

Research Article

Optimization of Cultural and Physical Parameters for Phenol Biodegradation by Newly Identified *Pseudomonas* sp. AQ5-04

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ABSTRACT

Phenol is widely used by many industries and it is one of the highly toxic environmental pollutants. Bioremoval is one of the most effective methods to remove phenol compared to other physio-chemical methods. Identification was carried out using 16s rRNA sequencing. Mineral salt media with 0.5 g/L phenol as the sole source of carbon. Factors influencing phenol degradation were optimised via one-factor-at-a-time and response surface methodology. Optimum degradation was achieved at pH 7.5, the temperature of 30°C and ammonium sulphate at 0.4 g/L. Using Response surface methodology the incubation period was reduced to 36 h compared to the OFAT approach where it takes 72 hours. The effect of 10 heavy metals at various concentrations was tested. The optimum values used for temperature, pH, ammonium sulphate and salinity for both the OFAT and RSM have correlated with the only pH displayed the slighted difference of 7.0 for OFAT and 7.5 for RSM. This shows the closest optimum conditions for both methods. The strain is also resistance to some heavy metals usually found in polluted environments together with phenol. Therefore, it can be clearly stated that *Pseudomonas* sp. strain AQ5-04 is the potential candidate for phenol bioremediation and further studies in the field of bioremediation. The bacterium can degrade phenol in the presence of between 1 to 3 ppm of the heavy metals As, Cd, Co, and Zn while growth and degradation were inhibited by Hg, Ag, Cu and Ni at 1 ppm. The isolate is a potential strain for further bioremediation studies.

Keywords: Phenol-degrading, Pseudomonas sp., Response surface methodology heavy metals, One factor at a time

Introduction

Phenols and phenolic compounds are poisonous to organisms even at low concentrations, with many of them are categorized as dangerous pollutants due to their toxicity towards human health for various reasons [1]. Some of the phenolic compounds include chlorophenols, nitrophenols, methyl phenols, alkylphenol, aminophenol, butylhydroxytoluene, nonylphenol and bisphenols A [1]. In Malaysia, the 2014 Environmental Quality Report showed that nearly all groundwater monitoring stations had phenol concentrations exceeding the National Guidelines for Drinking Water Quality Standard (0.002 mg/L). Phenol and phenolic compounds continue to be the top scheduled

wastes generated in Malaysia as the demand for phenol by the industries are increasing annually [3].

There are various physicochemical methods for the removal of phenol pollution from the environment, such as polymerisation [4], electrocoagulation [5] photodecomposition [6], advanced oxidation [7] and ion exchange [8]. However, a biological method is still considered as the preferable way of controlling phenol pollution since it is effective at low concentrations of phenol, cost-efficient. It does not produce secondary pollutants, as in the case of some physicochemical methods [1, 9]. The ability of some microorganisms to utilize phenol and other phenolic compounds is consider-

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ed as a tool for the disposal of toxic waste [10]. In fact, the attempt to use microorganisms to remove phenol and other organic environmental pollutants have been increasing in recent years. There are number of microorganisms that coexist in all-natural environments that can degrade organic compounds by producing intracellular or extracellular xenobiotics-degrading enzymes [11, 12, 13].

Many *Pseudomonas* species are used in biodegradation of phenol and phenolic compounds. For example, *Pseudomonas* sp. CF600, *Pseudomonas* sp. SA01 and *Pseudomonas* sp. strain JS150, which can degrade phenol by hydroxylat [14, 15, 16]. Other *Pseudomonas* species, such as *Pseudomonas* sp. strain ADP are able to degrades phenolic compounds like atrazine as a sole source of nitrogen [17].

Phenol biodegradation is affected by various factors such as temperature, pH, nitrogen source, and salinity [12]. Optimization of these parameters is important in the scaling up of the microorganism for bioremediation. Response Surface Methodology (RSM) is a statistical tool used in the modeling and analyzing interest response when subjected to the influence of several independent factors. It is a more appropriate method for optimization than one factor at a time approach due to its time and materials effectiveness. Also, it indicates the presence of interactions between the various parameters [18, 19]. The major components of RSM are; the selection of independent variables, the choice of experimental design, the statistical treatment, verification of the model fitness, obtaining the optimal values for each variable, and the validation of the results [18]. The design and optimization of biochemical processes require an investigational quantitative data. Therefore, this research is aimed to identify the phenol-degrading bacteria as well as to optimize the media and cultural conditions for phenol degradation and to test the effect of some heavy metals on the biodegradation process.

