Open Journals of Bioscience Research (OJBR)

Article Details:
openjournalsnigeria.org.ng/pub/ojbr20200103.pdf
Article Ref. No.: OJBR20200103
Volume: 1; Issue: 1, Pages: 28-43 (2020)
Accepted Date: March 05, 2020

Open Journals Nigeria (OJN)
Open Access | Bi-annual | Peer-reviewed
www.openjournalsnigeria.org.ng
editorial@openjournalsnigeria.org.ng

RESEARCH ARTICLE **OJBR20200103**

EFFECT OF TEMPERATURE AND PH ON PHENOL BIODEGRADATION BY A NEWLY IDENTIFIED *SERRATIA* SP. AQ5-03

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ABSTRACT

Phenol is mainly used by the industries to produce a variety of chemical products such as resins, textiles, pesticides, plastics and explosive. The wide use of phenol and other phenolic compounds by industries, has resulted in an increased presence of these toxic compounds in the environment as pollutants. Bio-removal of phenol by microorganisms especially bacteria has been demonstrated to be the most effective and economical approach compared to physio-chemical methods. The search for efficient phenol-degraders especially local sources to remediate local phenol pollution is important as indigenous bacteria usually have better survival and resilient to local geographical conditions. In this study, a phenol-degrading microorganism was isolated from local soil and waste water bodies. Identification was carried out using gram staining, 16s rRNA gene sequencing and molecular phylogeny analysis using the Phylip software. The isolates were inoculated in mineral salt media with 0.5 g/L phenol as the sole source of carbon. Phenol degradation was determined using 4-amino antipyrine method. Physical and cultural conditions influencing phenol degradation such as pH and temperature were optimized via one-factor-at-a-time. Through phylogeny analysis, the isolate was identified as Serratia sp. and the sequence was deposited the NCBI Genebank and accession number KT693287 was assigned to the bacteria. The highest degradation was achieved at pH 7.5 (phosphate buffer) and temperature of 30°C. Ammonium sulphate was established to be the best nitrogen source at the concentration of 0.4 g/L and a sodium chloride concentration of 0.15 g/L.

Keywords: *bio-removal, pH, temperature phylogeny, phenol, 16s rRNA*

INTRODUCTION

Environmental pollution is one of the major concerns in the 21st century; where billions of tonnes of harmful chemicals are produced by industries such as petroleum, paints, food, rubber, and plastic. These toxicants finds their ways into the environment through air, soil, and water. Combustion of fuel, burning activities and power stations are the major sources of air pollution where volatile hydrocarbons are released into the air (DOE, 2009). Air pollutions can lead to many respiratory, cardiovascular and liver diseases (Brook *et al.*, 2004; Ko and Hui, 2010). The discharge of untreated harmful compounds and heavy metals are the primary sources of water and soil pollution. In addition, oil spillage from petroleum industries contributes significantly to the global incidence of soil and water pollutions (Hossain, *et al.*, 2009).

Among the phenolic compounds, phenol is the most commonly used by industries and is the precursor for the synthesis of many industrial chemicals. Plastic, coke and petroleum industries produce the highest effluents containing phenol (up to 7 g/L). (Luo *et al.*, 2009; Luo *et al.*, 2011; Maheshwari and Gupta, 2016). Oil spills, deck overspill leakages from vessels, pipelines and storage tanks and offshore disposal of waste are also major sources of water pollution (Aghalino and Eyinla, 2009; Matkin, *et al.*, 2008). These industrial waste are treated with physiochemical methods. However, physiochemical methods alone are not efficient due to its high cost and also the generation of secondary pollutions (González, *et al.*, 2006; Suhaila, *et al.*, 2013b). The existence of these microorganisms can be used for the biodegradation of phenol and other phenolic compounds.

Bioremediation as an alternative method to physicochemical methods is a very cost-effective method, and an environmentally friendly way of controlling pollutions (Ali, *et al.*, 2009; Desai, *et al.*, 2010). To optimise the bioremediation ability of microorganisms to biodegrade phenol, an appropriate inoculum size, pH and temperature are crucial factors (Pradeep *et al.*, 2015).

Although a lot of research has been carried out on the degradation of phenol by microorganisms yet there is need for identifying more organisms that are capable of degrading phenols. To date, there are very few locally-isolated phenol-degrading microorganisms (Ahmad *et al.*, 2011; Fereidoun, *et al.*, 2007). There is a need to increase the reservoir of a phenol-degrading microorganism to prepare for phenol remediation in the current and future scenario.

MATERIAL AND METHODS

CHEMICALS, REAGENTS, AND EQUIPMENT

All the chemicals, reagents, and equipment used in this study are listed in Appendix I.The chemicals and reagents are of analytical grades.

