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RESEARCH ARTICLE

Phenol degradation by *Acinetobacter* sp. in the presence of heavy metals

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Abstract: The purpose of this study was to investigate the ability of *Acinetobacter* sp. strain AQ5NOL 1 immobilised in gellan gum beads to degrade phenol in the presence of heavy metals. Seven different heavy metals, namely, As^{5+}, Cu^{2+}, Cd^{2+}, Ni^{2+}, Cr^{6+}, Pb^{2+}, and Hg^{2+} at 1 ppm were tested. Results of the study showed that degradation of phenol by free cells was inhibited by Hg^{2+}, Cu^{2+} and Cr^{6+} after 48 hours of incubation by 97.91 %, 77.58 % and 75.26 %, respectively. Only Hg^{2+} and Cr^{6+} inhibited phenol degradation by immobilised *Acinetobacter* cells in 18 hours by 67.55 % and 53.19 %. Phenol degradation by immobilised cells was affected when Cr^{6+} and Hg^{2+} concentrations exceeded 0.5 and 0.1 ppm, respectively. However, inhibitory effects of heavy metals can be overcome by prolonging the incubation time for immobilised *Acinetobacter* sp. strain AQ5NOL 1 from 18 hours to 24 and 30 hours for Cr^{6+} (46.80 %) and Hg^{2+} (21.40 %), respectively.

Keywords: *Acinetobacter* sp., biodegradation, heavy metals, immobilised cells.

INTRODUCTION

Heavy metals can be toxic to microorganisms even at low concentrations but several microorganisms have been shown to have an exceptional ability to adapt and colonise noxious metal-polluted environments (Yusuf *et al*., 2016). Certain bacteria have developed capabilities to protect themselves from heavy metal toxicity by various mechanisms (Shukor *et al*., 2010; Halmi *et al*., 2013; Karamba *et al*., 2015). Bioremediation is effective in degrading pollutants and thus able to get rid of unwanted substances from industrial wastes such as phenolic compounds, dye, crude oil and hydrocarbons (Suhaila *et al*., 2013; Ahmad *et al*., 2014; Chander *et al*., 2014; Azri *et al*., 2015).

In a previous study (Ahmad *et al.*, 2011; 2012), it was reported that *Acinetobacter* sp. strain AQ5NOL 1 utilised phenol as the sole carbon source with optimum temperatures ranging between 25 to 35 °C. It has the ability to degrade 100 % phenol up to 1100 mg/L and 1900 mg/L by free and immobilised cells, respectively in 10 days. As both heavy metals and phenol are harmful pollutants and often occur together, this study was carried out to determine the effects of selected heavy metals on phenol degradation by the bacterium *Acinetobacter* sp.. This investigation would be significant in designing effective bioremediation strategies.

METHODOLOGY

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 was isolated in Malaysia by Ahmad *et al*. (2011) as previously described. The bacterium was 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; $MnSO_4.H_2O$, 0.01; $Fe_2(SO_4).H_2O$, 0.01;

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$NaMoO_4.2H_2O$, 0.01; $(NH_4)_2SO_4$, 0.4 at pH 7.5. The MSM was supplemented with 0.5 g/L phenol as the sole carbon source.

Immobilised cells

In this study, gellan gum gel was selected as the cell immobilisation matrix following the method described by Ahmad *et al*. (2012). A 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 (3.5 g/L of bacteria) was tested. Gellan gum beads without bacteria in MSM medium with phenol was used as the control. Phenol degradation was monitored by colourimetric assay for phenol using 4-aminoantipyrene as the reagent (APHA, 1998).

The effects of heavy metals

The effect of heavy metals on phenol degradation by the bacterium *Acinetobacter* sp. strain AQ5NOL 1 was determined using both free and immobilised cells. In this experiment, MSM containing 0.5 g/L phenol was separately supplied with different heavy metals [arsenic (As^{5+}), chromium (Cr^{2+}), cadmium (Cd^{2+}), cuprum (Cu^{2+}), nickel (Ni^{2+}), lead (Pb^{2+}), and mercury (Hg^{2+})] each at 1 ppm concentration. Bacterial cultures in free and immobilised cells (3.5 g/L) were incubated in a rotary shaker at room temperature at 150 rpm. Based on this study (Figure 1), degradation of 0.5 g/L phenol was analysed after incubation for 18 hrs for immobilised cells and 48 hrs for free cells, because these are the optimal incubation periods for 100 % removal of phenol. Simultaneously, phenol degradation and bacterial growth were verified using 4-aminoantipyrene and colony forming units methods, respectively.

