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Isolation, Identification and Characterization of Elevated Phenol Degrading *Acinetobacter* sp. Strain AQ5NOL 1

[1]Siti Aqlima Ahmad, [1]Mohd Arif Syed[†], [1]Noorliza Mat Arif, [1]Mohd Yunus Abdul Shukor and [2]Nor Aripin Shamaan.

[1]Department of Biochemistry, Faculty of Biotechnology and Molecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia;
[2]Faculty of Medicine and Health Sciences, Universiti Sains Islam Malaysia, 13th Floor, Menara B, Persiaran MPAJ, Jalan Pandan Utama, Pandan Indah, 55100 Kuala Lumpur, Malaysia.

Abstract: The increasing phenol and phenolic wastes necessitates the screening of bacteria that are able to degrade phenol. 115 bacterial isolates from several industrial sites and farms in Malaysia were screened for phenol degrading activity in minimal salt media (MSM) containing 0.5 gL^{-1} phenol. Thirty seven bacterial isolates exhibited phenol degrading activity and of this total, 6 isolates showed high phenol activity after 8 days of incubation. The isolate with the highest phenol degrading activity was subsequently identified as *Acinetobacter* s*p.* Strain AQ5NOL 1 based on Biolog™ GN plates and partial 16S rDNA molecular phylogeny. The optimum conditions for achieving high phenol degradation were 0.04% (w/v) $(NH_4)_2SO_4$, 0.01% (w/v) NaCl, pH 7, and temperature of 30°C. *Acinetobacter* s*p.* Strain AQ5NOL 1 was found to degrade phenol of up to 1500 mgL^{-1} concentrations under the optimized conditions. The isolation of *Acinetobacter* sp Strain AQ5NOL 1 provides an alternative for the bioremediation of phenol and phenolic wastes.

Key words: Isolation; Characterization; Elevated phenol degrading activity; *Acinetobacter* sp.

INTRODUCTION

Phenol and phenolic wastes from industrial effluents is becoming a growing concern in Malaysia as it heads toward industrialization (Idris and Saed, 2002; Chan and Lim, 2006). In tandem with the increase in phenolic wastes generated from industries, the Department of Environment, Malaysia (2006) reported that the benchmark for phenol of 0.002 mgL^{-1} in raw drinking water has been exceeded in the municipal water supply, agricultural areas, landfills, golf courses, ex-mining and industrial areas. The unsafe levels of phenol may pose a threat to community health (Hooived *et al.*, 1998).

Phenol is resistant to most biological processes because it is toxic to bacteria even at low concentration (Yang and Lee, 2007; Lin *et al.*, 2007). Numerous methods have been developed to treat phenols in wastewater including biodegradation (Adav *et al.*, 2007; Wang *et al.*, 2007), membrane separation (Kujawski *et al.*, 2004), adsorption (Rengaraj *et al.*, 2002; Roostaei and Tezel, 2004), oxidation (Idris and Saed, 2002) and extraction by liquid membrane (Lin *et al.*, 2007). The physico-chemical methods have their own limitations viz. reaction inefficiency, high energy consumption, production of sludge containing iron, and insufficient capacity (Chen *et al.*, 2007). However, biodegradation as a technology for decontaminating phenols is gaining attention due to its eco-friendly characteristics and cost-effectiveness. For biodegradation of phenol to be feasible, screening for microorganisms capable of degrading phenols needs to be done first.

Although there are numerous reports on bacteria with low phenol degrading activity, those describing high phenol degrading activity are generally lacking (Al-Sayed *et al.*, 2003). The first report of a bacteria able to degrade 300 mgL^{-1} phenol was described by Tibbles and Baecker (1989). This was followed by Adav *et al.*, (2007) who reported that strain ATCC 11171 (DQ 831531) is able to degrade phenol at concentrations of up to 1000 mgL^{-1} while Wang *et al.*, (2007) reported that *Acinetobacter* sp. strain RD12 (AY 673994) is able to degrade phenol at 1100 mgL^{-1} .

Corresponding Author: Professor Dr Mohd Arif Syed, Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
E-mail:: marifsyed@gmail.com

In this study, we report the isolation and characterization of a bacterial strain that is able to degrade elevated concentrations of phenol. This bacterial strain was subsequently identified as *Acinetobacter* sp. strain AQ5NOL 1 and is able to degrade phenol at a concentration of more than 1500 mgL^{-1}. The findings enable the utilization of this strain in the bioremediation of phenol and phenolic wastes in industrial effluents.

MATERIALS AND METHODS

All chemicals used were of analytical grade commercially available.

Screening and Isolation of Phenol Degrading Bacteria:

Water and soil samples were collected from several locations in Malaysia involved in the printing, manufacturing, dyeing, textile, electroplating, meat-processing, pharmaceutical and leather tanning activities, and several farms.