BACTERIAL SAMPLING AND SCREENING

Soil and wastewater samples were obtained from industrial areas from industrial areas in Selangor Johor, Kedah, Sarawak, and Melaka in Malaysia. The samples were kept in a 50 mL propylene centrifuge tubes in ice (0ºC) while being transported to the laboratory. The samples were stored in a -20ºC freezer until required for use. The phenol-degrading bacteria were isolated based on their growth on mineral salt media with 0.5 g/L phenol as the sole source of carbon and incubated at 27ºc with constant shaking at 180 rpm for 24 hours (Bai *et al.*, 2007).

MAINTENANCE OF THE ISOLATES

The isolates were sustained on nutrient agar slant culture at 4ºC for routine use and in glycerol stock at -20 º C for long-term storage.

GRAM STAINING

A light suspension of cells from 24 hour nutrient broth culture was prepared. A drop of the cell suspension was added to a clean glass slide and spread with a loop over the slide surface and allowed to air dry. The slide was covered with methanol and allowed to evaporate at room temperature. Heat-fixed smear slides were placed on staining tray and were gently flooded with crystal violet and allowed to stand for 1 minute. The slide was then tilted slightly and gently rinsed with distilled water using a wash bottle, then gently flooded with Gram's iodine and allowed to stand for 1 minute. The smeared slides were slightly and gently rinsed with distilled water using a wash bottle. The smear appeared as a purple circle on the slide. It was then decolourised using 95% ethyl alcohol applying the alcohol dropwise for 10 seconds and rinsed immediately with distilled water. It was then gently flooded with safranin to counter-stain and allowed for 1 minute and rinsed with distilled water. It was viewed under an under oil immersion microscope.

ANALYTICAL METHOD

Phenol degradation was determined using 4-amino antipyrine, a colourimetric assay based on the reaction of the reagent with phenol in the presence of potassium ferric cyanide under alkaline pH. The absorbance was read at 510 nm (Arif, *et al.,* 2013).

MINERAL SALT MEDIA

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

PROCEDURE FOR 4-AMINOANTYPRINE ASSAY

Sample (1.5 ml) was first centrifuged for 15 minutes at 10,000 × *g* and the supernatant was collected. One hundred µL each of 4- amino antipyrine and potassium ferric cyanide solutions were added to the 1 mL of the supernatant, and the pH was adjusted to 10 using 1 M NaOH. The mixture was incubated at room temperature for 15 minutes, and the absorbance was read against the blank at 510 nm using a spectrophotometer.

MOlECULAR IDENTIFICATION OF THE ISOLATE

GENOMIC DNA EXTRACTION

The genomic DNA was extracted using bacterial DNA extraction protocol according to the manufacturers' (Thermo Scientific GeneJet Genomic DNA Extraction) instruction. Pellets from a 24h old nutrient broth bacterial cells were

utilised for the extraction. The cells were harvested by centrifugation at 10000 × *g* for 10 min, and then 180 µL of digestion solution was added followed by 20 µL proteinase K solution and mixed exhaustively by vortexing until a homogenous solution was obtained. The resultant solution was then incubated at 56 ºC in a water bath for 1 min for the cells to lyse entirely. RNase A solution (20 µL) was added and mixed thoroughly and incubated at room temperature for 10 min. Then, 200 µL of lysis solution was then added to the mixture and was mixed thoroughly using a vortex mixer to attain homogeneity. By pipetting, 400µL of ethanol (50%) was added and mixed. The sample was then transferred into the GeneJet genomic DNA purification column with a collection tube and centrifuged for 1 min at 6000× *g*. The flow-through solution and the collection tube was discarded. Into a new tube, the GeneJet genomic DNA purification column was positioned and 500 µL of wash buffer I was introduced and centrifuged for 1 min at 8000 × *g* followed by the addition of 500 µL of wash buffer II to the column and centrifuged for 3 min. The step was repeated to get maximum yield. The collection tube was discarded, which contained the flowthrough of the solution. The GeneJet genomic DNA purification column was transferred into a 1.5 mL sterile microcentrifuge tube. Elution buffer (200 µL) was pipetted at the centre of the GeneJet genomic DNA purification column membrane and incubated at room temperature for 2 min and centrifuged at 8000 × *g* for 1 min to elute the DNA. The purification column was discarded and the DNA collected in a 1.5 ml centrifuge tube. The DNA was stored at -20ºC and 10 µL of DNA was loaded onto a 1% (v/v) agarose gel electrophoresis using λHindIII DNA marker (Fermentas, USA) as a template for the polymerase chain reaction (PCR).