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

Cell immobilisation is a promising approach in phenol biodegradation compared to free cells. In this study, immobilised *Acinetobacter* sp. strain AQ5NOL 1 in gellan gum beads showed faster phenol degradation than free cells at 0.5 g/L of phenol (Figure 1). Complete degradation of phenol by free and immobilised cells occurred after 48 and 18 hours of incubation, respectively, while gellan gum beads without cells showed no phenol degradation. Gellan gum gel was selected as the immobilisation matrix based on its established superior characteristics compared to carrageenan, agar, and alginate (Sanderson *et al*., 1989; Nawawi *et al*., 2015; Yusuf *et al*., 2015).

Heavy metals affect phenol degradation by impeding bacterial growth and/or inhibiting enzymes that play major roles in phenol degradation (Nair *et al*., 2008; Arif *et al*., 2013; Norazah *et al*., 2015). Heavy metals directly affect the membrane structure by disturbing the electron transport chain (Hall, 2002; Russak *et al*., 2008). Freely-suspended cells are exposed directly to the heavy metals and easily react, thus slowing down the rate of degradation by disturbing the membrane structure. The effect of different heavy metals (1 ppm) on phenol biodegradation by freely-suspended and immobilised *Acinetobacter* sp. strain AQ5NOL 1 is given in Table 1. The results revealed that Cr^{6+}, Cd^{2+}, and Hg^{2+} inhibited phenol-degrading activity by inhibiting bacterial growth at 5.5, 5.3 and 0.0 Log_{10}(CFU), respectively compared to the control 8.2 Log_{10}(CFU) ($p < 0.05$). However, this strain has been shown to have an exceptional ability to adapt and protect itself from Cu^{2+}, As^{5+}, Ni^{2+}, and Pb^{2+} ($p > 0.05$) (Nawawi *et al*., 2015). Hg^{2+} gave the highest inhibition on phenol degradation with only 2.09 % phenol degraded after 48 hours followed by Cd^{2+} (24.74 %) and Cr^{6+} (22.42 %). However with immobilised cells, only Hg^{2+} and Cr^{2+} inhibited phenol-degrading activities ($p < 0.05$) up to 32.45 % and 46.81 % phenol being

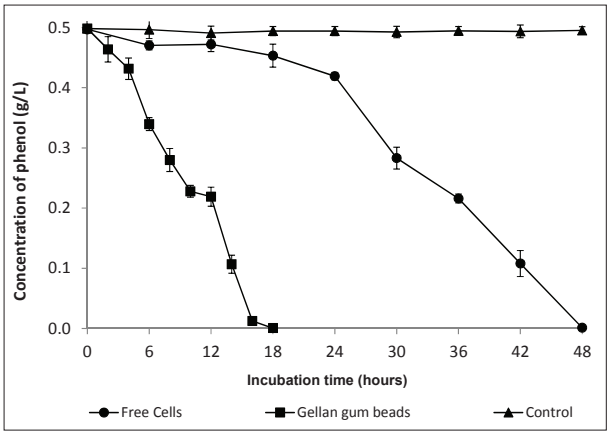

Figure 1: Degradation of phenol at 0.5 g/L by free (●) and immobilised (■) *Acinetobacter* sp. strain AQ5NOL 1. Gellan gum beads without the presence of bacteria were used as control (▲).

degraded, respectively after 18 hours. Immobilised cells displayed better phenol degrading efficiencies than freely suspended cells after 18 hours. The immobilised cells were protected by the polymeric gel and less exposed to heavy metals, thus reducing the chance of heavy metals interrupting the degradation process (Chung *et al*., 2003). The application of immobilised cells in bioremediation exhibits many advantages over free cells, including the stability of active cells, recovery of cells, and reusability of the immobilised system (Yun *et al*., 2009; Ahmad *et al*.,

Table 1: Comparison of the kinetic parameters of phenol degradation by free and immobilised *Acinetobacter* strain AQ5NOL 1 in the presence of various heavy metals at 1 ppm. Values are mean ± standard deviation (n = 3)

