Journal of Fundamental and Applied Sciences

ISSN 1112-9867

Available online at http://www.jfas.info

Research Article

PHENOL BIODEGRADATION AND OPTIMISATION OF CULTURAL AND PHYSICAL CONDITIONS BY THE FREE CELLS OF NEWLY ISOLATED PHENOL DEGRADING *ALCALIGENES* SP. AQ5-02

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Received: 06 July 2020 / Accepted: 13 August 2021 / Published online: 01 September 2021

ABSTRACT

Biological treatment is understood to be the most efficient technique for phenol removal paralleled to other physio-chemical methods. 16s rRNA sequencing for the identification. Mineral salt media with 0.5 g/L phenol as the only carbon source. Temperature, pH, salinity and nitrogen source were optimised. The effects of heavy metals on the percentage of phenol degradation with were tested. Accession number of KT693288 was assigned after identification. Temperature of 25- 35°C, pH 7-8 phosphate buffer were the optimum and ammonium sulphate was established to be the paramount nitrogen source at 0.4 – 0.5 g/L for isolate. The optimised conditions were found reducing the incubation period to 48 h with the ability to tolerate up to 0.2 g/L sodium chloride and degraded 50% and 1.1 g/L phenol. Meanwhile, the isolate AQ5-02 can also effectively degrade 1000 g/L phenol in the presence of heavy metals such as Cr, Zn, and Fe.

Keywords: 16s rRNA, *es* sp, phenol, physico-chemical, biological treatment, biodegradation

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doi: http://dx.doi.org/10.4314/jfas.v13i3.13

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1. INTRODUCTION

Environmental pollution is the one the primary concern in the 21st century. There are billions of tonnes of harmful chemicals produced by industries such as petroleum, paints, food, rubber, and plastic. These toxicants enter the environment through air, soil, and water. Furthermore, the combustion of fuel, burning activities and power stations are the major sources of air pollution where volatile hydrocarbons are exhausted into the air (1). Air pollution can result in many respiratory, cardiovascular and liver diseases (2). Discharging unprocessed harmful wastes including heavy metals is inclined to be the primary source of water and soil contamination in addition to oil spills from petroleum industries. Among the phenolic compounds, phenol is the most commonly used by industries and is the precursor for the synthesis of many industrial chemicals. Phenols are injurious to organisms even at even at low concentration with many of them categorised as dangerous pollutants due to their harmful effects on human well-being as the mentioned reason is considered as the primary concern.

Phenol is one of the most significant industrial effluents discharged by the processing industries such as oil refineries, dye, pesticide, plastic, plant and pharmaceutical industries (3). It is a critical environmental pollutant that is noxious to livings even at low concentration (4). The approved level of phenol in water is <0.002 mg/L, while lethal dose 50-500 mg/kg (5). Acute exposure to phenol could result in central nervous system disorders(6). It can potentially lead to coma, muscle weakness, burning effect on skin and renal damage. Other irregularities include irritation to the eye, headache, liver damage and gastrointestinal disturbance. Phenol exposure may also cause cancer and tumours. Chronic exposure to phenol is associated with the increased risk of insufficient blood to the heart and also coronary artery diseases (7). This exposure can occur through the skin, inhalation and ingestion. In fact, inhalation is accounted for almost 80% cases of phenol exposure (8).

There are a large number of microorganisms that virtually exist in all natural environments including polluted soil and water (9,10). The existence of these organisms in contaminated environment demonstrates their capacity to utilise or transform both organic and inorganic pollutants. The transformation can either be done by an extracellular enzyme secretion or by the uptake intracellular metabolism or catabolism via uptake (11,12) .This study was aimed at

identifying the isolate AQ5-02 and optimising the physical and cultural conditions for bacterial growth as well as phenol degradation rates.

2. RESULTS AND DISCUSSION

The 16s rRNA region of the isolate was amplified and sequenced; homology was searched in the GeneBank databases and the isolate was found with 99% similarity to *Alcaligenes species* Table 1. The sequence was then deposited at the NCBI GenBank and registered as *Alcaligenes* sp. AQ5-02 with accession number KT693286.

Table 1. First 10 sequences generating significant alignment with Alcaligenes sp. AQ5-02 from NCBI blast

Accession Number	Max Score	Total Score	Query Cover	E Value	Identity
NR113606.1	2403	2403	100%	0	99%
NR043445.1	2362	2362	100%	0	99%
NR104977.1	2353	2352	100%	0	98%
NR025357.1	2335	2335	100%	0	98%
NR042830.1	2276	2276	97%	0	97%
NR117261.1	2119	2119	100%	0	95%
NR133804.1	2117	2119	100%	0	95%
NR115804.1	2117	2117	98%	0	96%
NR116812.1	2098	2098	100%	0	95%
NR117030.1	2089	2089	98%	0	95%
NR116103.1	2085	2085	98%	0	95%
NR108569.1	2078	2078	98%	0	95%
NR137342	2067	2067	98%	0	95%
NR125538	2067	2067	98%	0	95%
NR137343	2061	2061	98%	0	95%
NR025280.1	2061	2061	100%	0	94%
NR043129.1	2060	2060	100%	0	94%
NR121780.1	2058	2058	98%	0	95%
NR137341.1	2056	2056	100%	0	94%
NR121716.1	2056	2056	100%	0	94%

The evolutionary history was concluded using the Neighbour-Joining technique. The optimal tree with a sum of branch length = 20.24931903 was presented. The percentage of replicate

trees in which the related taxa were clustered together in the bootstrap trial (1000 replicates) was revealed next to the branches (13) . The tree was drawn to scale, with branch distances in the same pieces as those of the evolutionary distances used to deduce the phylogenetic tree (14).The evolutionary reserves were calculated using the Maximum Composite Likelihood method in the unit of quantity of base substitutions per site. Meanwhile, evolutionary analyses were conducted in PHYLIP 9(15) as shown in Fig 1.