Material and Methods

Isolation of phenol-degrading bacteria

A total of 90 wastewater samples (freshwater) were collected in areas exposed to industrial activities within Malaysia including Sarawak and Peninsular Malaysia. Samples were collected between 2012 and 2013. The phenol-degrading bacterium AQ5-04 was isolated from a wastewater sample

obtained at the GPS location N°3°01.658', E°101°33.777'. This location receives water effluents from the pharmaceutical industry nearby. Four millilitre of wastewater sample was mixed with 40 mL of sterilized minimal salt medium (MSM) containing 0.5 g/L phenol and incubated at 25°C on a shaking incubator at 150 rpm for three days [20]. The cultures were streaked onto mineral medium agar plates supplemented with phenol and incubated at 25°C for three days. Isolates exhibiting distinct colonies were further isolated using five cycles of repeated subculturing into mineral medium agar plates supplemented with 0.5 g/L phenol. Every single pure colony of isolates was inoculated in a 0.5 g/L liquid phenol medium respectively and was daily monitored for phenol-degrading activity using the 4 aminoantipyrine colourimetric assay at 510 nm. Meanwhile, the bacteria growth was measured using OD600 nm. The isolate that displays the highest phenol degradation (percentage) using aminoantipyrine (AAP) method was selected for further study [20].

Molecular identification of the strain.

AQ5-04 was identified through a molecular 16s rDNA gene sequence phylogenetic analysis. The genomic DNA was extracted using a commercial kit (GeneJet Genomic DNA purification kit, ThermoScientific, Lithuan) and amplified using the following PCR universal primers; forward: 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse: 5'-TAC GGT TAC CTT GTT ACG ACT T-3' [21]. The polymerase chain reaction (PCR) was done under the following conditions: 1 cycle of initial denaturation at 96°C for 4 minutes; 30 cycles of 94°C denaturing for 1 min, annealing at 58°C for 1 min, and 72°C extension for 1 min) and a final extension at 72°C for 7 minutes. Phylogenetic tree analysis and evolutionary relationship of taxa: Twenty (20) 16s rDNA sequences of related *Pseudomonas* species were obtained from Genbank and aligned using Clustal W using PHYLIP.

Optimization of physical and medium factor influencing phenol degradation one factor at a time (OFAT)

The optimization was done using 0.5 g/L phenol as the sole source of carbon.

Effects of temperature

To evaluate the effect of temperature on phe-

not degradation by *Pseudomonas* sp. AQ5-04, the bacteria were grown in the MSM under various temperatures (15 to 45°C).

Effects of pH

To determine the best and optimal pH bacterial growth and phenol degradation by *Pseudomonas* sp. AQ5-04, various pHs were used, with overlapping buffering systems, acetate buffer (pH 4.0 to 6.0), phosphate buffer (pH 6.0 to 7.5), and Tris-HCl (pH 7.0 to 9.0) was used. The percentage of phenol degradation and bacterial growth was measured at the end of the incubation time.

Effects of nitrogen sources.

Various nitrogen sources were evaluated based on phenol degradation and bacterial growth by *Pseudomonas* sp. AQ5-04 to find the best nitrogen source. The nitrogen sources used for this study are ammonium sulphate, sodium nitrate, and nitrogen bicarbonate. Later, the best nitrogen source was optimised at concentrations of up to 0.8 g/L in phenol-MSM to evaluate the best concentration for the growth and phenol degradation by *Pseudomonas* sp. AQ5-04.

Effects of salinity.

Sodium chloride was used at concentrations of up to 0.3 g/L to evaluate the effect of salinity on phenol degradation and bacterial growth by *Pseudomonas* sp. AQ5-04.

Effect of different phenol concentrations.

The effect of phenol concentrations on the bacterial growth and the percentage of phenol degradation were evaluated, various levels of phenol were used (0.2 to 2.4 g/L) as the sole source of carbon in the MSM.

Effect of heavy metals.

To examine the effect of heavy metals on phenol degradation and bacterial growth, ten different heavy metals were tested at various concentrations. The heavy metals involved are arsenic (As), cadmium (Cd), cobalt (Co), copper (Cu), chromium (Cr), lead (Pb), mercury (Hg), nickel (Ni), silver (Ag) and zinc (Zn).

Statistical optimisation

Central composite design (CDD) and response surface methodology (RSM) were used in this study. RSM is a statistical tool used in modelling

and analyzing interest response when subjected to the influence of several independent factors. The main objective of using RSM in this study is to optimize phenol degradation (as a response). There are four major factors and interactions were analyzed and optimized by RSM. The selected major factors are temperature, pH, salinity, and $(\text{NH}_4)_2\text{SO}_4$. This design consists of 30 experimental runs. All four independent factors were studied at five different levels: $-\alpha$, -1 , 0 , $+1$, $+\alpha$. All experiments were carried out in triplicate and the average phenol degradation (response) in each run was recorded. The data obtained were fitted to a second-order polynomial equation, which generates a model equation that relates Y to the independent factors. By using analysis of variance (ANOVA) tool in Expert-Design software version 6.0, p-values and confidence levels of the data obtained were calculated. Optimal values from linear and interactions of factors were estimated using 3D plots. The predicted values from RSM were validated by a set of experiments.