POLYMERASE CHAIN REACTION

Biometra T-Gradient Thermocycler was used for conducting the PCR. The Universal primers**:** *27F: 5'-AGA GTT TGA TCC TGG CTC AG-3'* forward and *1492R: 5'-TAC GGT TAC CTT GTT ACG ACT T-3'*, reverse were used for the polymerase chain reaction (PCR) with Genomic DNA as the template (Karamba *et al.,* 2015). The 25 µL PCR mixture composed of 1 µL forward primer, 1 µL reverse primer, 1 µL DNA template, 9.5 µL master mix, 12.5 µL deionized water and 1 µL of the reverse and forward primers of 16S rRNA, respectively. The polymerase chain reaction (PCR) was carried out under the following conditions: 1 cycle of initial denaturation at 96ºC for 4 min; 30 cycles (94ºC denaturing for 1 min, 52.3ºC annealing for 1 min, 72ºC extension for 1 min) and one cycle of final extension at 72ºC for 7 min. Preservation was done at 10ºC. The presence of the amplified 16S rRNA was confirmed by using 1.0% agarose gel electrophoresis (Fermentas, USA) and was viewed with UV transilluminator (UPV, USA).

SEQUENCE ANALYSIS

The amplified DNA was sent for sequencing at MyTACG Bioscience Laboratories SDN BHD. The sequences were edited using MEGA version 6 and aligned for homology by the use of BLAST 2 (Basic Local Alignment System Tool) sequences**.**

PHYLOGENETIC TREE ANALYSIS AND EVOLUTIONARY RELATIONSHIPS OF TAXA

Phylogenetic trees were constructed using the PHYLIP suite of programs (Felsenstein, 1992). The program DNADIST was used to compute the evolutionary distance in the neighbour-joining method (Saitou and Nei, 1987). The distance

matrixes were written as an output file. SEQBOOT component checked and analysed the confidence levels of branches within the trees in the PHYLIP package, and 1000 bootstraps were used (Felsenstein, 1985). A CONSENSE program was used to construct the consensus tree for the topologies (Margush and McMorris, 1981). The TreeView program was used to view the tree (Page, 1996). Four models for base substitution were used; (Jukes and Cantor, 1969), Kimura (Kimura, 1980), F84 model (Felsenstein, 1992; Kishino and Hasegawa, 1989) and LogDet distance (Lockhart, *et al.*, 1994).

EFFECT OF PH AND TEMPERATURE

The preceding literature on biodegradation studies reported that bacteria have a preference of pH ranges of 6.5 to 7.5 for both bacterial growth and biodegradation rates (Demoling and Bååth, 2008). pH ranging from 4 to 9 was selected for this research. The buffers used were acetate buffer from pH 4 to 6, phosphate buffer from pH 6 to 7.5 and Tris-HCl buffer (pH 7 to 9). Temperature has being shown to be among the most vital factors in bioremediation (Margesin, *et al.*, 2005). In this study, temperatures ranging from 15 to 45ºC with an interval of 5 ºC was used to determine its effect on both the bacterial growth and phenol degradation rates.

STATISTICAL ANALYSIS

All conducted experiments were carried out in triplicates. Experimental errors were shown in Error bars in all graphs represented as standard errors (SE) from three determinations. All data were analyzed using GraphPad Prism 5. A one-way ANOVA (95% confidence interval) and t-test (LSD) for biodegradation were utilised to evaluate the differences among parameters and $p < 0.05$ is deemed statistically significant.

RESULTS

GRAM STAINING OF ISOLATE AQ5-03

Figure 1 shows the gram staining result of isolate AQ5-03. The bacterial cell was pink in colour, demonstrating a characteristic of Gram-negative. It is rod-shaped, motile through flagella and occurs in sets. The microscopic analysis exemplifies that the colony is creamy white.