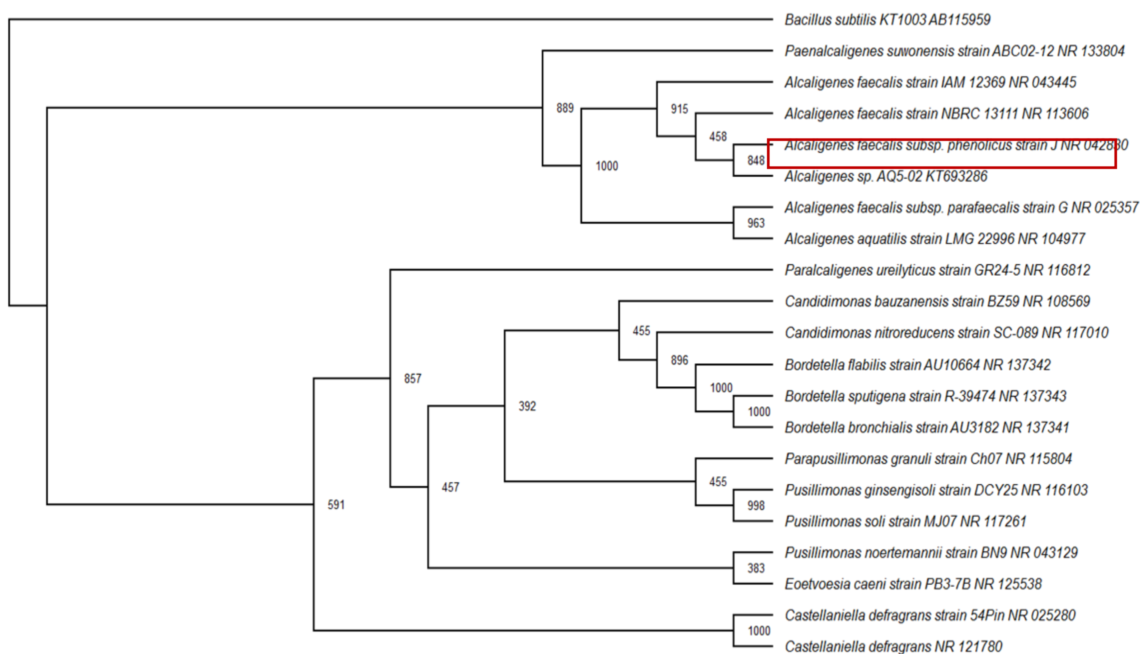


Fig.1. Neighbour-joining tree based on partial 16S RrRNA sequence demonstrating the phylogenetic relatedness of strain *Alcaligenes sp*. AQ5-02 within the species *and Bacillus subtilis* strain KT1003 AB115959 was used as an outgroup

Among the factors that are affecting the growth of bacteria and other microorganisms, the temperature is one of the most important factors and thus considered as a key player in bioremediation. Some studies have reported a temperature range of 10 to 55 ^{o}C (16) for phenol biodegradation. By that, an optimum temperature range from 20 to 35°C was reported for phenol degradation by *Acinetobacter* sp. strain AQ5NOL1(17). Some researchers published that 30 ^{o}C is the optimum temperature for phenol degradation by *Alcaligenes faecalis.* On the other hand, the findings from this study revealed that *Alcaligenes* sp. strain

AQ5-02 has an optimum temperature of 25 to 35ºC for both phenol degradation and bacterial growth. There is no significant difference (p>0.05) discovered between the percentages of phenol degradation with temperatures of 25, 30 and 35 ºC. However, 40ºC and above, there is an increase in both phenol degradation and bacterial growth; this may be due to the denaturation of key phenol degrading enzymes and some other essential proteins for a healthy metabolic activity of the bacteria. Many studies reported that the optimum temperature for phenol biodegradation is between 20 to 35ºC for a mesophilic bacterium. *Alcaligenes* sp. strain AQ5-02, a mesophilic bacterium in this study, has demonstrated a result similar to that of other researchers as shown in Figure 2.

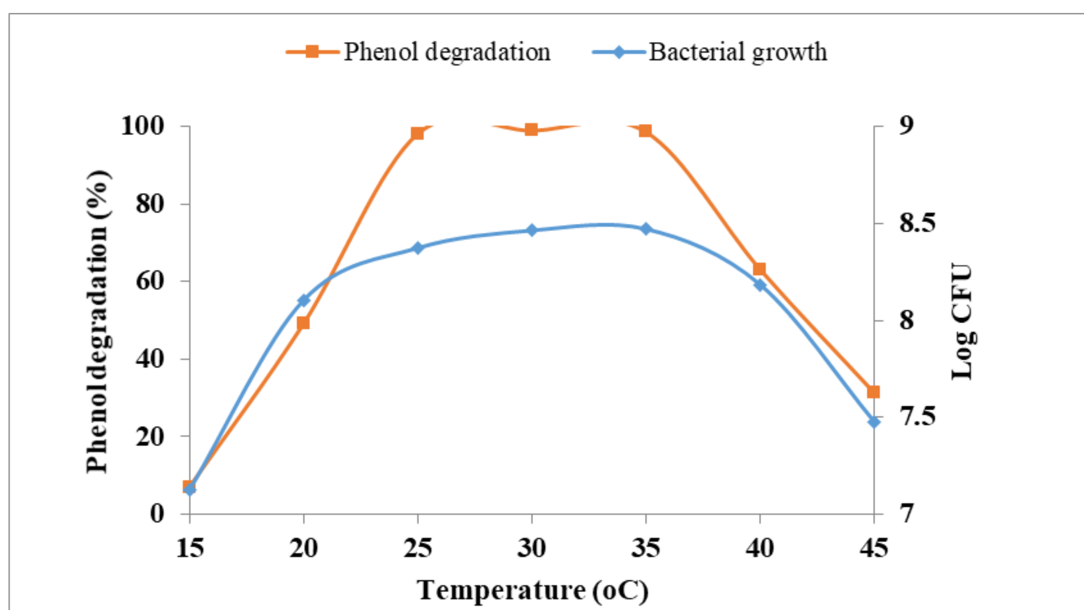


Fig. 2. Effect of temperature on phenol degradation and concentration and growth of *Alcaligenes sp*. AQ05-02

Enhanced phenol degradation was observed at pH 7.5. Both bacterial growth and percentage of phenol degradation are very high over a wide pH range using phosphate buffer (6 - 7.5); however, there was a decline found in both growth and degradation rate (Fig. 3) when using acetate and Tris-HCl. A decrease in bacterial growth values was observed at low, extreme acidic and alkali conditions. The growth and phenol degradation by *Alcaligenes* sp. AQ05-02 were identified to be optimal within the pH range of 7 -7.5 (phosphate buffer).

