloride, and atrazine and 1ppm of respiratory inhibitors such as potassium cyanide (KCN), sodium azide (NaN_3) and rotenone were used.

The effect of 1 ppm of different pesticides and respiratory inhibitors on phenol biodegradation by freely-suspended and Immobilised *Acinetobacter* sp. strain AQ5NOL 1 was given in Table 1. From the results obtained, only KCN inhibited phenol-degrading activities by freely-suspended cells after 48 hours. Phenol degradation and bacterial growth were significantly different compared to the control ($p<0.05$) in the presence of KCN (Fig 2). Other pesticides (carbofuran, paraquat dichloride, and atrazine) and respiratory inhibitors (NaN_3 and rotenone) have not showing any effect ($p>0.05$). KCN gave inhibition on phenol degradation, only 28.887% was degraded within 48 hours. However, in Immobilised cells, 47.310 % phenol was degraded within 18 hours. NaN_3 and rotenone have not showing any significant effect on phenol-degrading activities ($p>0.05$). Moreover, the Immobilised cells displayed better phenol-removing efficiencies compared to freely suspended cells in 18 hours. In Immobilised cells, only KCN inhibited phenol-degrading activities. According to a previous report, the free suspension (*Klebsiella oxytoca)* systems revealed that the cell viability was highly affected by initial KCN concentration, while the Immobilised cell systems could tolerate higher levels of KCN concentration[29]. Cyanide found major uses in industrial activities involving metal plating, mining, aluminum electrolysis, coal coking, ore leaching, coal gasification, pharmaceuticals, plastics and synthetic fibres[30,31,32]. Cyanide is highly toxic to living organisms related to its physicochemical specification. It is involved in inactivating the respiratory system by tightly binding to terminal oxidase[31,33]. Cyanide made the cells of an organism to be unable to use oxygen, primarily through the inhibition of cytochrome c oxidase[34,35]. According to previous study on the methaogenic degradation on phenolic compounds, cyanide can affects a phenolic-degrading consortium by causing an accumulation in the end products of non-methanogenic fermentation[36]. Therefore, different concentrations of KCN on phenol degradation were studied to verify the inhibition of phenol degrading activity.

The effect of different KCN concentrations ranging from 0.1 to 1.0 ppm on phenol degradation by Immobilised *Acinetobacter* sp. strain AQ5NOL 1 after 18 hours is shown in Fig 3. KCN concentration from 0.1 to 0.7 ppm does not have any effects on phenol-degrading activities ($p>0.05$). However, phenol-degrading activities were affected when KCN concentration has exceeded 0.8 ppm ($p<0.05$). In previous studies, KCN showed inhibitions on hydroxylases activity such as hydroquinone hydroxylase[37], purine hydroxylase[38], m-cresol hydroxylases, m-hydroxybenzyl alcohol hydroxylase[39] and 4-hydroxycinnamic acid hydroxylase[40]. This entire problem can be overcome by prolonging the incubation time from 18 hours to 20 hours (Fig 4). A lag phase was observed at 0.8 to 1.0 ppm concentration of KCN before sharp a decrease in

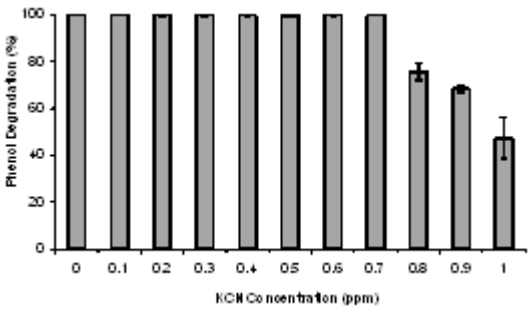

Fig. 3. Effect of KCN on phenol degradation of immobilised *Acinetobacter* sp. strain AQ5NOL 1

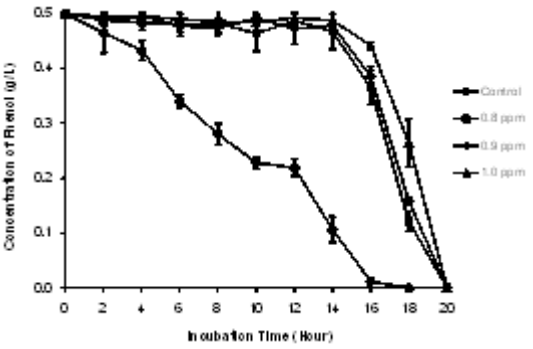

Fig. 4. Effect of KCN (0.8, 0.9 and 1.0 ppm) on phenol degradation of immobilised *Acinetobacter* sp. strain AQ5NOL 1.

phenol concentration. The inhibition of 0.8, 0.9 and 1.0 ppm KCN concentration on phenol-degrading activities was completed at 20, respectively.

CONCLUSION

Gellan gum Immobilised *Acinetobacter* sp. strain AQ5NOL 1 provided a unique feature in the growth of bacteria and degradation of phenol in the presence of 1 ppm carbofuran, paraquat dichloride, atrazine, KCN, NaN_3 and rotenone. However, the sensitivity of this strain to KCN indicated that future isolation of phenol-degrading microbes should be taken into account of heavy metals' tolerance as phenolic wastes often contain metallic pollutants.

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