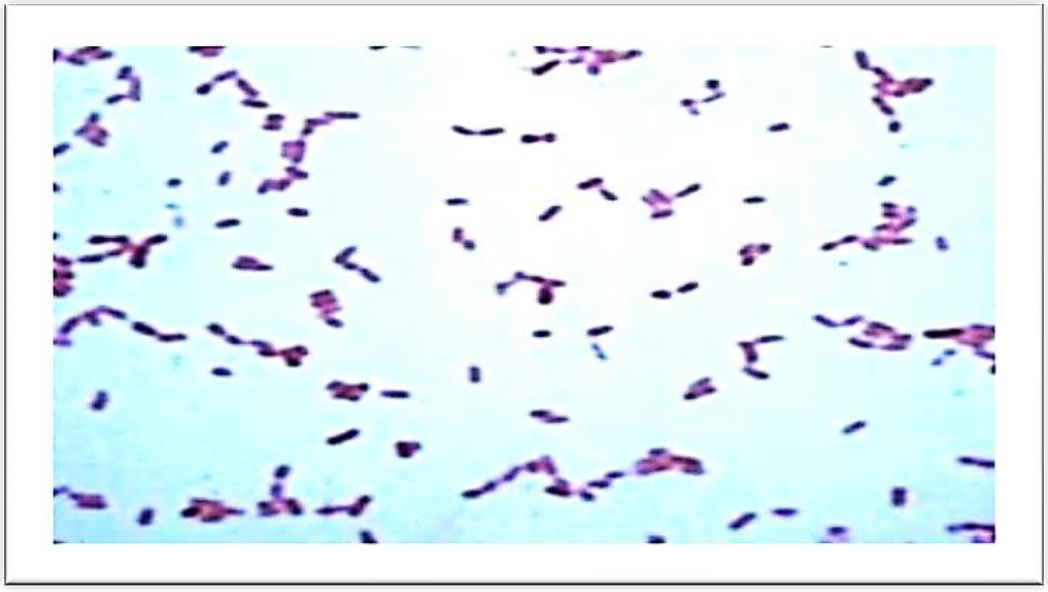

Figure 1: Gram stain smear of isolate AQ5-03 under 1000 x magnification on a light microscope.

GENOMIC DNA EXTRACTION

Genomic DNA of the isolate was successfully extracted. Clear bands with high intensity for the bacteria were obtained and was estimated at 23130 bp, which is an indication of good quality DNA concentration and were further used as the templates for the PCR (Zeng *et al.*, 2008). A Hind III digest of lambda DNA was used as the marker for the agarose gel electrophoresis (**Figure 2**).

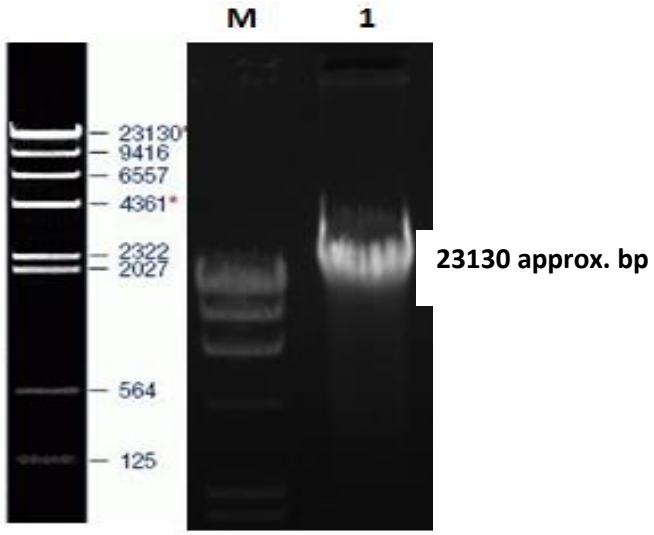

Figure 2: Agarose gel electrophoresis image of genomic DNA.
Lane M: λ Hind III™ Marker in bp; Lane 1 is AQ5-03 Genomic DNA (23130 bp).

POLYMERASE CHAIN REACTION (PCR)

The genomic DNA from isolates AQ5-02, AQ5-03 and AQ5-04 were used as the template for the 16S rDNA amplification. The primer used is based on reports of the highly conserved region for all bacteria as they completely amplified the 16S rRNA region of the gene corresponding to 1500 bp (Acinas *et al.,* 1997). The amplified 16S rRNA gene of the three isolates results to fragments with an estimated size of about 1500 bp as shown in Figure 3.

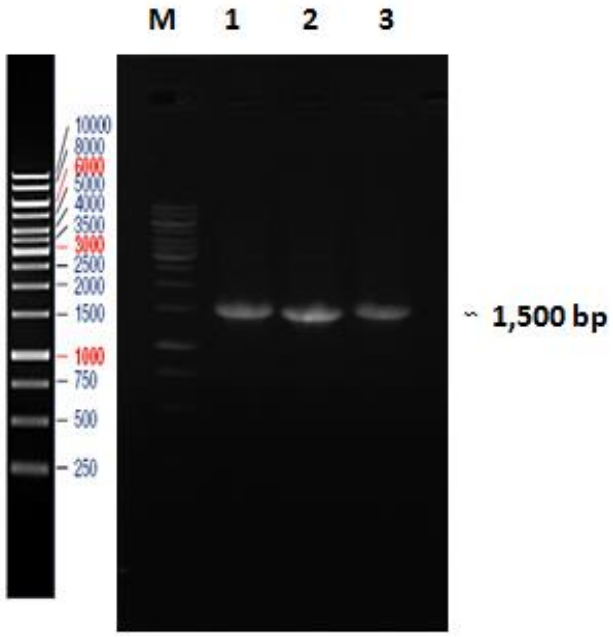

Figure 3: PCR product of 16S rRNA gene of isolate AQ5-02, AQ5-03, and AQ5-04.
Lane M: GeneRuler 1 kb DNA Ladder markers in bp: Lane 1 ,2 and 3 AQ5 -03 PCR product (1500 bp).