Results and Discussions

Molecular identification of the strain

Isolate AQ5-04 was identified using the 16S rDNA sequencing; the nucleotide sequence was compared to those on the NCBI Gene Bank database. rRNA sequence indicated a 99% similarity with *Pseudomonas* sp. The sequence was further deposited at the NCBI Gen bank and assigned an accession number of KT693288. The evolutionary history was inferred using the Neighbour-Joining method [22]. An optimal tree with the sum of branch length = 57.80900656 was observed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches [23]. The tree is drawn to scale with branch lengths of the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [24] in the units from the number of base substitutions per site. The analysis involved 17 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd. All positions containing gaps and missing data were eliminated. There was a total of 1377 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [25], as shown in Figure 1. The analysis revealed that the bacterium

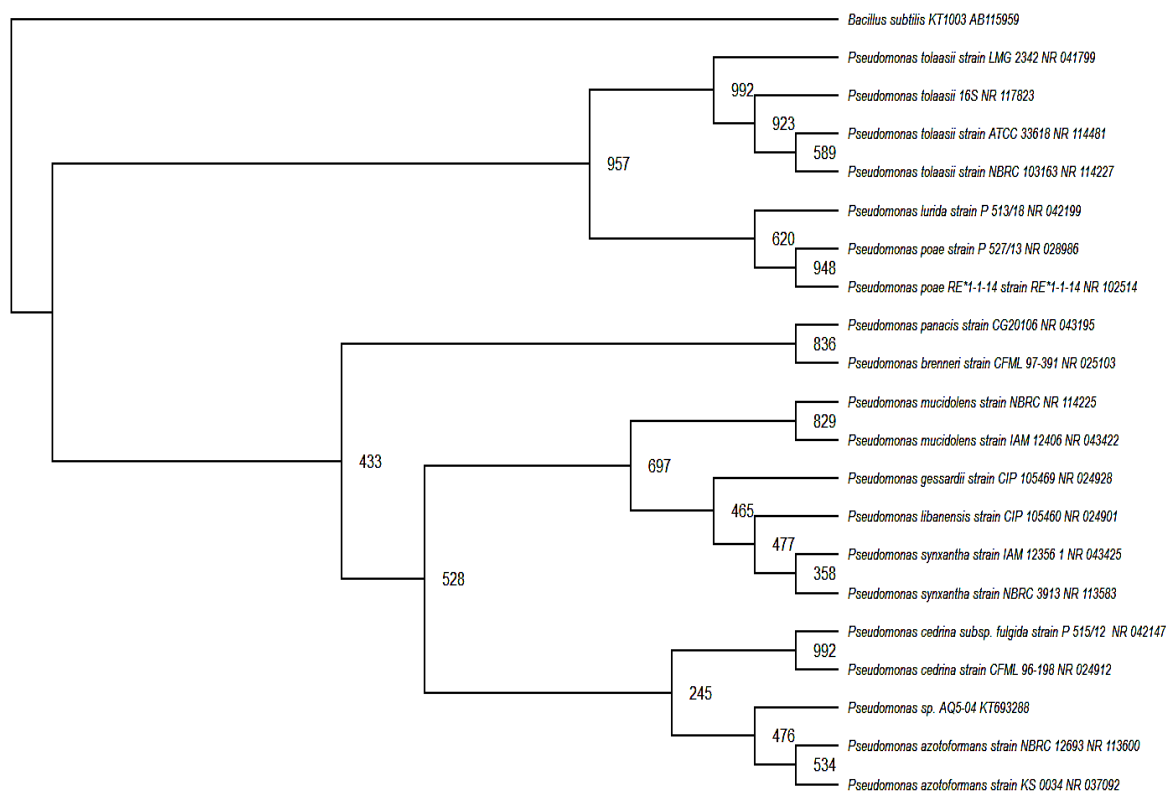


Figure 1. Neighbour-joining tree based on partial 16S RrRNA sequence demonstrating the phylogenetic relatedness of strain AQ5-04 within the *Pseudomonas* species and *Bacillus subtilis* strain KT1003 AB115959 was used as an outgroup

exhibited similarity with another *Pseudomonas* sp. The phylogenetic tree also exhibited that, the bacterium is associated with a clade that harbours other *Pseudomonas* sp. such as *Pseudomonas azotoformans* strain NBRC 12693 NR_113600, *Pseudomonas synxantha* strain NBRC 3913 NR_113583, *Pseudomonas mucidolens* strain NBRC NR_114225 and *Pseudomonas cedrina* strain CFML 96-198 NR_024912 as shown in Fig.1 The bacterium was therefore tentatively identified as *Pseudomonas* sp. AQ5-04.