Kinetic parameters/ performance	Heavy metals (1 ppm)							
	Control	As^{5+}	Cu^{2+}	Cd^{2+}	Ni^{2+}	Cr^{6+}	Pb^{2+}	Hg^{2+}
Free cells (18 hrs)								
Maximum cell [Log_{10}(CFU)]	8.2 ± 0.15	8.1 ± 0.04	8.1 ± 0.09	5.3 ± 0.28	8.1 ± 0.13	5.5 ± 0.18	8.2 ± 0.10	0.0 ± 0.00
Phenol degraded (g/mL)	47.00 ± 0.02	26.00 ± 0.01	62.00 ± 0.02	30.00 ± 0.01	71.00 ± 0.03	32.00 ± 0.03	39.00 ± 0.04	3.00 ± 0.00
Phenol degradation rate (g/mL/hr)	2.61 ± 0.00	1.44 ± 0.00	3.44 ± 0.00	1.67 ± 0.00	3.94 ± 0.00	1.78 ± 0.00	2.17 ± 0.00	0.17 ± 0.00
Percentage of phenol degradation (%)	9.40 ± 5.11	5.20 ± 0.55	12.40 ± 5.0	6.00 ± 2.08	14.20 ± 8.33	6.40 ± 8.19	7.80 ± 1.80	0.60 ± 0.22
Free cells (48 hrs)								
Maximum cell [Log_{10}(CFU)]	10.0 ± 0.51	9.8 ± 0.66	9.9 ± 0.51	4.2 ± 0.08	10.0 ± 0.44	3.3 ± 0.12	10.0 ± 0.59	0.0 ± 0.00
Phenol degraded (g/mL)	499.00 ± 0.00	478.00 ± 0.00	478.00 ± 0.00	124.00 ± 0.00	478.00 ± 0.00	112.00 ± 0.02	468.00 ± 0.00	10.00 ± 0.02
Phenol degradation rate (g/mL/hr)	10.40 ± 0.00	9.96 ± 0.00	9.96 ± 0.00	2.58 ± 0.00	9.96 ± 0.00	2.33 ± 0.00	9.75 ± 0.00	0.21 ± 0.00
Percentage of phenol degradation (%)	99.80 ± 0.06	95.60 ± 0.71	95.60 ± 0.26	24.80 ± 5.74	95.60 ± 0.88	22.40 ± 4.74	93.60 ± 0.86	2.00 ± 1.73
Gellan gum (18 hrs)								
Phenol degraded (g/mL)	500.00 ± 0.00	500.00 ± 0.00	499.00 ± 0.00	499.00 ± 0.00	499.00 ± 0.00	234.00 ± 0.02	499.00 ± 0.00	162.00 ± 0.02
Phenol degradation rate (g/mL/hr)	27.78 ± 0.00	27.78 ± 0.00	27.72 ± 0.00	27.72 ± 0.00	27.72 ± 0.00	13.00 ± 0.00	27.72 ± 0.00	9.00 ± 0.00
Percentage of phenol degradation (%)	100.00 ± 0.04	100.00 ± 0.07	99.80 ± 0.05	99.80 ± 0.09	99.80 ± 0.03	46.80 ± 4.84	99.80 ± 0.11	32.40 ± 3.58

Note: Phenol media without the presence of heavy metals were used as control
Phenol degradation expressed as a percentage of 0.5 g phenol

2012). Since Cr^{6+} and Hg^{2+} inhibited phenol-degrading activities of immobilised cells, investigations were carried out to find the effect of different concentrations of Cr^{6+} and Hg^{2+} (0.1 to 1.0 ppm) on phenol degradation after 18 hours.

In this experiment, at all concentrations studied, a lag phase was observed at the beginning before phenol degradation was observed (Figure 2). The completion of phenol degradation at 0.6 and 0.7 ppm Cr^{6+} concentrations occurred at 20 and 22 hours, respectively. At 0.8, 0.9 and 1.0 ppm concentrations complete degradation occurred at 24 hours. The results indicate that immobilisation of cells in gellan gum beads protected the *Acinetobacter* sp. strain AQ5NOL 1 from chromium toxicity, with higher concentrations requiring an additional incubation period for complete degradation. In this case, the incubation time needed to be increased from 18 to 24 hours. The presence of a lag phase indicates that cells were adjusting to the toxicity of chromium through metabolic adaptation before phenol-degrading metabolism started. Gellan gum offers protection to heavy metal toxicity through various ways (Yusuf *et al*., 2016). The gellan gum itself can adsorb some of the heavy metals, reducing the bioavailability of the toxic heavy metals (Nawawi *et al*., 2015; Ibrahim *et al*., 2016). In addition, cells on the outer layer exposed to heavy metals adsorb the heavy metals, and become a protective layer to cells immobilised deep in the matrix (Ahmad *et al*., 2012). A summary of the phenol degradation kinetics is shown in Table 2.