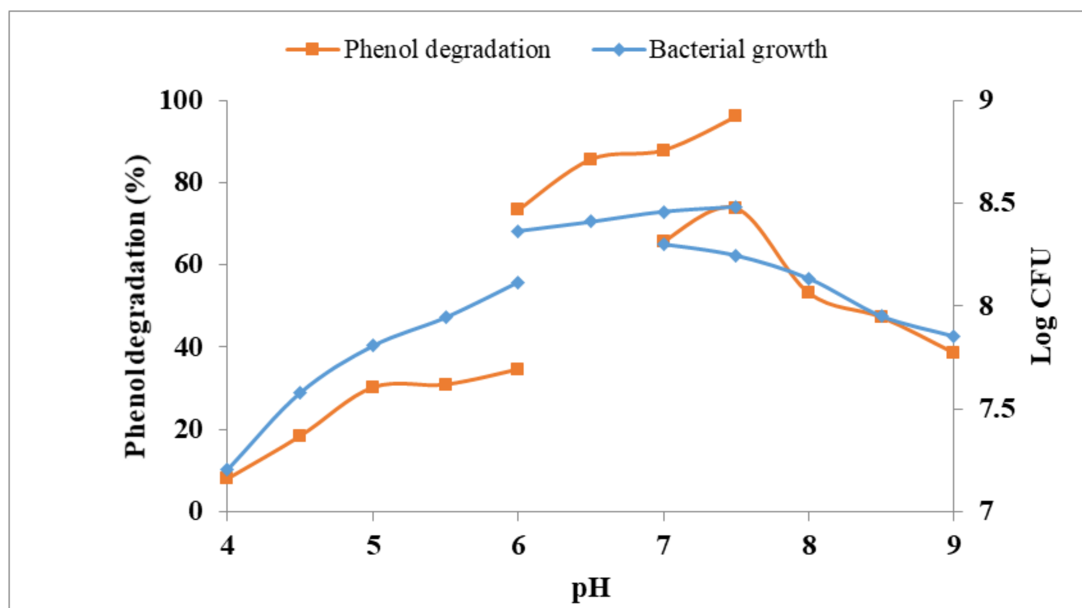


Fig.2. Effect pH on phenol degradation and concentration and growth of *Alcaligenes sp*. AQ5-02

Effects of sources of nitrogen were presented in Fig. 4. Ammonium sulphate is the best nitrogen source compared to other organic and inorganic nitrogen sources such as ammonium carbonate, alanine, glycine, and sodium nitrate. Using ammonium sulphate in the culture media had given 98.7% phenol degradation and the highest bacterial growth. Many studies have referred ammonium sulphate as the best nitrogen source for phenol biodegradation by bacteria such as *Rhodococcus* UKM-P (18), *Acinetobacter* sp. strain AQ5NOL 1 (19) and *Pseudomonas putida* (20). Ammonium sulphate can be easily assimilated and absorbed by the cells (21). The effects of ammonium sulphate concentration were also tested within a range of 0 – 0.8 g/L. Ammonium concentration of 0.4 g/L was found to be the optimum concentration for both phenol degradation and bacterial growth; there is no significant $p > 0.05$ between 0.4 and 0.5 g/L. There was a decline in phenol degradation and bacteria growth at low concentration and also high concentration as shown in Fig. 5,this is agreement with other reported studies (22)

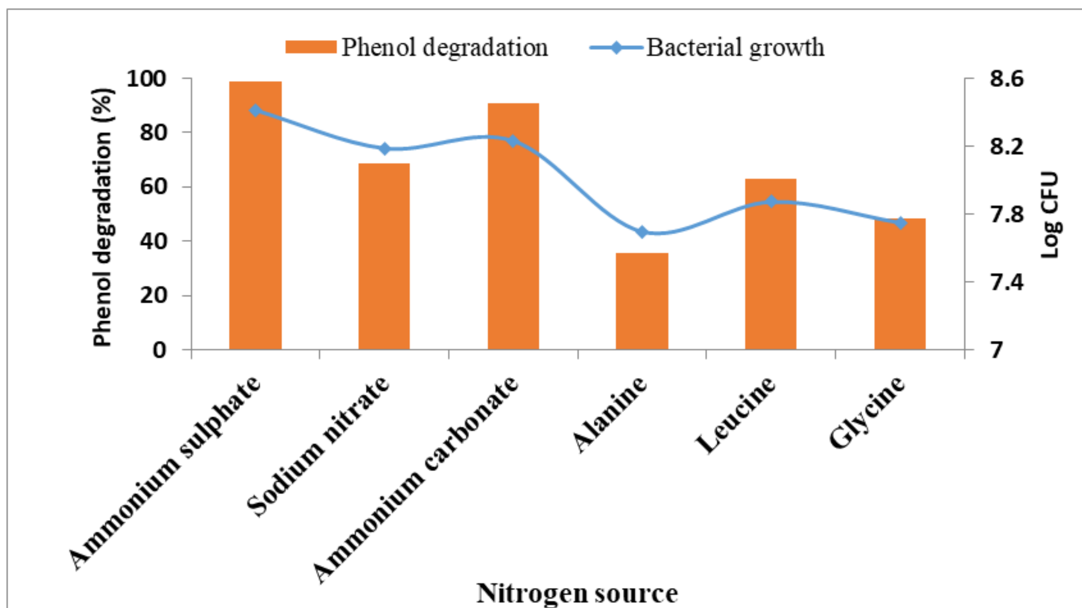


Fig. 4. Effect of nitrogen source on phenol degradation and concentration and growth of *Alcaligenes sp*. AQ5-02