Effect of temperature

Temperature is one of the factors affecting the growth and ability of a microorganism to be used in bioremediation. Studies on phenol degradation reported temperatures ranging from 10 to 55°C [26]. The optimum temperature for *Pseudomonas pictorum* and *Pseudomonas* sp. NBM11 was found to be 30°C [27, 28]. The results of this study revealed that *Pseudomonas* sp. Strain AQ5-04 sp. has an optimum temperature of 25°C to 35°C for both phenol degradation and bacterial growth (Figure 2a). There was no significant difference (p

> 0.05) in phenol degradation percentage for the temperatures of 25, 30, and 35°C. However, both the phenol degradation and bacterial growth declined at temperatures higher than 35°C, which may be due to the inactivation or denaturation of the phenol degrading enzyme and some other proteins. Phenol degradation has been seen to be greatly affected by temperature [29]. Many studies have reported that the optimum temperature for phenol biodegradation is between 20°C to 35°C for mesophilic bacteria. *Pseudomonas* sp. strain AQ5-04 is a mesophilic bacterium; this further suggests the similarity between the obtained result and that of other researchers. The highest temperature limit for phenol degradation by mesophilic bacteria is 45°C [30], a thermophilic *Bacillus* sp. A2 was reported to grow in media containing phenol as the sole source of carbon at temperatures up to 70°C [31]. Phenol degradation efficiency of A cold-tolerant *Arthrobacter* sp. AG31 was reported.

Effect of salinity

Effect of salinity on the bacterial growth and to degrade phenol efficiently at 10°C [32].

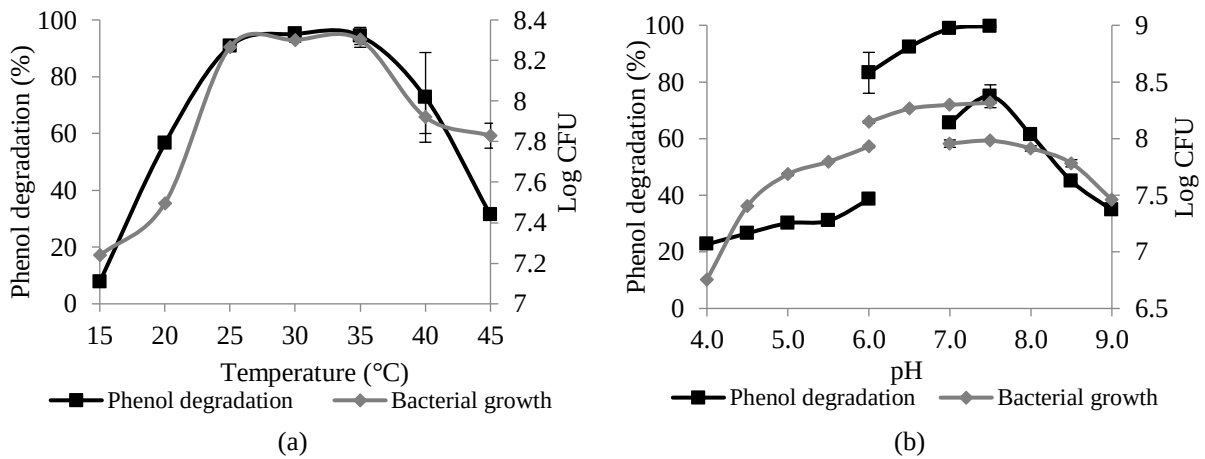


Figure 2. (a) Effect of temperature on phenol degradation and concentration and growth of *Pseudomonas* sp. AQ05-04 (b) Effect of pH on phenol degradation and concentration and growth of *Pseudomonas* sp. AQ05-04. Error bars indicates mean \pm SEM, n = 3.

