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ISOLATION AND OPTIMISATION OF PHENOL DEGRADATION BY ANTARCTIC ISOLATE USING ONE FACTOR AT TIME

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Abstract

Phenol has been classified as a priority pollutant by the United States Environmental Protection Agency (US EPA) due to its high toxicity. The increased level of phenol concentration in the Antarctic environment causes a significant risk to the aquatic and terrestrial lives there due to its persistence, biomagnification and accumulation in the food chain. The biodegradation of phenol in Antarctica is considered highly challenging owing to its harsh and extremely cold climate. Therefore, actions of bioremediation are crucial to overcome this problem. To date, little data is available regarding the biodegradation of phenol by aboriginal Antarctic bacteria, and reports on the utilisation of phenol as sole carbon source by strains isolated from Antarctic soil are scarce. In the present study, bacteria isolated from Antarctic soil and identified as *Arthrobacter* sp. strain AQ5-15 based on 16S rRNA sequence was screened and optimised through conventional method for efficient phenol degradation. Based on preliminary screening, AQ5-15 strain was capable of completely degrading 0.5 g/L phenol within 108 h at 10°C. A study on the effects of significant factors including nitrogen source and concentration, salinity, pH and temperature was carried out to optimise the conditions for phenol degradation. Finding revealed that this strain is a psychrotolerant with optimum temperature at 20°C and prefer neutral or near-neutral condition for phenol degradation. The basic knowledge obtained from this study will provide the benefits on custody of the Antarctic environment especially in removing phenol and its derivatives at low temperature.

INTRODUCTION

Regardless of the fact that Antarctic continent is conspicuously free from high negative impact of human activities, literature data can be the source for concluding that it is not a place free from anthropogenic pollutants, which mostly connected with long-range atmospheric transport (LRAT) as well as deposition in this particular area. Although there are various toxic compounds and numerous persistent pollutants transferred to this Antarctic continent via natural processes by mass flows in the atmosphere and oceans, factors such as population growth and improper waste management in the research stations there, marine transportations, tourism activities as well as industrial activity in far countries of both the Southern and Northern also resulted in a notable level of anthropogenic pollutants in Antarctica [1, 2].

Antarctica is mainly affected by occurrence of oil spill due to the transport of fuels to powerhouses as well as fuel spill during refilling fuel tanks and vehicles. The fuels usually used in Antarctica are gasoline, lubricant oil and diesel which comprising high content of phenolic compounds [3]. There are several major events that have been reported in association with the oil spill, which led to phenol pollution in Antarctica. For instance, a major fuel spill occurred in 1989 when the Argentine Polar Transporter, Bahia Paraiso, which was *en route* to resupply Argentine research station, ran aground and sank near Palmer Station, Antarctica releasing over 150 000 gallons of diesel [2]. Besides, a Chilean ship known as the Patriarche, undergone shipwreck and spilled 1500 L of diesel off the north-west Antarctic coast in the year 2001 (CEP-IV-IP62, 2001). Petroleum derived pollutants in Antarctica tend to be persistent and mobile in soil, air, water, sediments as well as ecological receptors even at low concentrations [4] due to incredibly low temperatures and frequent dryness of the continent.

Phenol is known as a by-product of many industrial processes involving oil refineries, pesticides, insecticides, and

pharmaceutical [5] which and has been listed as priority hazardous pollutant by US Environmental Protection Agency (US EPA) that can give toxic threat and diseases [6]. Although low concentration of phenol has the ability to inhibit the microbial growth present in biological wastewater treatment system, they also promote a major reduction in the rate of degradation of contaminant compounds and cause severe destruction to the environment and living beings [7]. Many aquatics lives like plants, fishes and microorganisms are at risk of its mutagenic, teratogenic and carcinogenic effects. The toxicity of phenol also may lead to damage of blood, liver and kidney along with cardiac toxicity, resulting in weak pulse, cardiac depression and reduced blood pressure in human [8]. Due to this, the US Environmental Protection Agency (USEPA) and World Health Organization (WHO) have recommended a limit of 1 µg/L of phenol concentration in tap water [9, 10].