Water (5 mL) and soil (3 g) samples were mixed in 10 mL sterile nutrient broth containing peptone (3.0 gL^{-1}) and beef extract (5.0 gL^{-1}) and incubated at 25°C on a shaking incubator at 150 rpm for 24 h. Bacterial cultures were isolated by repeated culturing in mineral salt medium (MSM) containing in gL^{-1}: 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 in a 250 mL conical flask (Bai *et al.,* 2007) and supplemented with filter-sterilized phenol (0.5 gL^{-1}) as a carbon source. The isolates were incubated at 25°C with shaking at 150 rpm. After five cycles of culturing, serial dilutions of the cultures were prepared and streaked onto mineral medium agar plates supplemented with phenol and incubated at 25°C for 3 days. Isolates exhibiting distinct colonies were further purified by repeated subculturing into basal salt medium and solidified basal salt medium.

15 mL of the selected bacterial cultures was inoculated on 150 mL MSM containing 0.5 gL^{-1} phenol and incubated in a shaking incubator at 25°C at 150 rpm. Phenol degradation was monitored daily using 4-aminoantipyrine following the method of the American Public Health Association (1998). Isolates showing high phenol degradation rates were selected and re-inoculated into media containing phenol at concentrations of 100 - 1300 mgL^{-1}. The isolate with the highest phenol degrading activity was then identified.

Identification of Phenol-degrading Bacterium:

Identification of the bacterial isolate exhibiting the highest phenol degrading activity was carried out using its cultural and morphological characteristics on Nutrient agar, Gram staining and Biolog™ Identification System followed by 16S rRNA sequence analysis.

Genomic DNA Isolation and Sequencing of 16S rRNA:

Extraction of genomic DNA from bacterial isolate SA28a(i) was carried out using DNeasyR Blood and Tissue Kit supplied by Qiagen according to the manufacturers' recommendation. PCR amplification was performed using BiometraR T-gradient thermocycler. The PCR mixture contained 0.5 µL of deoxynucleotide triphosphates, 2 µL of PCR buffer, 2 µL of $MgCl_2$, 0.5 µL of 100 µM forward and Reverse primers, 0.5 µL of Taq DNA polymerase, 1 µL of template DNA and 43 µL of nuclease-free water. The 16S rDNA gene from the genomic DNA was amplified by PCR using the following forward and reverse primers of 16S rDNA respectively; 5'-AGAGTTTGATCATGGCTCAG-3' and 5'-ACGGTTACCTTGTTACGACTT-3'. PCR was performed under the following conditions: initial denaturation at 94°C for 4 sec, followed by 94°C for 30 sec, 56°C for 30 sec, and 2 steps of 34 cycles of 72°C for 4 sec and a final extension at 72°C for 4 min. Bases were compared with the database in GenBank at http://www.ncbi.nlm.nih.gov/BLAST/.

Phylogenetic Analysis:

The Phylogenetic position of the phenol degrading bacteria isolated in this study was determined by sequencing analysis of PCR-amplified bacterial small subunit (16S) rRNA gene. The nucleotide sequence from the gene was aligned using CLUSTAL W, version 1.6 (Thompson *et al.*, 1994). A multiple alignment of 20 16S rRNA gene sequences that closely matches isolate SA28a(i) were retrieved from the GenBank. Construction of the phylogenetic tree was carried out using PHYLIP, version 3.573 (J.Q. Felsenstein, PHYLIP—phylogeny inference package, version 3.573, Department of

Genetics, University of Washington, Seattle, WA. (http://evolution.genetics.washington.edu/phylip.html), with *Bacillus subtilis* as the outgroup in the cladogram. DNADIST algorithm program was used to compute the evolutionary distance matrices for the neighbor joining/UPGMA method. The model of nucleotide substitution is those of LogDet. The neighbor joining method of Saitou and Nei (1987) was used to deduce the phylogenetic tree. Bootstrap analyses with 1000 re-samplings were performed with the SEQBOOT program in the PHYLIP package to obtain confidence estimates for the phylogenetic tree topologies (Felsenstein, 1985). Majority rule (50%) consensus trees were constructed using the CONSENSE program (Margush and McMorris, 1981) and the tree was viewed using TreeView (Page, 1996).

Characterization of Bacterial Growth and Phenol Degradation:

In all the experiments, 15 mL bacterial isolate was inoculated onto 150 mL MSM containing 0.5 gL^{-1} phenol and incubated in a shaker incubator at 150 rpm at 25°C for over 3 days. Bacterial growth was monitored daily by colony count and phenol degrading activity by the colorimetric assay for phenol using 4-aminoantipyrene as the reagent. The temperature, pH, salinity, nitrogen source and phenol concentration were varied accordingly in the optimization experiments; temperature range of 10 – 55°C, pH range of 4.0 – 9.0 in 50 mM acetate, phosphate and Tris-HCl buffers, salinity range set at 0 - 0.30 gL^{-1} NaCl, nitrogen sources each at 0.4 gL^{-1}; $(NH_4)_2SO_4$, NH_4Cl, $NaNO_3$, proline, cysteine, leucine, glycine, phenylalanine, alanine and histidine, and phenol concentration range of 0.3 – 2.0 gL^{-1}, respectively.