16S rRNA GENE SEQUENCING

The 16S rRNA gene has some exceptionally conserved regions treasured for attaining decent sequence alignment. The resultant 1352 bases for the isolate AQ5-03 was compared with the available 16S sequences at the NCBI GeneBank database utilizing the blast server (http://www.ncbi.nlm.nih.gov/BLAST). This analysis clarifies that the sequence of the isolate AQ5-03 is closely related to *Serratia* species (Tables 1)

Table 1: Foremost ten sequences producing the best alignment with *Serratia* sp. AQ5-03 from NCBI blast.

Accession Number	Max Score	Total Score	Query Cover	E-value	Identity
NR044385	2352	2352	100%	0	98%
NR036886	2351	2351	100%	0	98%
NR114043	2340	2340	100%	0	98%
NR113236	2338	2338	100%	0	98%
NR041980	2329	2329	100%	0	98%
NR042356	2246	2246	100%	0	97%
NR041979	2230	2230	100%	0	97%
NR025338	2228	2228	99 %	0	97%
NR112005	2226	2226	100%	0	97%
NR037110	2224	2224	100%	0	97%

PHYLOGENETIC TREE FOR *Serratia* sp. AQ5-03

The phylogenetic relationships between *Serratia* sp. strain AQ5-03 sp. and other related species of the *Serratia* genus constructed using their 16S rRNA gene sequences shows that the closest relative of strain AQ5-03 is *Serratia marcescens* with 95% 16S rRNA sequence similarity (Figure 4).