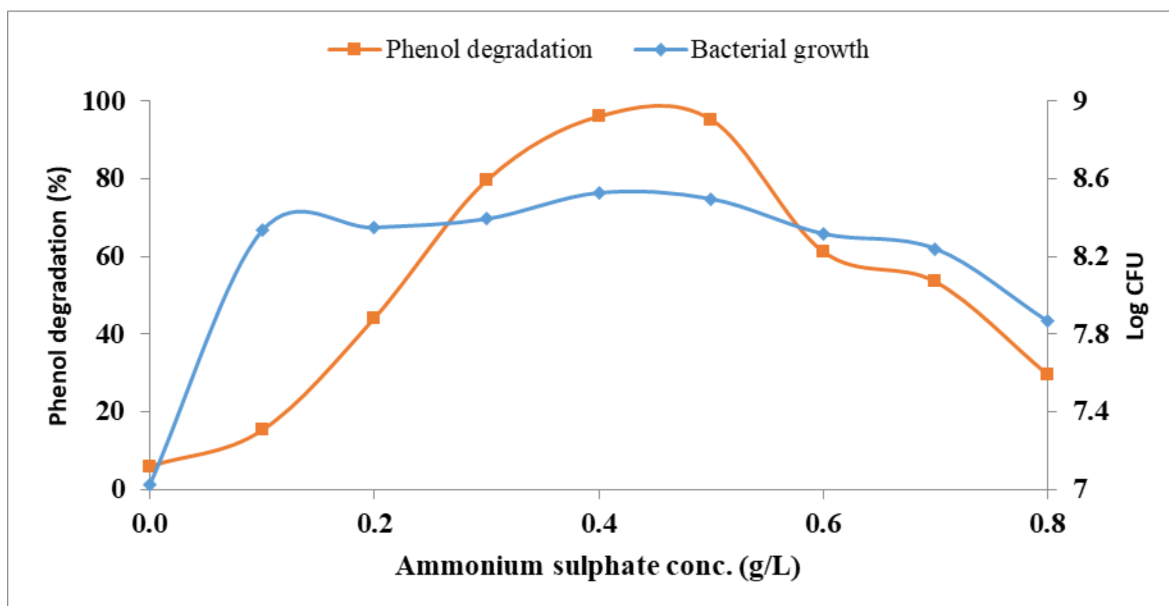


Fig.5. Effect of ammonium sulphate concentration on phenol degradation and bacterial growth on *Alcaligenes sp*. AQ5-02

Fig. 6 illustrates the effects of salinity on the ability of *Alcaligenes* sp. strain AQ5-02 to degrade phenol and also its effects on the growth of *Alcaligenes* sp. strain AQ5-02 to ascertain whether or not *Alcaligenes* sp. strain AQ5-02 can be used to remove phenol in salty areas

including ocean and near coastal regions where there are reported cases of phenol pollution. Sodium chloride concentrations of 0.1 and 0.15 g/L are the best for the growth of *Alcaligenes* sp. strain AQ5-02 and its phenol degrading capability. High NaCl concentration showed an inhibitory effect on the bacterial growth as well as phenol degradation that might be due to an osmotic stress leading to the rupture of bacterial cells, thus inhibiting the bacterial growth and phenol degradation (23)

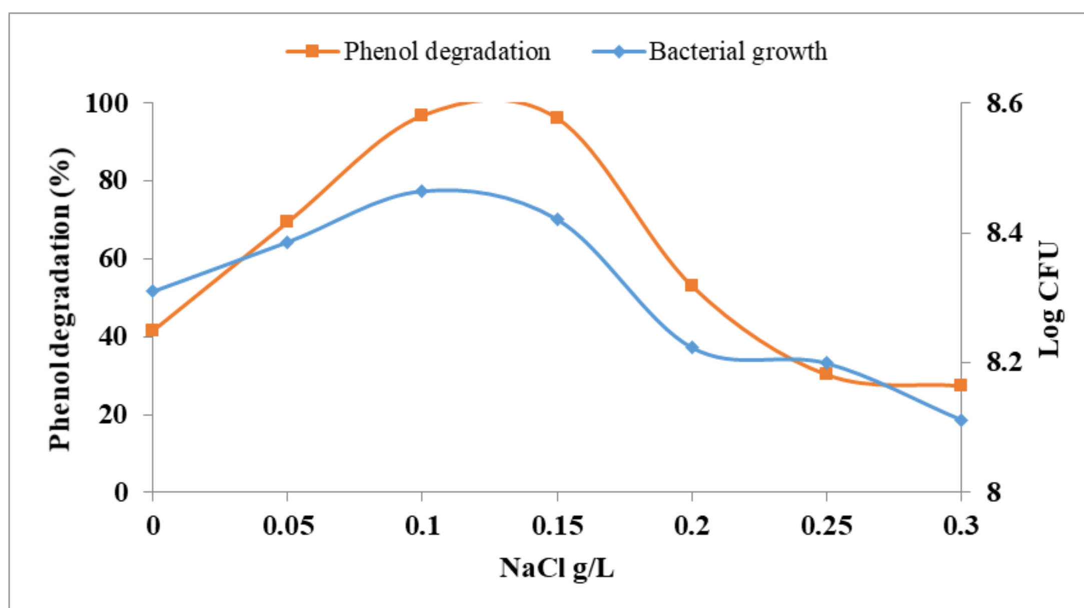


Fig.6. Effect of salinity on phenol degradation and concentration and growth of *Alcaligenes* sp. AQ5-02

Table 2. CCD response can be used to generate response surfaces and 3D contours to establish the effects of these factors on phenol degradation. Y is the response (percentage phenol degradation), A (temperature), B (pH), C (ammonium sulphate Concentration), D (Sodium chloride concentration).