In recent times, much attention has been given to the development of biological remediation technology compared to the physical and chemical methods due to its cost effectiveness, environmental friendliness and ability to eliminate various organic pollutants [11-15]. However, there are limitations when it comes to phenol and other hydrocarbons due to their hardly-biodegradable properties and highly toxic to microorganisms. In concordance, a number of studies have posited the application of indigenous microorganisms in hydrocarbon and phenol pollutants degradation particularly in Antarctica as they are already adapted to the contaminated environment for growth and have the tendency to survive in the cold and extreme climate of the continent [16-19]. Moreover, Antarctic Treaty forbids the use of foreign organisms in Antarctica; hence, bioremediation can only be instigated using native microbes in this continent [20]. To date, numerous hydrocarbon-degrading bacteria have been isolated from Antarctica, which are typically devoted to the genera *Sphingomonas*, *Acinetobacter*, *Pseudomonas*, *Rhodococcus*, and *Arthrobacter* [18, 20, 21].

Earlier studies reported that bacteria that are able to utilise hydrocarbon depend strongly on physical and environmental factors [22-24]; hence, considering these factors affecting microbial degradation became one of the great research interests in the current scenario. In addition, contamination by hydrocarbon can engender detrimental changes in soil properties including alterations in maximum surface temperature, pH, as well as nitrogen and carbon levels [16]. Considering these effects, an optimisation process must be carried out to attain maximum degradation of hydrocarbons like phenol. In the conventional approach, optimisation is carried out using individual factors by altering one variable at a time while maintaining other variables at a constant value.

In this study, cold-adapted bacteria from Antarctic soil has been isolated, screened and identified for phenol degrading ability. Degradation of phenol was then assessed using conventional 'one-factor-at-a-time' to ascertain the effectiveness of the degradation of phenol.

MATERIALS AND METHODS

Media preparation

Minimal salt medium (MSM) was prepared by adding the following in 1 L of dH_2O (g/L): K_2HPO_4 (0.4), KH_2PO_4 (0.2), NaCl (0.1), Mg_2SO_4, (0.1), $(NH_4)_2SO_4$ (0.4), $MnSO_4.H_2O$ (0.01), $Fe_2(SO_4).H_2O$ (0.01) and $NaMoO_4.H_2O$ (0.01) with pH adjusted to 7.5 using 1 M NaOH solution. Sterile phenol was added to the cooled medium after autoclaved at 121°C for 20 min using Hirayama autoclave machine [25]. The medium was stored in a chiller at a temperature of 10°C prior to the inoculation.

Isolation and screening for phenol-degrading strains

Bacteria was isolated from Antarctic Base soil obtained on 10 February 2018 by co-author Dr. Ahmad from King George Island, Antarctica (62º11'47.645"S, 58º58'11.485"W). Soil sample weighing 2 g was suspended in 50 mL of cold sterile MSM consisting 0.5 g/L phenol and incubated on a shaking incubator at 150 rpm for 4 days at 10°C. Aliquot of soil suspension was subcultured into MSM with 0.5 g/L phenol every 4 days for 12 days. The aliquot then was spread onto MSM agar plates supplemented with 0.5 g/L phenol as a sole carbon source and incubated for 14 days at 10°C. A single colony of the resulting bacterial colonies was inoculated in nutrient broth and incubated on a shaking incubator at 150 rpm and 10°C overnight. The incubation temperature of 10°C was chosen to signify the summer soil surface temperature that can go up to 18°C [18, 26, 27]. MSM containing 0.5 g/L of phenol was inoculated with 10.0% (v/v) of overnight grown bacteria from nutrient broth and incubated on the shaking incubator at 150 rpm and 10°C for 108 h. Uninoculated sterile MSM was used as a control for confirming the ability of the strain to utilise phenol as a sole carbon source. Phenol degradation was monitored for every 12 h using 4-aminoantipyrine colorimetric assay following the method provided by the American Public Health Association [28] and bacterial growth using optical density 600 (OD_{600}) [29].

Identification using Gram staining and 16S rRNA sequencing

The morphological properties of potential strain were examined using Gram-staining method and observed using optical microscope. For 16S rRNA sequencing, genomic DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Netherlands). The DNA extracted was then used as template for PCR amplification performed using forward and reverse primers, 27F (5'-AGAGATTGATCCTGGCTCTG-3') and 1492R (5'-GGTTTCCTTGTTACGACAT-3') on a GS-96 Thermal Cycler (Hercuvan Lab System, Malaysia) under the following conditions: initial denaturation at 94°C for 3 min; 34 cycles of denaturation at 94°C for 1 min, annealing at the temperature of 53°C for 1 min and elongation at 72°C for 2 min followed by final elongation at 72°C for 10 min. The PCR products were then confirmed by running gel electrophoresis on 1.0% agarose gel stained with ViSafe Red Gel Stain (Vivantis Technologies, Malaysia). The nucleotide sequences obtained from MyTACG Bioscience Sdn. Bhd., Malaysia were then compared with bacterial 16S rRNA sequences for closely related published species in GenBank using National Centre for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) program [30] and aligned with ClustalW. Neighbour-joining phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) program version 7.0 [31]. The reliability of the reconstruction was analysed by performing bootstrap using 1000 replications.