Statistical Analysis:

The data obtained were analysed statistically using One-way ANOVA.

Results:

Screening and Isolation of Phenol Degrading Bacteria:

Out of a total of 115 soil and water samples, 37 bacterial isolates were found to exhibit phenol degrading activity. From these, six were found to exhibit high phenol degrading activity (Figure 1). The isolates were tagged accordingly, with isolate SA28a(i) showing the highest phenol degrading activity followed by isolate N72, H8, SA28a(ii), SA15a and SA35, respectively. The bacterial isolates were then inoculated onto MSM containing different phenol concentrations and left for 3 days (Table 1). Isolate SA28a(i) exhibited phenol degrading activity at 1300 mgL^{-1} as compared with the others and subsequently selected for further study.

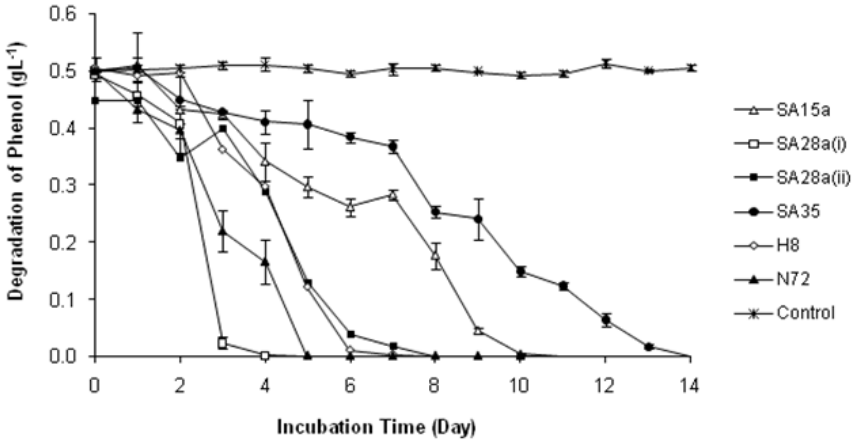

Fig. 1: Bacterial isolates with phenol degrading activity incubated in minimal salt media over 14 days at 25°C in a shaking incubator. Control contained no bacteria. Values shown represent the mean ± SEM, n=3.

Identification of the Phenol-degrading Bacterium:

Isolate SA28a(i) was found to be Gram-negative and aerobic cocci-shaped bacterium. The isolate could utilize 39 types of carbon substrates from the 95 tested. The identification system used showed

that isolate SA28a(i) is similar to four genuses of *Acinetobacter* (0.302 similarity). A moderate bootstrap value (39%) links isolate SA28a(i) to *Acinetobacter sp.* PRGB16 [EF195346], indicating a moderate phylogenetic relationship (Figure 2). The strain is grouped in the clade harboring different species of *Acinetobacter,* particularly *Acinetobacter sp.* PRGB 15 [EF195345]. Thus, isolate SA28a(i) is identified tentatively as *Acinetobacter* sp. strain AQ5NOL 1.

Table 1: Degradation of phenol by bacterial isolates in different concentration of phenol. [(+) Phenol degrading activity (-) No activity].

Isolates	Phenol concentration (mgL^{-1})							
	100	300	500	700	800	900	1100	1300
SA15	+	+	+	-	-	-	-	-
SA28a(i)	+	+	+	+	+	+	+	+
SA28a(ii)	+	+	+	+	+	+	-	-
SA35	+	+	+	-	-	-	-	-
H8	+	+	+	-	-	-	-	-
N72	+	+	+	+	+	+	+	-