Table 2: Central composite design and its experimental and predicted values of phenol degradation by *Alcaligenes sp.* AQ5-02

Trial Run	Factor 1	Factor 2	Factor 3	Factor 4	Response	
	Temperature (°C)	pH	Nitrogen source g/L	Salinity g/L	Actual value (%)	Predicted value (%)
1.	20	7.5	0.45	0.15	36.08	37.79
2.	30	7.5	0.45	0.15	98.30	98.47
3.	35	6.0	0.10	0.00	83.81	84.98
4.	35	6.0	0.80	0.30	42.00	43.27
5.	30	7.5	0.45	0.15	97.90	98.47
6.	35	9.0	0.10	0.30	59.00	61.34
7.	30	7.5	0.45	0.45	93.90	92.69
8.	35	9.0	0.80	0.30	33.81	34.68
9.	30	7.5	0.45	0.15	98.00	98.47
10.	35	6.0	0.10	0.30	77.94	77.73
11.	25	6.0	0.80	0.30	82.00	80.90
12.	25	6.0	0.10	0.30	94.44	97.02
13.	30	7.5	0.45	0.15	100	98.47
14.	30	7.5	0.45	-0.15	68.67	68.54
15.	25	9.0	0.10	0.30	86.40	84.37
16.	35	6.0	0.80	0.00	60.00	62.16
17.	30	10.5	0.45	0.15	77.52	77.50
18.	30	7.5	0.25	0.15	69.25	67.78
19.	35	9.0	0.80	0.00	67.56	66.19
20.	25	9.0	0.80	0.00	44.30	44.64
21.	30	7.5	0.45	0.15	99.40	98.47
22.	30	7.5	0.45	0.15	98.00	94.47

23. 25	9.0	0.10	0.00	41.40	41.34
24. 40	7.5	0.45	0.15	43.10	40.04
25. 30	7.5	1.45	0.15	36.50	36.62
26. 30	4.5	0.45	0.15	87.44	86.11
27. 35	9.0	0.10	0.00	80.00	81.23
28. 25	6.0	0.10	0.00	42.10	41.36
29. 25	6.0	0.8	0.00	38.00	36.87
30. 55	9.0	0.8	0.30	76.00	76.05

The F-value (309.55) inferred that phenol degradation by Acaligenes sp.AQ5-02 is a significant model $p < 0.0001$ and also the lack of fit is not significant $p > 0.0001$ the R^2 value of 0.9966, Adj R^2 value of 0.9933 (Table 3) are all indicating that the model has a good fit (24).

Table 3: Analysis of variance (ANOVA) for the phenol degradation response surface reduced quadratic model

Source	**Sum of squares**	**DF**	**Mean square**	**F Value**	**Prob>F**
Phenol degradation					
Model	15740.60	14	1124.33	309.55	<0.0001*
Residual	54.48	15	3.33		
Lack of fit	48.93	10	4.89	4.41	0.0577
Pure error	5.55	5	1.11		
Cor Total	15795.08	29			
Std. Dev	1.91	R-Square	0.9966		
Mean	70.40	Adj R-Squared	0.9933		
C.V	2.71	Pred R-squares	0.9817		
Press	289.83	Adeq Precision	47.33		

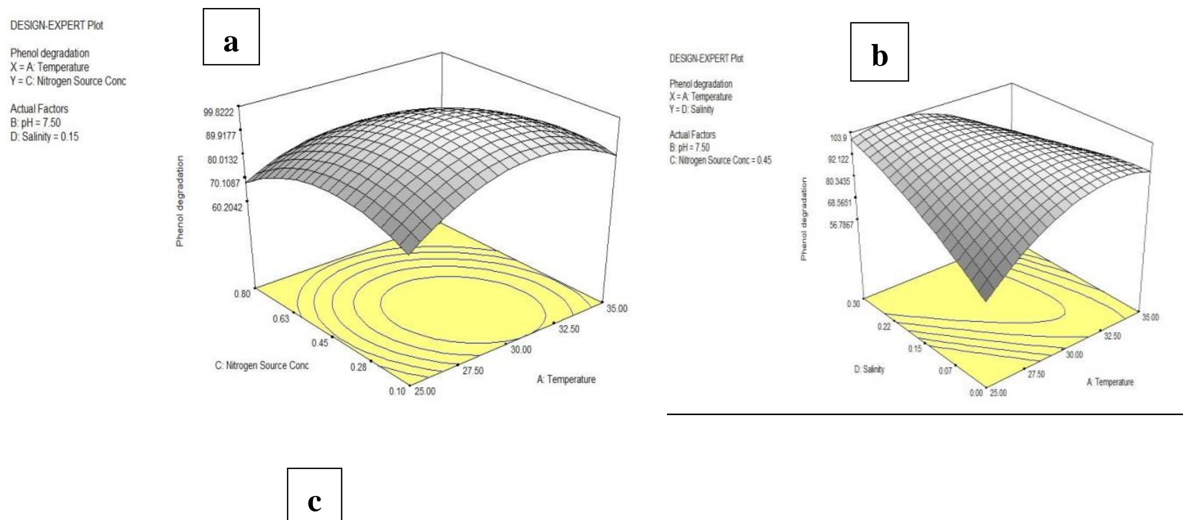
The results from mathematical optimisation were reasonably promising. Statistical analysis is resolute that approximately 98 % phenol removal was attained under optimal conditions. As shown in Table 3, pH, Ammonium sulphate concentration, and salinity were found to be statistically significant $P < 0.0001$.

Table 4: Model coefficient and their significances estimated by multiples linear regression for percentage phenol degradation

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
A	7.6	1	7.62	2.10	0.1682
B	111.20	1	111.20	30.61	<0.0001*
C	1455.80	1	1455.80	400.81	< 0.0001*
D	874.59	1	874.59	240.79	< 0.0001*
A^2	6079.20	1	6079.20	1673.72	< 0.0001*
B^2	475.81	1	475.81	131.00	< 0.0001*
C^2	3669.34	1	3669.34	1010.24	< 0.0001*
D^2	546.52	1	546.52	150.47	< 0.0001*
AB	13.95	1	13.95	3.84	0.0689
AC	336.17	1	336.17	92.55	< 0.0001*
AD	3958.30	1	3958.30	1089.79	<0.0001*
BC	60.68	1	60.68	16.71	0.0001*
BD	159.52	1	159.52	43.92	<0.0001*
CD	135.26	1	135.26	37.24	0.0001*