Cr^{6+} is a unique transition metal ion, which has been established to be biologically significant on living organisms at all levels. However, at high concentrations, it inhibits the biodegradation of organic pollutants (Kourtev *et al*., 2006; Al-baldawi *et al*., 2015) including phenol (Al-Defiary & Reddy, 2014). In the environment, pollution due to Cr^{6+} and its compounds is widespread because of their application in industry as dyes, pigments, and refractory material (Zakaria *et al*., 2012). It appears that this source of pollution is the same as phenol pollution. Additionally, chemical pollution due to phenol and Cr^{2+} in the ocean has been increasing in recent years in the China Sea (Zhang *et al*., 2007). According to a previous study, Cr^{2+} is more toxic on Gram-negative bacteria than Gram-positive bacteria at 1 ppm (Ross *et al*., 1981) and the toxicty of chromium to *Acinetobacter* sp. strain AQ5NOL 1, a Gram negative bacterium utilised in this study affirms this observation.

Hg^{2+} is considered the most toxic non-radioactive metal in the environment and is toxic in any form

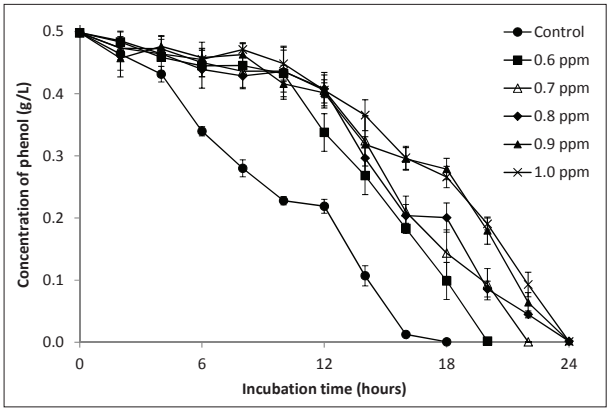

Figure 2: The effect of 0.6 to 1.0 ppm of chromium on phenol biodegradation by immobilised *Acinetobacter* sp. strain AQ5NOL 1

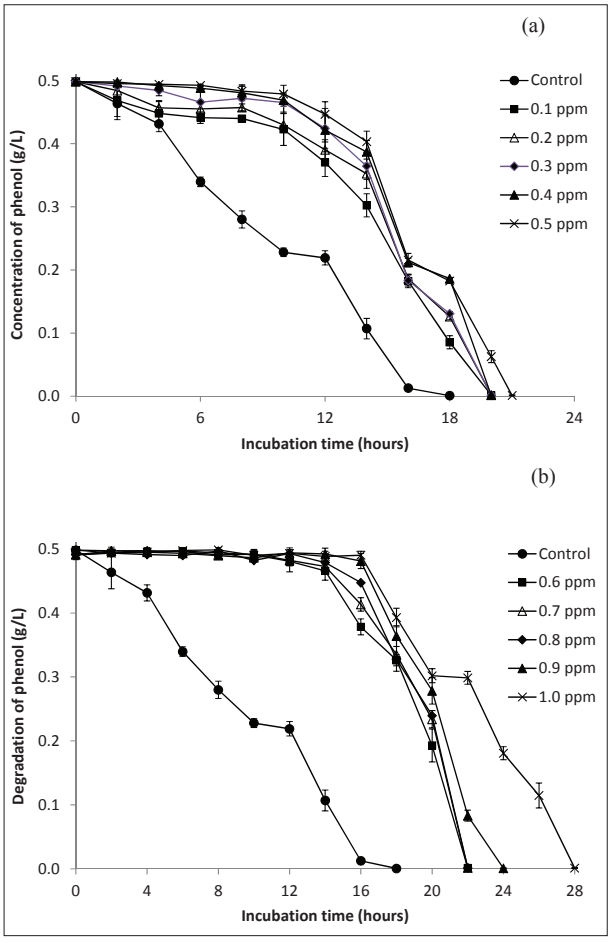

Figure 3: The effect of (a) 0.1 to 0.5 ppm and (b) 0.6 to 1.0 ppm of mercury on phenol biodegradation by immobilised *Acinetobacter* sp. strain AQ5NOL 1

Table 2: The effect of different concentrations of chromium and mercury on phenol biodegradation by immobilised *Acinetobacter* sp. strain AQ5NOL 1 at 18 hours. Values are mean ± standard deviation (n = 3)