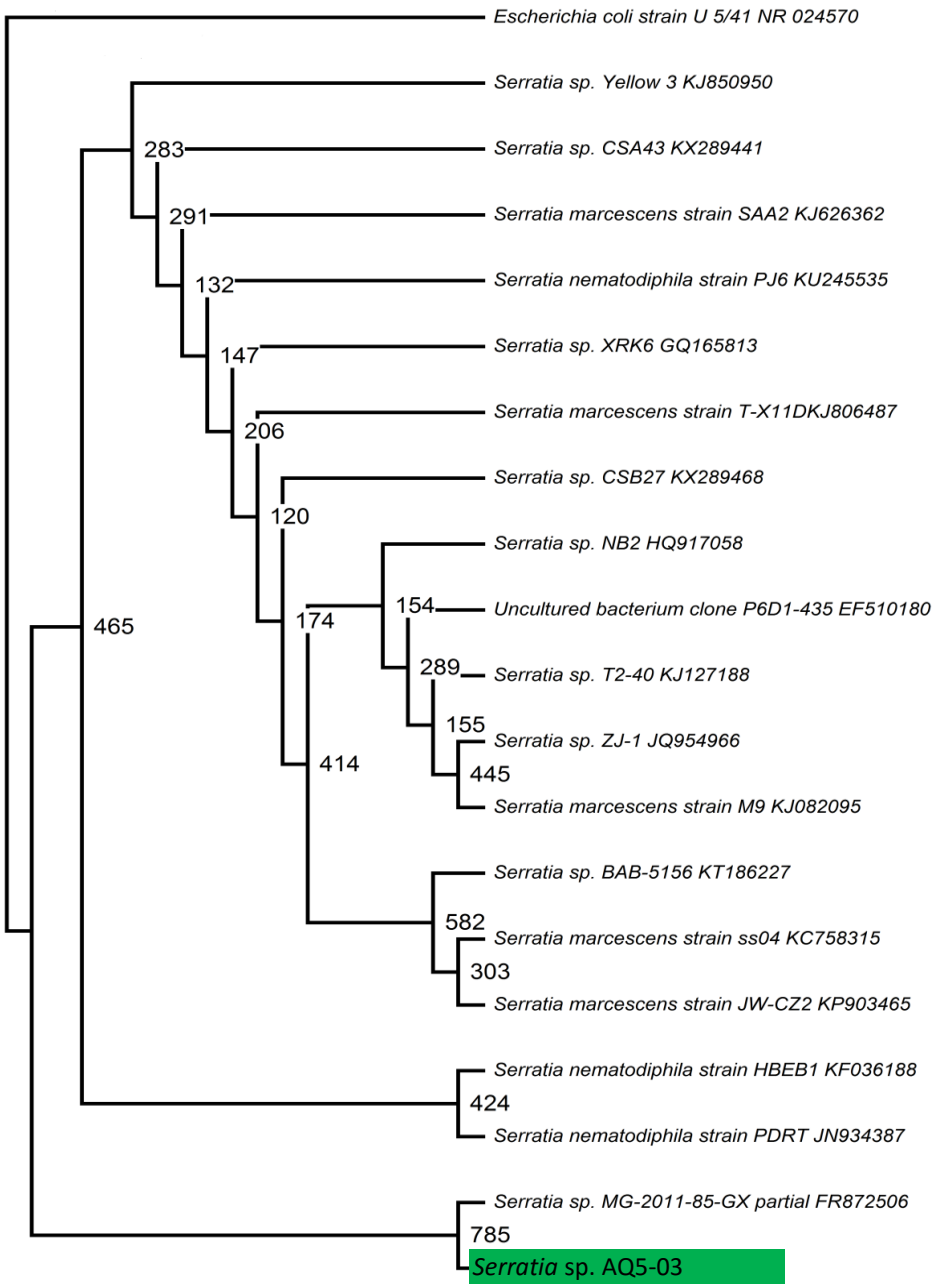

Figure 4: Phylogenetic tree showing the position of *Serratia* sp. AQ5-03 strain among *Serratia* genera and other bacteria.

EFFECT OF TEMPERATURE

As shown in Figure 5, the highest percentage of phenol degradation was between the temperatures of 30 and 35 ^{o}C, where more than 90 % of degradation was achieved.

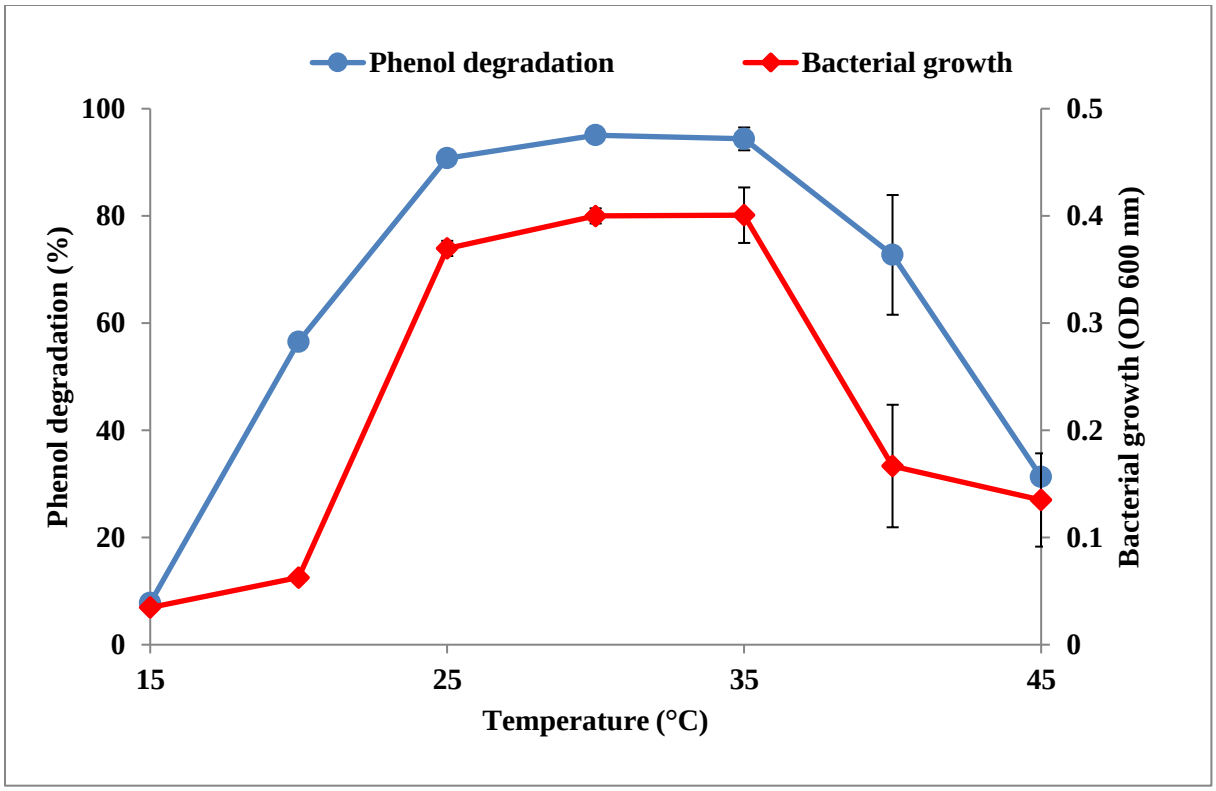

Figure 5: Effect of temperature on phenol degradation bacterial growth by *Alcaligenes* sp. *Serratia* sp. AQ5-03