3D contour plots of response surface disclose the interaction of two factors at a time maintaining all other factors at a constant level (Fig. 7). It enlightens how the various

parameters interact with each other. The interaction between temperature and nitrogen source concentration reveals the correlation in which the response low at both extremes, and at 30 ^{O}C and 0.45 g/L ammonium sulphate the response was precise high (Fig. 7a). The interaction between temperature and salinity revealed the interrelation between temperature and salinity in which a contour was established. It shows a high response at a temperature of 30 ^{O}C with a decline the response at both extreme temperatures, but for salinity, the highest response is up to 0.30 g/L NaCl (Fig. 7b). There was an excellent interaction between pH and nitrogen source concentration. At all extremes, the percentage degradation was very low, but at the pH size of 7.50 and ammonium sulphate 0.45 g/L, the percentage phenol degradation inclines to be very high (Fig. 7c). As shown in (Fig. 7d) the interaction between pH and salinity concentration with highest phenol degradation at pH 7.50 with both upper and lower pH reduces the capability of bacteria to degrade phenol but can degrade effectively at 0.3 g/L NaCl. Also, 0.45 g/L ammonium sulphate and salinity of 0.15 to 0.30 shows a better degradation (Fig. 7e).



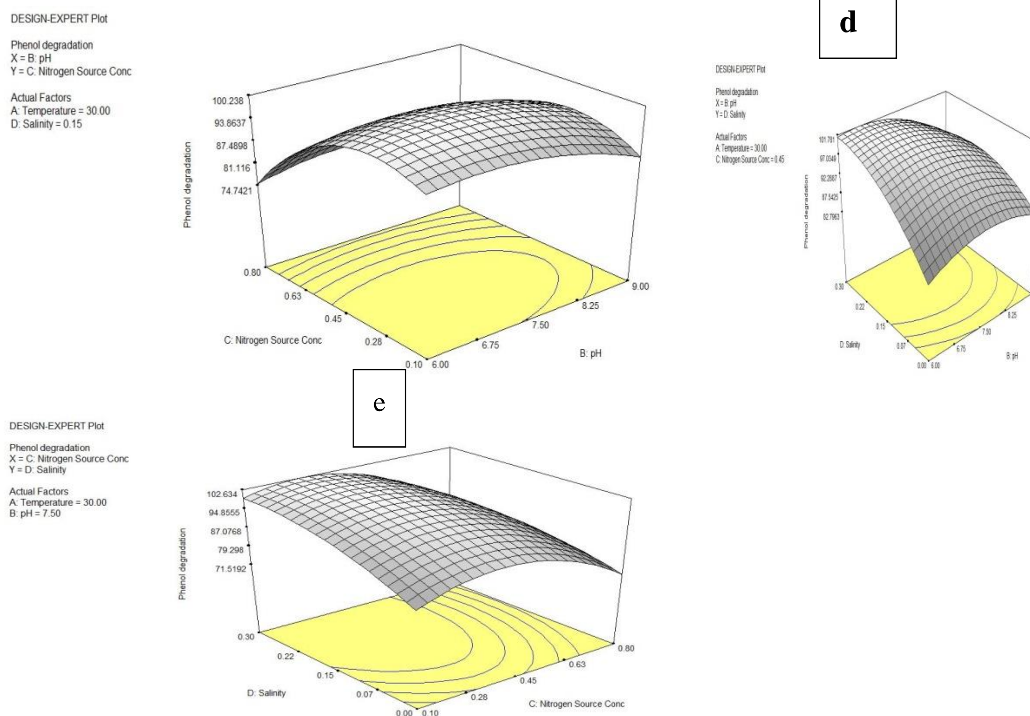


Fig.7. (a) Temperature vs Nitrogen source concentration, (b) Temperature vs salinity (c) Nitrogen source concentration vs pH (d) Salinity vs pH (e) Nitrogen source concentration vs salinity

Fig 7(a-d) demonstrated the effect of various phenol concentration on bacterial growth and the phenol degradation. Alcaligenes species are well recognised for their capacity to degrade phenol and other-other poly Aromatic Hydrocarbons (25,26). The isolate AQ5-02 can degrade 400, 800 and 1000 mg/L within 48, 60 and 72 hours respectively. As shown in fig 7d the isolate is unable to grow and mineralize phenol at concentrations 1200 mg/L, thus the concentration of 1200 mg/L has an inhibitory effect on the metabolic functions of the bacteria. Though the ability of Alcaligenes sp. to tolerate and utilised phenol as a sole source of carbon has been reported, strain AQ05-02 is among the few isolates that can degrade up to 1000 mg/L and at the same tolerance heavy metals to the best of our knowledge. Previous studies report an *Alcaligenes faecalis* can degrade 1.2 g/L phenol within 72 hours (27,28).Phenol is a very toxic organic compound that has an inhibitory effect on the bacterial growth. Thus, there is no phenol degradation noticed under high phenol concentrations. Also, some bacteria such

as *Rhodococcus* can degrade up to above 1100 mg/L (17)