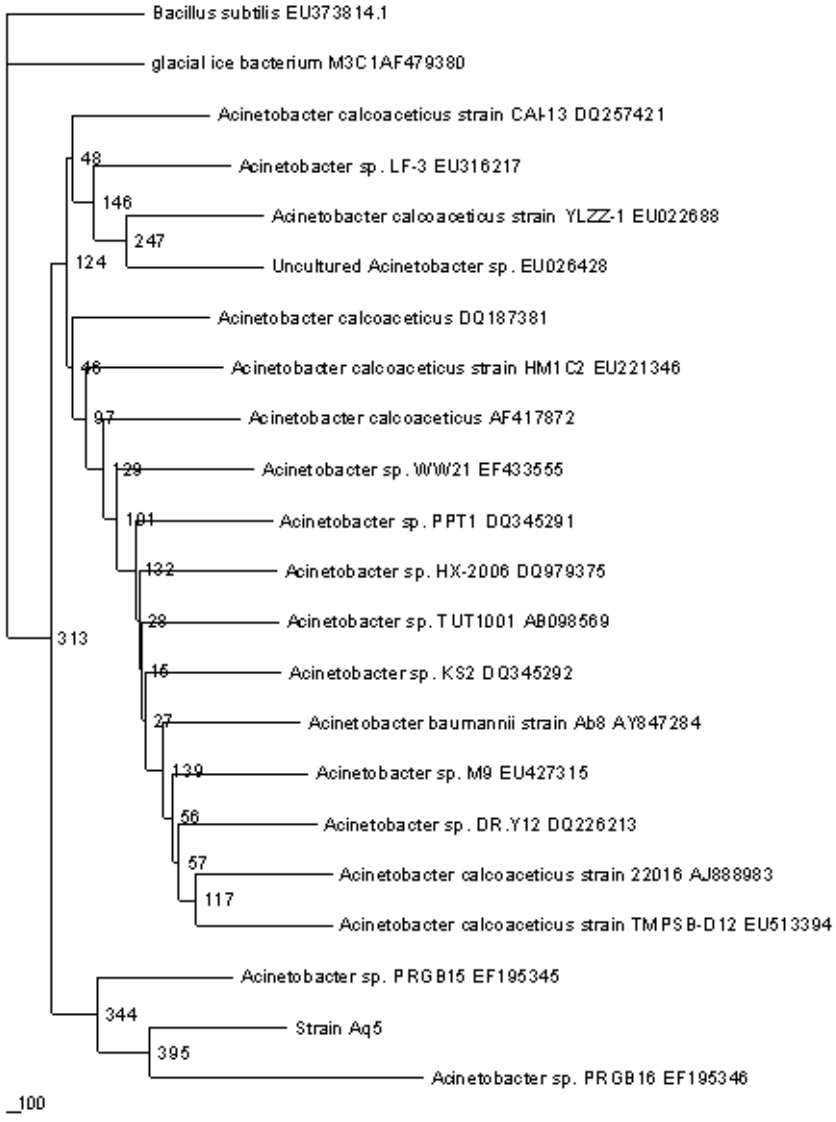

Fig. 2: The phylogenetic relationship of Strain AQ5NOL 1 and other related reference microorganisms based on 16S rRNA gene sequence.

Optimization of Conditions for Growth and Phenol Degradation:

The effect of temperature on the growth of *Acinetobacter* sp. strain AQ5NOL 1 in 0.5 gL^{-1} phenol was studied at temperatures ranging from 10 to 55ºC. The growth of Strain AQ5NOL 1 is minimal at temperatures below 20ºC, increased gradually to a maximum at 25 – 30ºC and then decreased gradually until 40ºC after which there is a drastic drop in growth (Figure 3). Phenol degrading activity was minimal at temperatures below 20ºC, high between 20 – 37ºC after which phenol degrading activity dropped drastically above 37ºC.

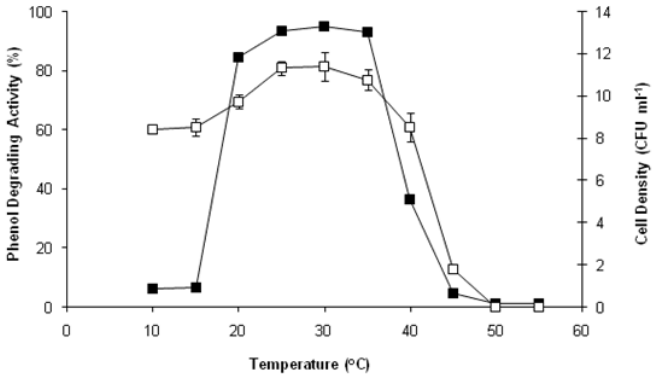

Fig. 3: The effect of temperature on growth of *Acinetobacter* sp. Strain AQ5NOL 1. The isolate was grown in MSM containing 0.5 gL^{-1} phenol and incubated in a shaking incubator at 25ºC for 3 days. (□), bacterial growth; (■), phenol degrading activity. Values shown are mean ± SEM, n=3.

Acinetobacter sp Strain AQ5NOL 1 showed high growth rates between pH 6.5 - 8.0 (Figure 4A). Growth was dramatically reduced at pH less than 6.5 and above pH 8.0. Phenol degrading activity showed a similar trend with the optimal phenol degrading activity in the range of pH 6.5 – 8.0.