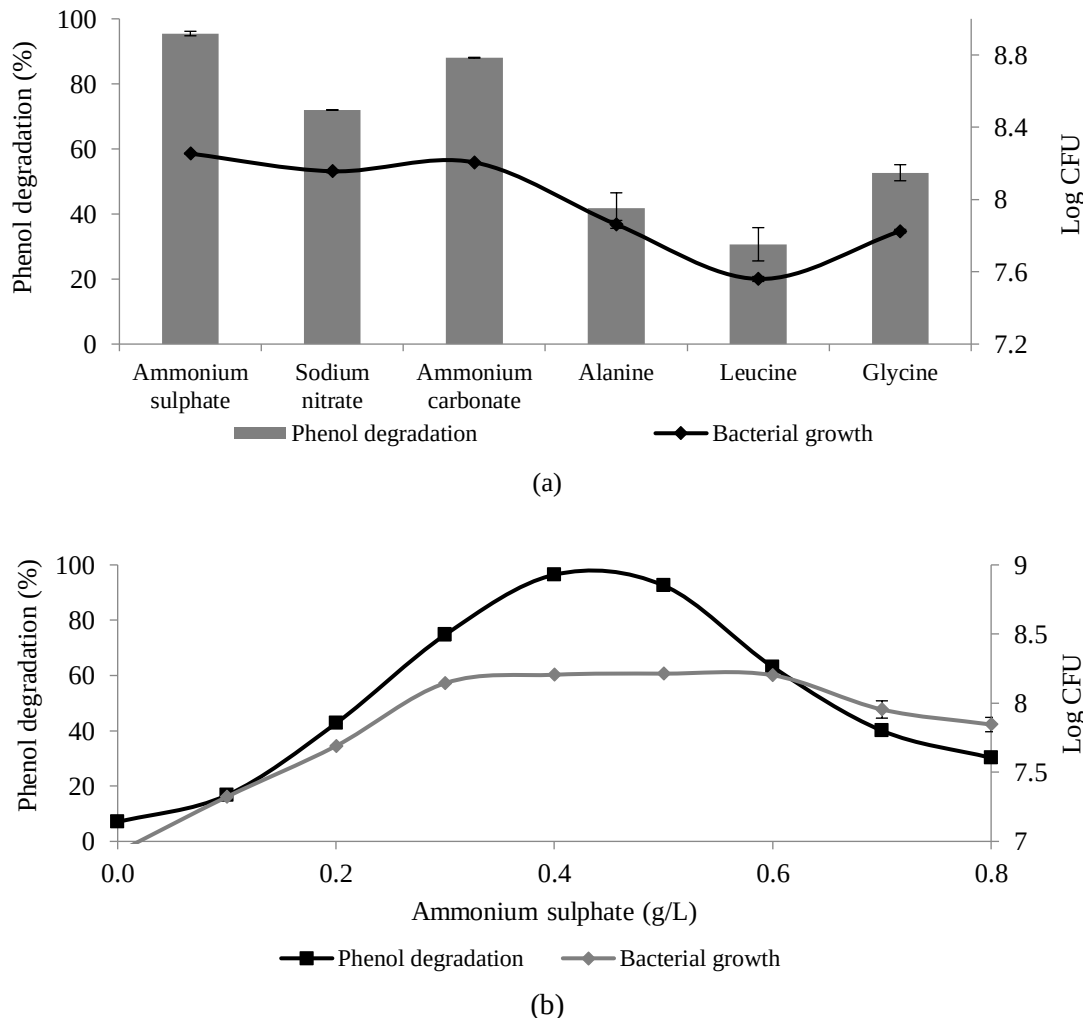


Figure 3. (a) Effect of nitrogen source on phenol degradation and concentration and growth of *Pseudomonas* sp. AQ05-04 (b) Effect of ammonium sulphate concentration on phenol degradation and concentration and growth of *Pseudomonas* sp. AQ05-04. Error bars indicates mean \pm SEM, n=3.

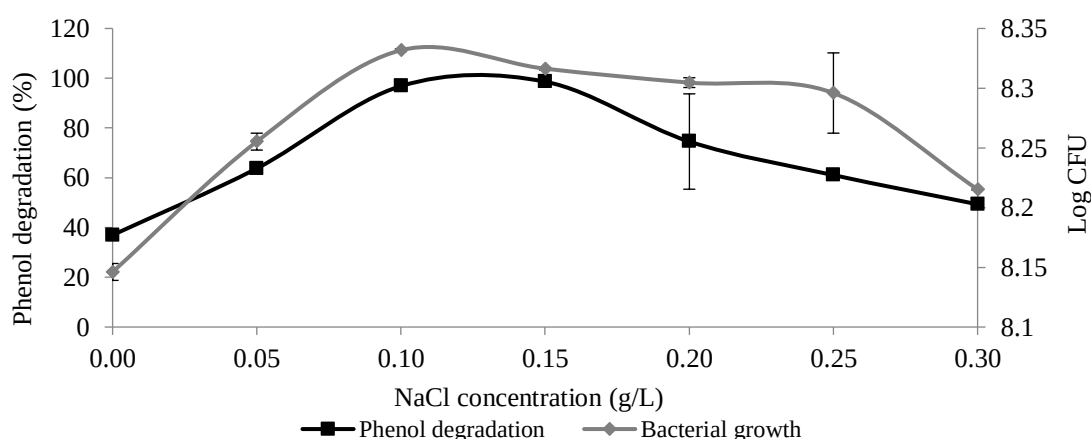


Figure 4. Effect of salinity on phenol degradation and concentration and growth of *Pseudomonas* sp. AQ05-04. Error bars indicates mean \pm SEM, n=3.