EFFECT OF DIFFERENT BUFFERS ADJUSTED TO DIFFERENT pH ON PHENOL DEGRADATION AND BACTERIAL GROWTH *Serratia* sp. AQ5-03 AND PHENOL DEGRADATION

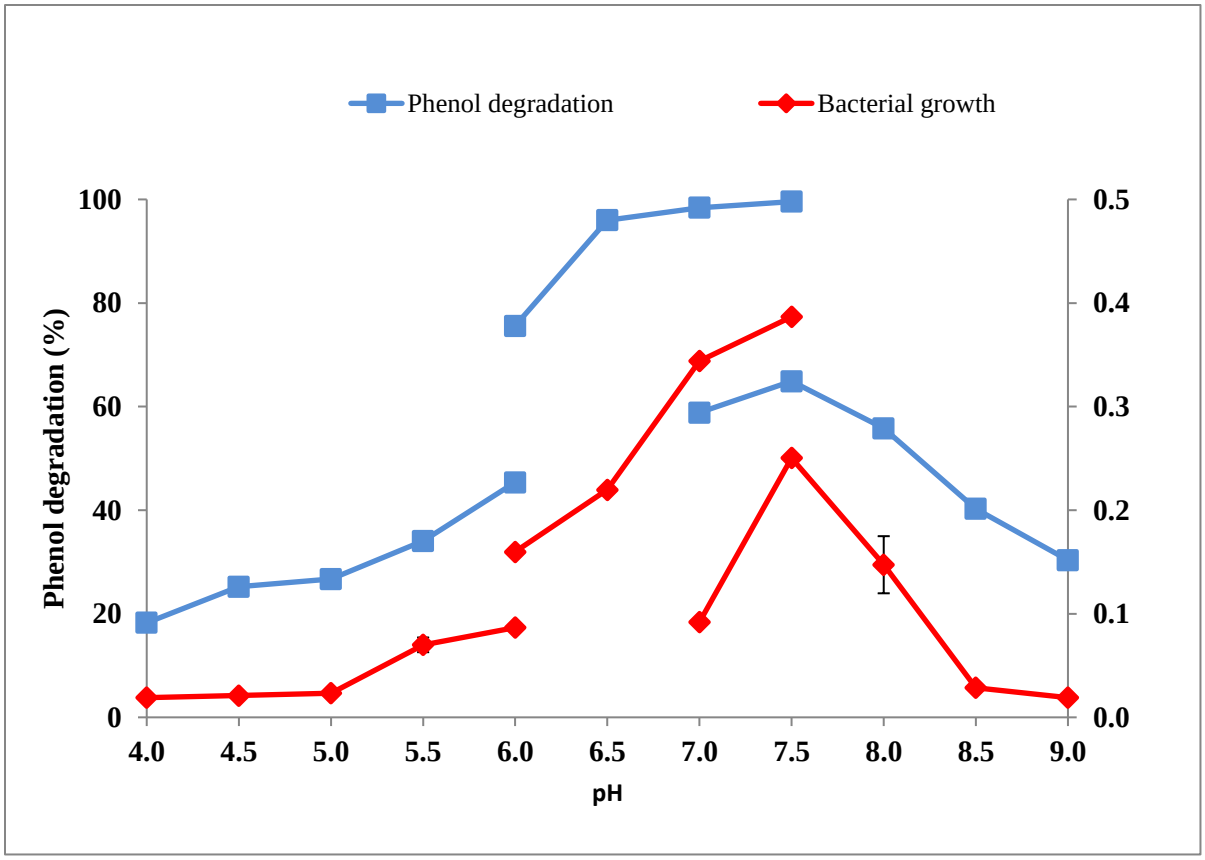

Figure 6: Effect of different buffers adjusted to different pHs on phenol degradation and bacterial growth by *Serratia* sp. AQ5-03.