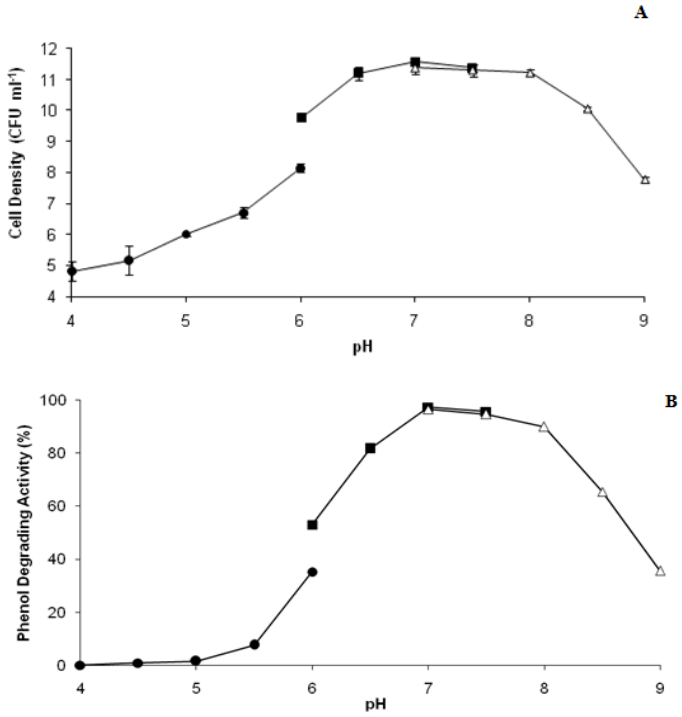

Fig. 4: Effect of pH on growth and phenol degrading activity of *Acinetobacter* sp. Strain AQ5NOL 1. Buffer systems (50 mM) used were (●), Acetate; (■), Phosphate; (?), Tris-HCl. (A), Bacterial growth; (B), Phenol degrading activity. Values shown are mean ± SEM, n=3.

Although most of the nitrogen sources tested supported growth, $(NH_4)_2SO_4$, sodium nitrate and ammonium chloride, cysteine and histidine recorded relatively high growth rates ($p<0.05$) after 3 days of incubation at 25°C, with $(NH_4)_2SO_4$ recording the highest growth rate (Figure 5A). Phenylalanine, alanine, glycine, leucine and proline recorded growth similar to control. Phenol degrading activity of the isolate also showed a similar trend to the growth rates. It was significantly ($p<0.05$) higher in $(NH_4)_2SO_4$, sodium nitrate and ammonium chloride, cysteine and histidine while for phenylalanine, alanine, glycine, leucine and proline, the activity was lower. Both growth and phenol degrading activity was highest in $(NH_4)_2SO_4$.

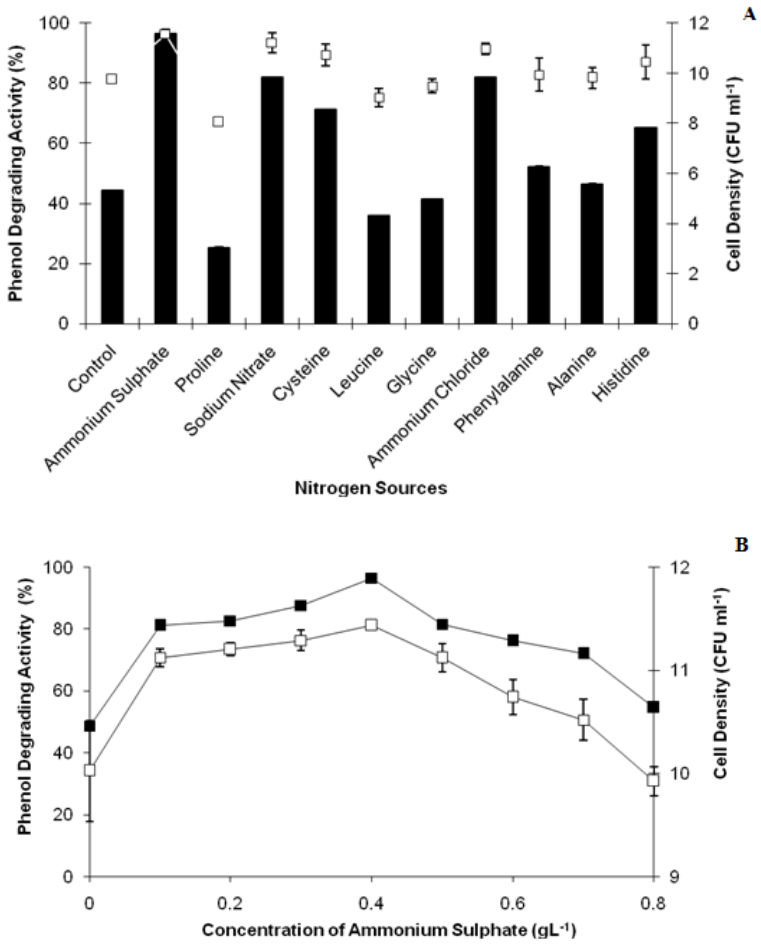

Fig. 5: Growth and phenol degrading activity of *Acinetobacter* sp Strain AQ5NOL 1.
A, Growth and phenol degrading activity in different nitrogen sources; B, growth and phenol degrading activity in different ammonium sulphate concentrations. (□), growth represented by cell density; (■), phenol degrading activity. Nitrogen sources were at 0.4 gL^{-1}. Values shown are mean ± SEM, n=3.