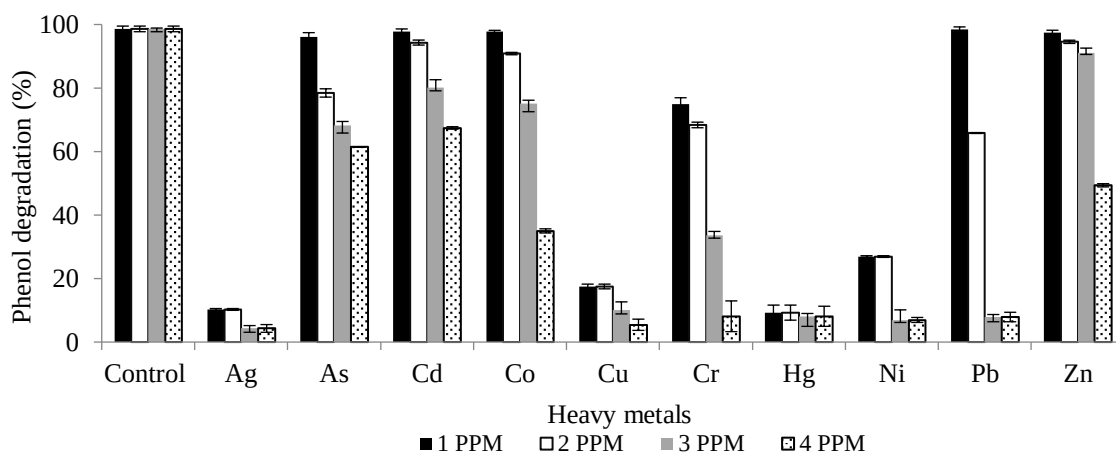


Figure 5. Effect of various heavy metals concentrations on the percentage phenol degradation by *Pseudomonas* sp. AQ05-04. Error bars indicates mean \pm SEM, n=3.

the phenol degradation was tested to determine whether or not isolate can be used to degrade phenol in the ocean or near coastal areas where there are reported cases of phenol pollution. The result revealed that sodium chloride between 0.1 and 0.15 g/L was the best concentrations of sodium chloride in regards to growth and degradation. Meanwhile, high NaCl concentration displayed an inhibitory effect on the bacterial growth as well as phenol degradation, which may be due to an osmotic stress leading to the rupture of bacterial cells, thus inhibiting the growth and decreasing the degradation (Figure 4). The bacteria were unable to survive under high phenol concentrations. Phenol is a very toxic organic compound with an inhibitory effect on bacterial growth. Thus, there is no phenol degradation observed under high phenol concentrations. In most studies reported that bacterial growth is severely affected by a NaCl con-

centration above 1.5 %. High salinity results in osmotic stress which hinder bacterial growth as while as biodegradation ability of the bacteria [26]. Some studies reported that *Pseudomonas aeruginosa* and *P. fluorescens* strains are able to grow in medium containing 1.5% NaCl [39]. Certain bacteria from Amazon rainforest are tolerant high salinity. For example, *Aliccaligenes faecalis* can withstand 56 g/L NaCl, *Candida tropicalis* a yeast also from Amazon is reported to resist 150 g/L NaCl [40]. Resistance to high salinity by bacterial is an advantage for bioremediation of phenol and other pollutants at maritime and coastal areas [41]. Among the three isolates, *Serratia* sp. AQ5-03 has the advantage of growing well at NaCl higher than most reported concentrations [42].

Effect of heavy metals

Mercury (Hg) is considered the most toxic he-

Table 3. Central composite design and its experimental and predicted values of phenol degradation by *Pseudomonas* sp. AQ05-04

Factor 1	Factor 2	Factor 3	Factor 4	Response	
Temperature (°C)	pH	Nitrogen source g/L	Salinity g/L	Actual value (and)	Predicted value (and)
30	7.5	1.10	0.15	93.9	93.61
20	7.5	0.5	0.15	5.50	2.35
30	10.5	0.5	0.15	33.20	34.74
30	7.5	0.1	0.15	88.30	87.82
30	4.5	0.5	0.15	82.00	79.70
30	7.5	0.5	0.15	93.40	95.43
40	7.5	0.5	0.15	64.80	67
30	7.5	0.5	0.15	96.70	95.43
30	7.5	0.5	0.25	57.66	58.90
25	6.0	0.8	0.30	66.40	66.05
35	9.0	0.2	0.30	49.20	46.76
25	9.0	0.2	0.30	40.90	38.65
25	6.0	0.2	0.00	43.90	42.86
35	9.6	0.8	0.30	55.70	54.03
25	9.6	0.8	0.30	44.30	46.76
30	7.5	0.5	0.15	93.01	95.43
35	6.0	0.2	0.00	99.40	100.42
35	9.0	0.8	0.00	71.80	71.39
25	6.0	0.8	0.00	33.87	40.05
30	7.5	0.5	0.15	95.20	95.43
25	9.0	0.8	0.00	86.92	88.32
35	9.0	0.2	0.00	77.12	74.76
35	6.0	0.8	0.00	98.57	98.11
30	7.5	0.5	0.45	70.10	69.50
30	7.5	0.5	0.15	73.00	72.83
35	6.0	0.8	0.30	96.70	97.15
25	6.0	0.2	0.30	53.00	56.89
30	7.5	0.5	0.15	96.10	95.43
25	9.0	0.2	0.00	38.00	41.03
35	6.0	0.2	0.30	92.90	91.81