Kinetic parameters/ performance	Concentration (ppm)										
	Control	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Cr											
Phenol degraded (g/mL)	499.00 ± 0.00	498.00 ± 0.00	498.00 ± 0.00	499.00 ± 0.00	499.00 ± 0.00	499.00 ± 0.00	401.00 ± 0.04	357.00 ± 0.05	299.00 ± 0.03	221.00 ± 0.04	234.00 ± 0.02
Phenol degradation rate (g/mL/hr)	27.72 ± 0.00	27.67 ± 0.00	27.67 ± 0.00	27.72 ± 0.00	27.72 ± 0.00	27.72 ± 0.00	22.28 ± 0.00	19.83 ± 0.00	16.61 ± 0.00	12.28 ± 0.00	13.00 ± 0.00
Percentage of phenol degradation (%)	99.80 ± 0.05	99.60 ± 0.20	99.60 ± 0.09	99.80 ± 0.06	99.80 ± 0.17	99.80 ± 0.10	80.20 ± 5.97	71.40 ± 7.48	59.80 ± 4.72	44.20 ± 6.17	46.80 ± 3.42
Hg											
Phenol degraded (g/mL)	500.00 ± 0.00	415.00 ± 0.02	374.00 ± 0.01	369.00 ± 0.00	314.00 ± 0.00	318.00 ± 0.00	174.00 ± 0.01	165.00 ± 0.04	172.00 ± 0.01	136.00 ± 0.02	107.00 ± 0.02
Phenol degradation rate (g/mL/hr)	27.78 ± 0.00	23.06 ± 0.00	20.78 ± 0.00	20.50 ± 0.00	17.44 ± 0.00	17.67 ± 0.00	9.67 ± 0.00	9.17 ± 0.00	9.56 ± 0.00	7.56 ± 0.00	5.94 ± 0.00
Percentage of phenol degradation (%)	100 ± 0.03	83.00 ± 2.08	74.80 ± 0.94	73.80 ± 0.69	62.80 ± 0.39	63.60 ± 0.76	34.80 ± 1.73	33.00 ± 5.24	34.40 ± 0.90	27.20 ± 3.22	21.40 ± 2.92

Note: Phenol media without the presence of heavy metals were used as the control
Phenol degradation expressed as a percentage of 0.5 g phenol

(Abdel-Salam *et al*., 2010). In Malaysia, the main sources of Hg^{2+} in river waters are urban and agricultural run-offs, effluent from industries, domestic discharges, sewage treatment plants, earthwork, constructions, and pig farms (DOE, 2002). This heavy metal also appears in phenol-polluted environments. Hence, the effect of Hg^{2+} on phenol degradation was studied. In this study, the presence of 1 ppm Hg^{2+} inhibited 100 % of bacterial growth and phenol degradation by free cells, and more than 70 % of phenol-degrading activities by immobilised cells. Hg^{2+} concentrations ranging from 0.1 to 1.0 ppm affected phenol degradation ($p < 0.05$) (Table 2). Above 0.5 ppm, the metal inhibited more than 60 % of phenol-degrading activity, and this can be classified as very detrimental (Ahmad *et al*., 2015). But, the inhibitory effect can be overcome by prolonging the incubation time for immobilised *Acinetobacter* sp. strain AQ5NOL 1 from 18 to 28 hours (Figure 3). The inhibition of phenol degrading activities by 0.1 to 0.4 ppm of Hg^{2+} was completed in 20 hours (Figure 3a), followed by 0.5 ppm at 21 hours, 0.6 to 0.8 ppm at 22 hours, 0.9 ppm at 24 hours, and 1.0 ppm at 28 hours (Figure 3b). In previous studies, Hg^{2+} has shown an inhibition of degradation of phenolic compounds by inhibiting hydroxylases activity such as 3-hydroxybenzoic acid-6-hydroxylase (Rajasekharan *et al*., 1990) and aryl 2,4-dichlorophenol hydroxylase (Radjendirane *et al*., 1991).

CONCLUSION

Gellan gum-immobilised *Acinetobacter* sp. strain AQ5NOL 1 provided excellent protection to the inhibitory effects of 1 ppm As, Cu, Cd, Ni, Cr, Pb and Hg on the growth of the bacterium and the degradation of phenol by this bacterium. The inhibitory effects of chromium and mercury on immobilised cells could be overcome by prolonging the incubation period. The range of concentrations of heavy metals utilised in this study is very similar to the concentration range of heavy metals detected in the environment according to several studies and reports (Shukor *et al*., 2008; DOE, 2010). The results indicate that the immobilised bacterium utilised in this study can be an efficient candidate for phenol bioremediation in sites co-contaminated with heavy metals.

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