The effect of different concentrations of $(NH_4)_2SO_4$ in the range of 0–0.8 gL^{-1} was evaluated (Figure 5B). The isolate required 0.4 gL^{-1} $(NH_4)_2SO_4$ for optimum growth and phenol degrading activity compared with the other concentrations ($p<0.05$). Growth was rapidly reduced when the concentration of the nitrogen source was increased above 0.4 gL^{-1}.

The effect of salinity on the growth of Strain AQ5NOL 1 in 0.5 gL^{-1} phenol was studied at NaCl concentrations ranging from 0 to 0.3 gL^{-1} (Figure 6) The results show that *Acinetobacter* sp. strain AQ5NOL 1 recorded the highest growth at 0.1 gL^{-1} NaCl. Phenol degrading activity was also highest at 0.1 gL^{-1} NaCl.

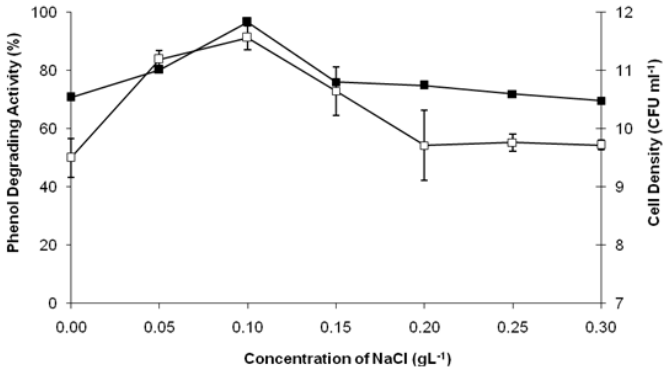

Fig. 6: Phenol degrading activity and growth of *Acinetobacter* sp Strain AQ5NOL 1 in different concentrations of sodium chloride. (□), growth represented by cell density; (■), phenol degrading activity. Values shown are mean ± SEM, n=3.

Growth and Phenol Degrading Activity of Acinetobacter sp. *Strain* ***AQ5NOL 1 in Different Phenol Concentrations:***

Strain AQ5NOL 1 was inoculated into media containing 300 – 2000 mgL^{-1} phenol and its growth and phenol degrading activity monitored for 10 days (Figure 7). The bacteria showed different phenol degrading activities and growth in the different concentrations of phenol. Phenol degrading activity was high at the lower phenol concentrations but gradually decreased as the phenol concentration increased. Phenol degrading activity was observed to taper off to a minimum at 1500 mgL^{-1} phenol concentration and almost no degrading activity above this concentration. Maximum growth was achieved within 3 days of incubation in the low phenol concentrations but a noticeable retardation of growth was observed at phenol concentrations higher than 1500 mgL^{-1}.

Discussion:

Bacteria collected from several phenol contaminated locations were reported to exhibit phenol degrading activity. Examples are *Bacillus stearothermophilus* FDTP-3 and Burkholderia *sp.* (Dong *et al.*, 1992; Cobos-vasconcelos *et al.*, 2006), several strains of *Pseudomonas putida* (Hutchison and Robinson, 1990; Kotturi *et al.*, 1991; Monteiro *et al.*, 2000), *Pseudomonas resinovorans* (Yang and Lee, 2007), *Alcaligenes eutrophus* (Leonard and Lindley, 1998), *Alcaligenes faecalis* (Bai *et al.*, 2007), *Bacillus stearothermophilus* (Dong *et al.*, 1992; Adams and Ribbons, 1988), *Bacillus thermoleovorans* (Milo *et al.*, 1999; Duffner *et al.*, 2000) and *Brevibacillus* sp. (Yang and Lee, 2007). *Acinetobacter* sp. Strain AQ5NOL 1 is no exception. It was isolated from a contaminated site and exhibits high phenol degrading activity.