avy metal, while Pb is relatively toxic to a living organism [43]. Also, a small amount of Zn and Cu are essential for basic metabolic activities in a microorganism but are toxic to the bacteria in high concentrations. Other metals like Hg, Pb, Cd and Cr have no known significant role in bacterial growth and development as they are known to be toxic to living organisms even at low concentration [44]. Strain AQ5-04 is effective in biodegrading phenol even with the presence of heavy metals. Hg, Cu, Ag, and Ni are the toxic heavy metals related to this strain. Even at the concentration of 1 ppm, the degradation percentages were found to be 9.2%, 10%, 17% and 26%, respectively. This sug-

gests the inhibitory effects contributed by these heavy metals on phenol degradation even at 1 ppm (Figure 5), which might be due to their ability to inhibit several key enzymes in biodegradation processes or because of their toxic property to some essential proteins and some cellular components in the bacterial biological systems [45]. Likewise, heavy metals, As, Cd, Co, Pb, and Zn show low effects at lower concentrations, but as the concentrations increases, they displayed strong inhibitory effects on the degradation rate. At 3 and 4 ppm, there is a decline in the degradation rate for the six heavy metals that have no significant inhibitory effect at lower concentrations; Nevertheless, in the

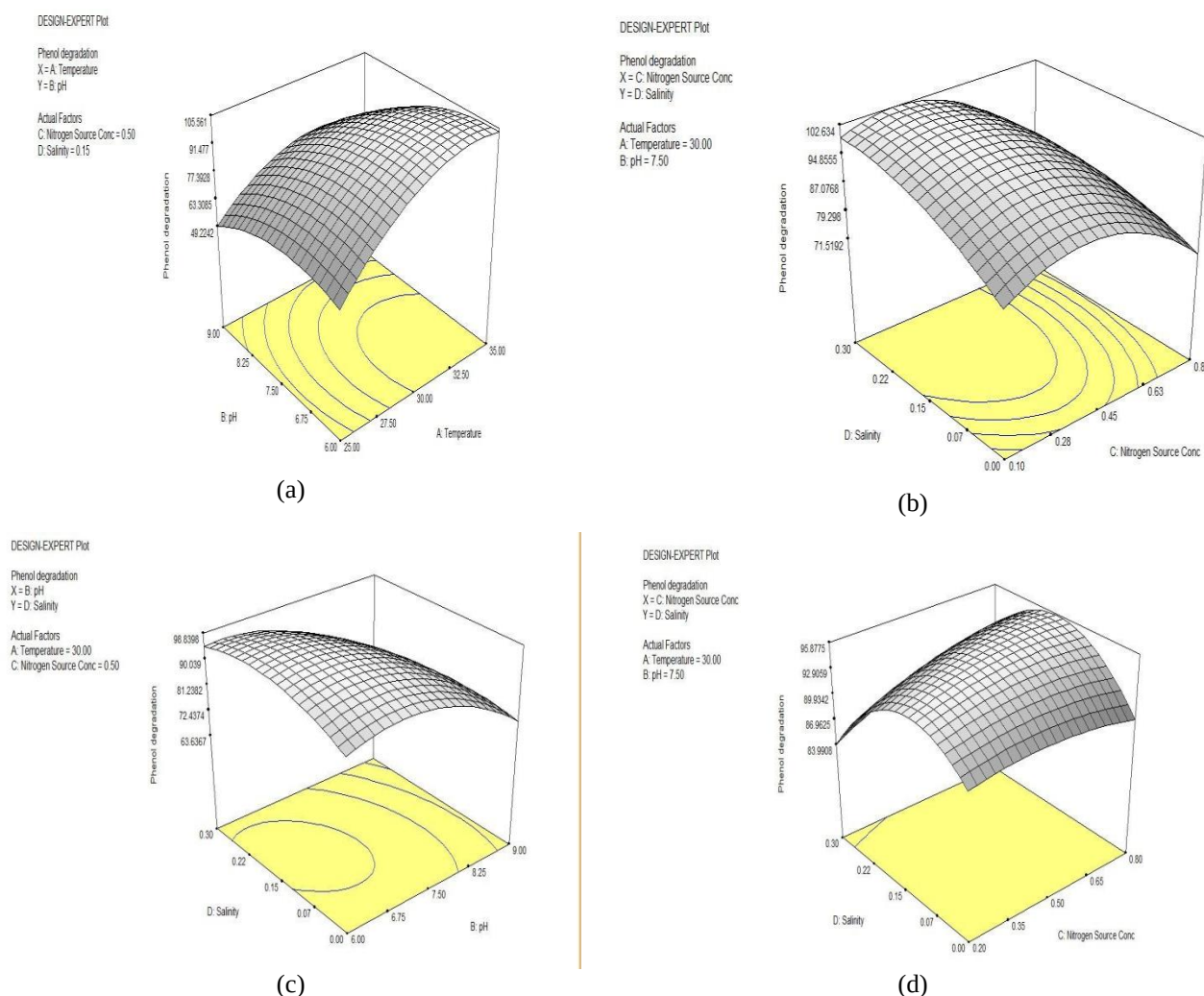


Figure 6. 3-D plots of phenol degradation for surface optimization a pH versus temperature; b salinity versus ammonium sulphate concentration ammonium sulphate concentration versus Temperature; c salinity versus temperature; d ammonium sulphate concentration versus pH;

presence of As and Cd at 3 and 4 ppm at degradation rate was found to be more than more than 60% as shown in Figure 5. Various isolates have been reported to tolerance phenols with some heavy metals, but this isolate can degrade phenol contaminated with up to 3 ppm of some known toxic heavy metals but inhibited by Hg and Ag. Previous studies indicated that Hg inhibits phenol degradation by microorganisms such as *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* [46], *Aureobasidium pullulans* [47], *Bacillus brevis* [48], *Alcaligenes* sp. [49] and *Sulfobacillus acidophilus* [50]. Ag also has been reported to inhibit phenol degradation and as well as bacterial growth by *Rhodococcus AQ5NOL1* [34].

Statistical optimization

The results from RSM revealed that the model

is significant (< 0.05) with the F-value of 196.57 (Table 1) where there is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Besides, the lack of fit for the model is not significant (> 0.05). Meanwhile, Values of "Prob $> F$ " less than 0.0500 indicated that the model terms are significant [51].