DISCUSSION

Neighbor-joining algorithm of phylogenetic analysis ascertained that the strain belonged to the *Serratia* sp. The evolutionary history was concluded using the Neighbour-Joining Technique (Saitou and Nei, 1987). The optimal tree with a sum of branch length = 40.34932914 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 (Felsentein, 1985). 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. The evolutionary reserves were calculated using the Maximum Composite Likelihood method (Koichiro *et al.*, 2004) in the unit of quantity of base substitutions per site. Evolutionary analyses were conducted in PHYLIP (Tamura *et al.*, 2013). *E.coli strain* 5/41 was used as the outgroup. Species names of bacteria were followed by their accession numbers. Bootstrap values were calculated based on 1000 resamplings**.**

Although there are reports of some bacteria that can degrade phenol at temperatures higher than 45 ^{o}C, the most widely reported range is between 25 and 35 ^{o}C, which is an ideal range for most of the bioremediation processes especially in the tropical region. Using the Tukey-Kramer Multiple Comparisons Test for *Serratia* sp AQ5-03, there

was no significant difference ($p>0.05$) between 25 and 35 ^{o}C, but there is a significant difference between 25 and 40 ^{o}C ($p<0.001$). Temperature affects general metabolic processes according to the Arrhenius where a double increase in the rate of degradation and growth is anticipated at a temperature increase of 10 ^{o}C. Very high temperatures denature enzymes and protein and are the cause of cessation of degradation and bacterial growth, respectively. On the other extreme, low-temperature phenol degradation has been reported (Kotturi *et al.,* 1991; Li, *et al.,* 2010) while thermophilic phenol degradation has also been reported with degradation observed at temperatures as high as 70 ^{o}C (Chen *et al.,* 2008; Mutzel *et al.,* 1996).

Studies on the effect of temperature on the optimal growth for all the isolates showed that the optimum points were achieved within the temperature range of 25-35 ^{o}C (Figure 5). This indicates that the isolate grows best within the same range with most of the reported mesophilic bacteria (Bakhshi *et al.,* 2011; Ahmad *et al.,* 2012; Norazah *et al.,* 2016). There was a decline in the bacterial growth at both of the extreme temperatures. Also, ANOVA analysis showed that there is a significantly difference ($p<0.001$) between 25 and 40 ^{o}C when to compare between 30 and 35 ^{o}C for the *Serratia* sp. AQ5-03 but no significant difference between 30 and 35 ^{o}C ($p>0.005$). Higher temperatures inhibit the growth of all the three bacteria this may be due to the denaturation of some key proteins which mediates the proper growth of microorganisms. The optimal temperature ranges revealed by this bacterial growth is suitable for use in temperate regions.

Among the factors that affect bioremediation, pH is one of importance. Thus, maintenance of pH in mineral salt media is very vital since pH affects bacterial physiological and biochemical activities which influences the growth and proliferation of bacteria. In this study, an overlapping buffering system comprising of acetate, phosphate, and Tris-HCl ranging from 4.0 to 9.0 were tested (Yadzir *et al.,* 2016). A low bacterial growth and percentage phenol degradation were recorded for all the three isolates at highly acidic values. Also at high alkaline pH, low degradation, and bacterial growth were observed. Figure 6 illustrated a healthy bacterial growth and phenol degradation by isolate AQ5-03 using phosphate buffer as the best buffering system for that particular experiment. The isolate degrades 0.5 g/L phenol at pH range of 6.5 to 7.5 with a very shape decline in both growth and phenol degradation capacity at pH 8 and above. At acidic pHs of from 4.0 to 5.5 (acetate buffer), there were minimum growth and degradation rates. Most microorganisms used for phenol bioremediation have a preference for a neutral or near neutral pH for effective phenol degradation. Many phenol-degrading bacteria shared optimal pH as shown in this study, such as *Bacillus cereus* at pH 7.0, *Pseudomonas putida* MTCC 1194 pH 7.1 (Kumar *et al.*,2005), *Acinetobacter* sp. strain PD12 pH 7.2 (Zhang *et al.,* 2007), *Alcaligenes faecalis* at pH 7.2 (Jiang *et al.,* 2007), *Rhodococcus* UKM-P at pH 7.5 (Suhaila *et al.*,2013a), *Ewingella Americana* at pH 7.5 (Khleifat, 2006). However, there are few reports of some bacteria that degrade phenol outside the pH range of 6.5 to 7.5 such as *Pseudomonas putida* ATCC that grows and degrades phenol at pH of 5.5 to 6.0 (Mordocco *et al.*,1999) and also *Ochrobactrum* sp. at the optimum pH of 8.0 (Kiliç, 2009). *Halomonas campisalis* is able to mineralised phenol within the pH range of 8.0 to 11 (Alva and Peyton, 2003). An optimum pH is favourable for phenol degrading enzymes, and it enhances the stability and the affinity of the enzymes towards the substrates.

CONCLUSION

Phenol degrading bacteria isolate AQ5-03 was identified as *Serratia* sp. AQ5-03 using Gram staining technique and 16S rRNA sequencing. The sequence has been deposited in Genbank with accession number KT693287. Optimisation for the best phenol biodegradation conditions was conducted by One Factor at a Time (OFAT) approach. *Serratia* sp. AQ5-03 is a good candidate for further research in bioremediation.

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