Several *Acinetobacter* sp. strains with the ability to degrade phenol have been described; *Acinetobacter* sp. strain RD12 (AY 673994) isolated from Tianjin Jizhuangzi Wastewater Treatment Facility in China (Wang *et al.*, 2007), *Acinetobacter* sp. (DQ 831531) and *Acinetobacter* sp. strain W-17 (Adav *et al.*, 2007; Beshay *et al.*, 2002), *Acinetobacter calcoacetius* NCIB 8250, *Acinetobacter calcoacetiu* and *Acinetobacter radioresistens* (Paller *et al.*, 1995; Kumaran and Paruchuri, 1996; Pessione and Giunta, 1997). These species were isolated from phenol contaminated locations and demonstrated the ability to degrade phenol. With the successful isolation and characterization of *Acinetobacter* sp Strain AQ5NOL 1, it can now be added to the growing list of bacteria exhibiting phenol degrading activity. This in turn will offer a greater possibility of bioremediation being made successful.

Acinetobacter sp. Strain AQ5NOL 1 is able to degrade phenol at up to 1500 mg L^{-1}. This is considerably higher than that reported by other groups; *Bacillus stearothermophilus* FDTP-3 and Burkholderia *sp.* degrading up to 500 mgL^{-1} phenol (Dong *et al.*, 1992; Cobos-Vasconcelos *et al.*, 2006), *Pseudomonas putida* Strain DSM 548 at 100 mgL^{-1} (Monteiro *et al.*, 2000), F1 ATCC 17484 (Hutchison and Robinson, 1990) and Q5 (Kotturi *et al.*, 1991) at 200 mgL^{-1}, CCRC14365 at 600 mgL^{-1} (Chung *et al.*, 2003) and MTCC 1194 at 1000 mgL^{-1} (Kumar *et al.*, 2005). However, *P. putida* F1 ATCC 700007 that is able to degrade 750-1750 mgL^{-1} phenol (Abuhamed *et al.*, 2004) compares favourably with *Acinetobacter* sp Strain AQ5NOL 1.

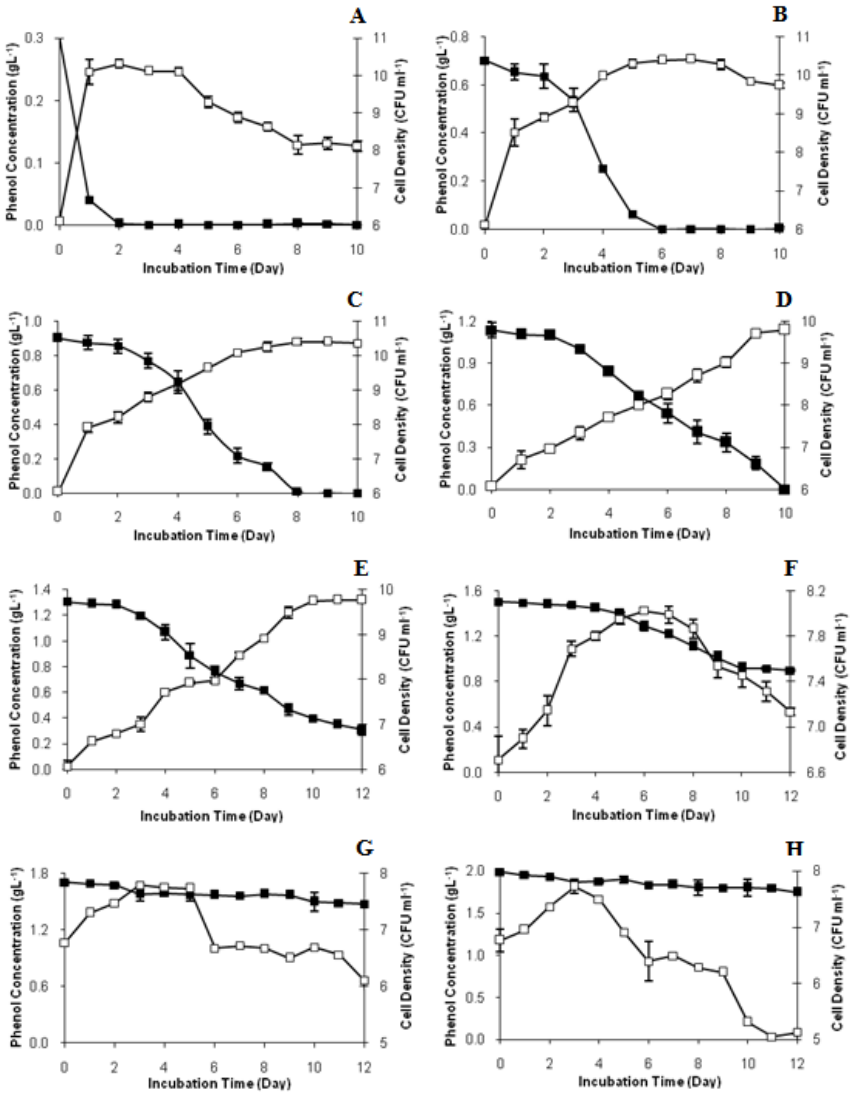

Fig. 7: Growth and phenol degrading activity of *Acinetobacter* sp. Strain AQ5NOL 1 in different concentrations of phenol. (A), 300 mgL^{-1} ; (B), 700 mgL^{-1} ; (C), 900 mgL^{-1}; (D), 1100 mgL^{-1}; (E), 1300 mgL^{-1} ; (F), 1500 mgL^{-1} ; (G), 1700 mgL^{-1} ; (H), 2000 mgL^{-1}. (□), Bacterial growth; (■), phenol degrading activity. Values shown are mean ± SEM, 3.