In this case, A, C, D, A^2 , B^2 , D^2 , AC, BC, CD are the significant model terms. On the other hand, values greater than 0.1000 indicated insignificant model terms (Table 1). It showed that the response (phenol degradation) depends on the product and quadratic product of X_a , X_b , X_c , and X_d . Lower estimation of 3.59% coefficient of variance (CV) additionally portrays the unwavering high quality of trial information [52]. Table 2 illustrates the model coefficient and their statistical significance by multiple regression for percentage phenol degra-

tion. The four variables (temperature, pH, ammonium sulphate concentration and salinity) influencing phenol degradation as reported by [53]. Table 3 illustrated the predicted responses and also the actual responses.

Analysis of 3D interactive plots presented that there is an interaction between temperature (A) and pH (B), temperature and salinity (D), PH and salinity, as well as between ammonium sulphate concentration and salinity (Figure 6a to 6d), which further displayed a higher phenol degradation achieved at the temperature of 30°C, pH 7.5 0.5 g/L ammonium sulphate and 0.15 g/L sodium chloride. The predicted data from the model was experimentally verified. A modified phenol basal media was formulated with predicted model values and percentage degradation was then detected. The results showed 99% phenol degradation in an incubation period of 36 h. RSM has reported being used for optimisation in phenol biodegradation, 100 % phenol degradation was achieved using the RSM optimised parameters by *Rhodococcus* sp. NAM 81 [12]. *Alcaligenes faecalis* was able to degrade 100 % of 2100 mg/L phenol using RSM optimised conditions [54]. *P. Aeruginosa* (MTCC 7814) remove 83.86% phenol using RSM validation experiment [37].

Conclusion

Pseudomonas sp. strain AQ5-04 has been identified and optimized for phenol biodegradation. The study reveals that the strain grows and degrades best when ammonium sulphate is used as a source of nitrogen sources. A pH value of 7 was identified as the best pH for phenol degradation by these bacteria. Also, the optimum temperature was found at 30°C, which is within the average range of temperature in Malaysia. The bacterium was able to degrade up to 98% of 5 mg/L phenol in mineral salt media. It has also degraded up to 70% of 8 mg/L phenol. The optimum condition for phenol remediation was further revealed with the help of response surface methodology. The optimal conditions were obtained from this model using the factors responsible for phenol degradation in central composite design. The optimum values used for temperature, pH, ammonium sulphate and salinity for both the OFAT and RSM have correlated with the only pH displayed the slighted difference of 7.0 for OFAT and 7.5 for RSM. This shows the closest optimum conditions for both

methods. The strain is also resistance to some heavy metals usually found in polluted environments together with phenol. Therefore, it can be clearly stated that *Pseudomonas* sp. strain AQ5-04 is the potential candidate for phenol bioremediation and further studies in the field of bioremediation.

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