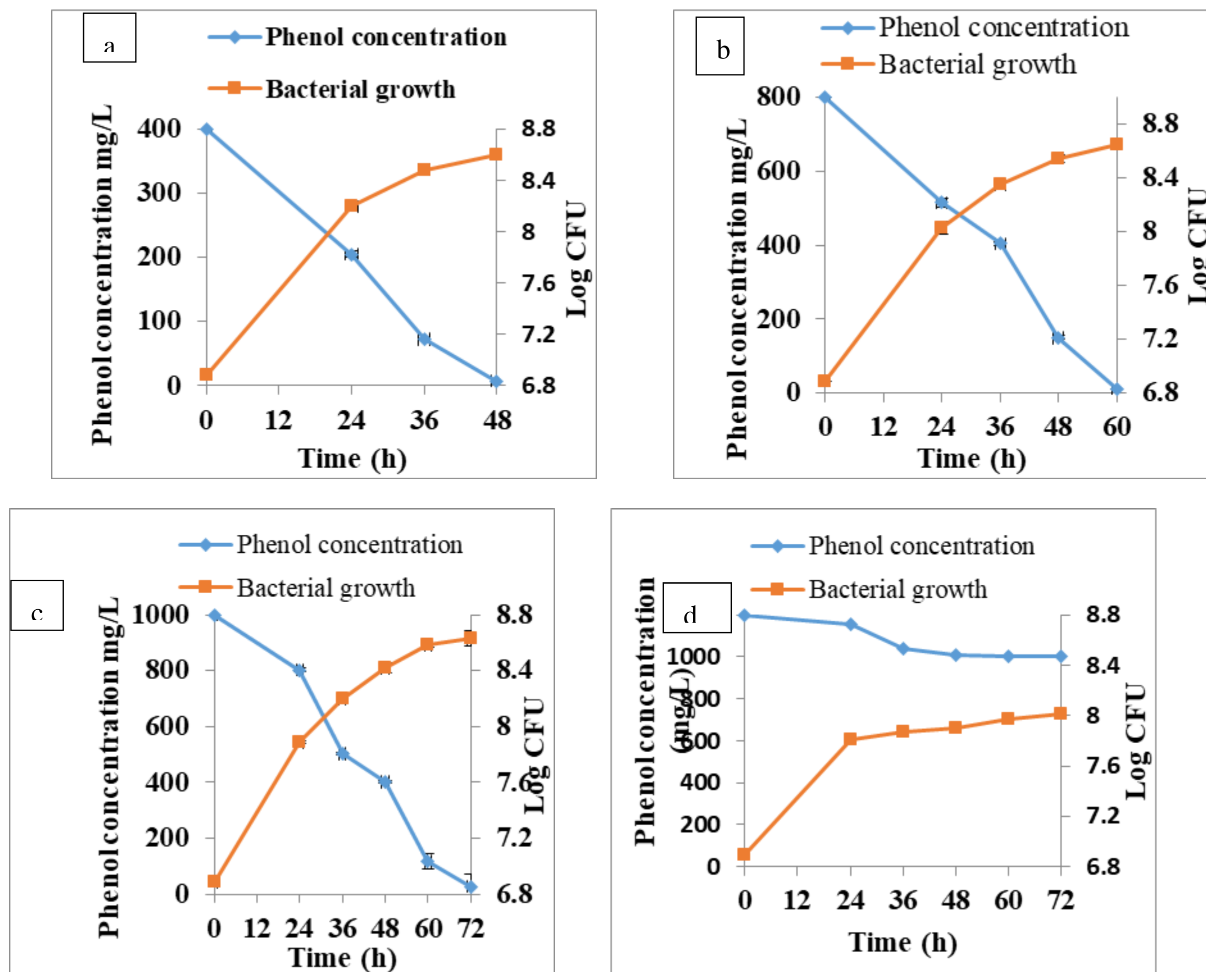


Fig.7. Effect of initial phenol concentration on the bacterial growth and percentage phenol degradation by *Alcaligenes sp*. AQ05-02 (a) 400 mg/L (b) 800 mg/L (c) 1000 mg/L (d) 1200 mg/L

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On the effects of 5 ppm heavy metals, there is more than 70 % degradation has been achieved within 48 hours even the media was supplemented with Cr and Iron, while only 60% removal was achieved within 72 hours using Cu. As and Nickel were reported to have a severe inhibitory effect on the percentage of degradation rate **Figure 8.** The result is in line with some studies that suggested microbes can degrade phenol co-containminated with heavy metals (30)

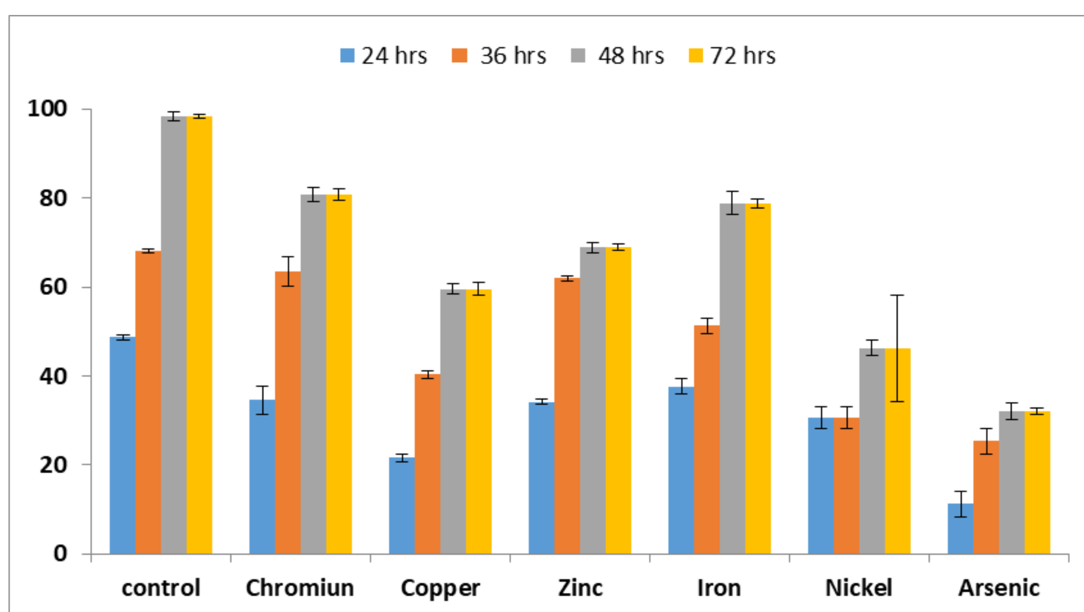

Fig.8. Effect of heavy metals on the bacterial growth and percentage phenol degradation by Alcaligenes *sp*. AQ5-02

3. EXPERIMENTAL

3.1 Sampling and Isolation

Wastewater sample was collected from an industrial area in Sarawak at GPS location Nº3º01.658', Eº101º33.777'. The phenol-degrading bacterium AQ5-02 was isolated from a

sample. Four millilitres of water sample was mixed with 40 ml minimal salt medium (MSM) containing 0.05 % phenol as the sole source and incubated at room temperature with shaking at 150 rpm for three days(31) .The culture was then inoculated on a mineral medium agar plate enhanced with phenol and incubated at room temperature for three days. Distinct colonies were subjected to further isolation by several cycles of sub-culturing on mineral medium agar plates until a single pure colony was obtained. The single colony was then inoculated in phenol mineral salt medium with 0.5 g/L phenol and percentage phenol degradation, and bacterial growth was monitored using 4-aminoantipyrine colorimetric assay at 510 nm and OD600 nm respectively(25).