For *Acinetobacter* sp, several reports have described the ability of this bacteria species to degrade high phenol concentrations. *Acinetobacter* sp. isolated from a landfill waste in South Africa is able to degrade phenol at 300 mgL^{-1} concentration (Tibbles and Baecker, 1989), *Acinetobacter* sp. strains W-17 and *Acinetobacter calcoacetius* are able to degrade phenol up to 500 mgL^{-1} (Beshay *et al.*, 2002; Kumaran and Paruchuri, 1996). At higher phenol concentrations, *Acinetobacter* sp. DQ 831531 degrades phenol up to 1000 mgL^{-1} (Adav *et al.,* 2007) while *Acinetobacter* sp. strain RD12 (AY 673994) is capable of degrading phenol up to 1100 mgL^{-1} (Wang *et al.*, 2007). Therefore, Strain AQ5NOL 1 is among the highest phenol degrader as it degrades phenol up to 1500 mgL^{-1} concentration.

Several other groups had also reported that their bacterial strains had optimum growth and phenol degrading activity between 25 – 30ºC (Bai *et al.*, 2007; Chung *et al.*, 2003; Saravan *et al.*, 2007). Being a tropical country, the soil temperature in Malaysia varies from 25 – 35ºC and thus, Strain AQ5NOL 1 seems to be a suitable indigenous bacterium for degradation of phenol.

The pH optimum of between pH 6.5 – 8.0 of Strain AQ5NOL 1 is similar to that other phenol degrading bacteria reported; *Alcaligenes faecalis* at pH 7.2 (Bai *et al.*, 2007), *Acinetobacter* sp. strain

RD12 at pH 7.2 (Wang *et al.*, 2007), *Pseudomonas putida* at pH 8 (Chung *et al.*, 2003) and an indigenous mixed microbial consortium at pH 7 (Saravan *et al.*, 2007). Information on the pH optimum for growth and phenol degrading activity would help in designing an effective bioremediation strategy (Davey, 1994).

Ammonium sulphate was reported to be the optimum nitrogen source for phenol degradation by *Pseudomonas putida, Acinetobacter* sp. and *Alcaligenes faecalis* (Chung *et al.*, 2003; Bai *et al.*, 2007; Wang *et al.*, 2007). Strain AQ5NOL 1 requires 0.4 gL^{-1} $(NH_4)_2SO_4$ for optimum growth and phenol degrading activity compared with the other concentrations ($p<0.05$). Wang *et al.*, (2007) and Bai *et al.,* (2007) reported that the optimum $(NH_4)_2SO_4$ concentration for phenol degrading activity in *Acinetobacter* sp. strain RD12 and *Alcaligenes faecalis* is 0.4 gL^{-1}.

In 2007, Bai *et al.*, (2007) had reported that phenol-degrading *Alcaligenes faecalis* has the optimum NaCl concentration of 0.1 gL^{-1}. In contrast, *Pseudomonas putida* has the lowest optimum NaCl concentration at 0.015 gL^{-1} (Chung *et al.*, 2003) while the other extreme is shown by the phenol degrading *Alcaligenes faecalis* isolated from Amazonian rain forest that showed optimum growth at 56 gL^{-1} NaCl concentration (Bastos *et al.*, 2004). Detection of salinity range provides the potential advantages of bioremediation (Duràn and Esposito, 2000). With the optimum salinity of 0.4 gL^{-1} shown by Strain AQ5NOL 1, it is more suitable in freshwater rather than in marine conditions.

Overall information on the optimized conditions for *Acinetobacter* sp Strain AQ5NOL 1 in growth and phenol degradation will be of great help in realizing its potential in the bioremediation of phenol.

Conclusions:

We have isolated and characterized a local phenol-degrading *Acinetobacter* sp. Strain AQ5NOL 1 that can degrade elevated concentrations of phenol of up to 1500 mgL^{-1}. The bacterial strain is potentially useful in the bioremediation of phenols in industrial effluents. Work is in progress to characterize the enzyme involved in phenol degradation and to study the potential of this bacterial isolate in the bioremediation of phenol wastes by optimizing the conditions for the bacteria in immobilized and free forms.

ACKNOWLEDGMENTS

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