3.2 Mineral salt media

K_2HPO_4 (400 mg/L), KH_2PO_4 (200 mg/L),$MgSO_4$, (100 mg), $Fe_2(SO_4).H_2O$ (10mg/L), NaCl (100 mg/L), $NaMoO_4.2H_2O$ (10 mg/L), $MnSO_4.2H_2O$ (10 mg/L) and $(NH_4)_2SO_4$ (400 mg/L) were put in 1 L of distilled water with the pH adjusted to 7.2 before being autoclaved at 121ºC for 15 min and 500 mg of phenol was introduced as the only source of carbon prior use (32).

3.3 Genetic identification of the strain

Cells from the exponentially growing culture of AQ5-02 were used for molecular identification. Genomic DNA was extracted and used as a template for 25 µL volume of polymerase chain reaction (PCR). The PCR reaction mixture comprises the composition of 12.5 µL master mix (contains Taq polymerase and dNTP), 9.5 µL ddH_2O, 1µL DNA template, 1µL forward primer (5’-AGA GTT TGA TCC TGG CTC AG-3’) and 1µL reverse primer(5'-TAC GGT TAC CTT GTT ACG ACT T-3') (33). The PCR conditions were: 4 min of initial denaturation at 94°C for 4 min; 94°C denaturing for 1 min (30 cycles), 1 min annealing at 58°C, and 1 min extension at 72°C and one cycle of final extension at 72°C for 7 min. Blast search was done in the National Centre for Biochemical Information (NBCI) and Phylogenetic tree analysis and evolutionary relationship of taxa: twenty (20) 16s rDNA sequences of related *Alcaligenes* species were obtained from gene bank and aligned using Clustal Omega by PHYLIP.

3.4 4AAP assay

Phenol degradation was determined using 4-aminoantypyrine, a colorimetric assay based on

the reaction of the reagent with phenol in the presence of potassium ferric cyanide under alkaline pH and potassium ferric cyanide. Absorbance was measured at 510 nm

3.5 Effects of temperature and pH

The optimum temperature for phenol degradation and bacterial by *Alcaligenes* sp. AQ5- 02 was proved by growing the bacteria under various temperatures ranging from 15 to 45°C. While pH. Ranging from 4.0 to 9.0 was used, acetate buffer was used for pH 4.0 to 6.0, phosphate buffer pH 6.0 to 7.5 and Tris-HCl for pH 7.0 to 9.0. At the end of incubation period (72 h), phenol degradation and bacterial growth were measured.

3.6 Effects of Nitrogen sources and best concentration

Various nitrogen sources for both organic and inorganic were used to determine the best nitrogen source. The nitrogen sources utilised for this study are ammonium sulphate, sodium nitrate, nitrogen bicarbonate leucine, glycine, and Alanine. Concentrations ranging from 0 to 0.8 g/L were used for ammonium sulphate in the MSM to evaluate the best concentration for growth and phenol degradation by *Alcaligenes sp*. AQ5- 02.

3.7 Effects of Salinity

Effects of salinity on phenol degradation and bacterial growth by *Alcaligenes* sp. AQ5- 02 was determined by growing the bacteria under various sodium chloride concentrations ranging from 0 to 0.3 g/L in 100 mL MSM.

3.8 Statistical optimisation

Central composite design (CDD) was used for the statistical optimisation in the study and it is one of the techniques in response surface methodology (RSM), it is a statistical tool used in modelling and investigating interest response when exposed to the effect of several independent factors. The main aim of using RSM in the research is to optimise the response (percentage phenol degradation). There are four major factors and interactions were analysed and optimised by RSM. The selected major factors are temperature, pH, salinity and $(NH4)_2SO_4$. This design comprises of 30 experimental trials. All four independent parameter were tested at five different levels: $-\alpha$, -1, 0, +1, $+\alpha$. The data obtained were fitted to a second-order polynomial equation, which generates a model equation that relates Y to the independent factors. Analysis of variance (ANOVA), p-values and confidence levels were

calculated using a tool in the Expert-Design software version 6.0. Optimal values from linear and interactions of factors were estimated using 3D plots were used to estimate the interactions or otherwise of the factors.

3.9 Effect of different phenol concentration

Phenols with concentrations ranging from 0.2 to 2.4 g/L were used in the MSM as the only source of carbon to ascertain the effects of various phenol concentrations on the bacterial growth as well as phenol degradation rate.

3.10 Tolerance to heavy metals

Heavy metals resistance was assessed by adding 5 mM of different heavy metals namely arsenic (As), cadmium (Cd), cobalt (Co), copper (Cu), chromium (Cr), lead (Pb), mercury (Hg), nickel (Ni), silver (Ag) and Zinc (Zn) with various concentrations to observe the effects of heavy metals on phenol degradation and the bacterial growth

4. CONCLUSION

In the furtherance of isolation for novel and competent microbial strain in phenol degradation, a new bacterial strain was reported, which is moderately capable of utilising a high concentration of phenol as the sole source of carbon and energy. This strain can also tolerate relatively high phenol concentrations and resist heavy metals such as Cr, Fe, and Cu. Hence, this strain is a very suitable candidate for further studies on the bioremediation of phenol and heavy metals.

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How to cite this article:
Aisami A, Ahmad SA, Yasid NA, Johari WLW, Shukor MY. Phenol biodegradation and optimisation of cultural and physical conditions by the free cells of newly isolated phenol degrading *alcaligenes* sp. AQ5-02. J. Fundam. Appl. Sci., 2021, *13(1), 1340-1360.*