Optimisation of phenol degradation and growth conditions using one-factor-at-a-time

This one factor at a time (OFAT) approach was proposed to screen for the best medium condition, which necessitates several individual parameters assigned based on the literature review. Parameters such as pH, salinity, nitrogen source and concentration, and temperature were tested individually for optimised condition [18]. Assessed factors were optimised by maintaining all other factors at a constant level for the usual scaling-up strategy. Each following parameter was investigated after considering the previously optimised parameter(s). Nitrogen sources optimised include ammonium sulphate, ammonium chloride, ammonium nitrate, sodium nitrate, leucine, glycine, and serine. The concentration of the selected nitrogen source was varied between 0.1 and 0.8 g/L. Tolerance to salt was determined by adding increasing amount of NaCl; 0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3 (g/L) to the medium. The optimised pH was measured using three different buffers: acetate buffer (pH 5–6), phosphate buffer (pH 6–7.5) and Tris–HCl buffer (pH 7–9). Effects of temperature were evaluated at 10, 15, 20, 25 and 30°C. These experiments were carried out in triplicate using a one-factor-at-a-time approach. All parameters were carried out within a time frame of 72 h while maintaining the same initial inoculum size of 10.0% (v/v).

RESULTS AND DISCUSSION

Antarctic soil collected was subjected to supplementation with 0.5 g/L phenol as the sole carbon source at 10ºC to screen for psychrophilic or psychrotrophic bacteria that are able to degrade phenol. After two weeks of enrichment, only one pure colony was observed on the phenol agar based on their morphological properties. Despite the fact that Antarctic soil comprised various bacterial communities [32, 33], antibacterial properties of phenol inhibited the growth of most microbial species at adequately high concentration even though they have the metabolic capability to use phenol as substrate [34, 35]. Accordingly, the colony obtained was classified as a strain that can withstand high concentration of phenol, which was then named as phenol-degrading strain AQ5-15 and stored in glycerol stock at -80°C for further use. Upon screening, this strain showed complete degradation of 0.5 g/L phenol at 10°C within the incubation time of 108 h (**Figure 1**). The ability of the strain to degrade phenol was monitored for every 12 h using 4-AAP assay and the incubation temperature of 10°C was used in this study to signify the maximum temperature in Antarctica during summer [36, 37]. The condensation of 4-AAP with phenol in the presence of alkaline oxidising agent ($C_6N_6FeK_3$ and NH_4Cl) resulted in the formation of red antipyrine dye (AAPPC), which was later detected with spectrophotometer [38]. Despite being sensitive, this assay has the advantage of giving negative blanks [39].

Colonies of this strain were yellow and opaque on both phenol and nutrient agar. Gram-staining showed that it was Gram-positive with rod-shaped bacterium (**Figure 2**). Based on 16S rRNA sequences, strain AQ5-15 was identified as *Arthrobacter* sp. The phylogenetic tree shown in **Figure 3** displays the affiliation of strain AQ5-15 with 20 closely related types of the genus *Arthrobacter* with *Pseudomonas* sp. used as outgroup. Analysis of 16S rRNA sequences revealed that strain AQ5-15 shared the highest similarity (99%) with *Arthrobacter sulfureus* (99.5%), *Arthrobacter oxydans* (99.4%) and *Arthrobacter psychrophenolicus* (99%). However, the low bootstrap values obtained were insufficient to classify both strains to species level with confidence. Therefore, the strain was here referred to as *Arthrobacter* sp. AQ5-15 and the nucleotide sequence was deposited in the NCBI database under the following accession number: *Arthrobacter* sp. strain AQ5-15 (MK744046).

Studies on Antarctic soil bacteria community revealed that psychrophilic or psychrotrophic *Arthrobacter* sp. was abundant along the transect [18, 40, 41]. Several studies reported the ability of *Arthrobacter* sp. as a phenol biodegrader [42-45]. This bacterial species also has the capability to degrade phenol at low temperature as reported in some studies [18, 46, 47].

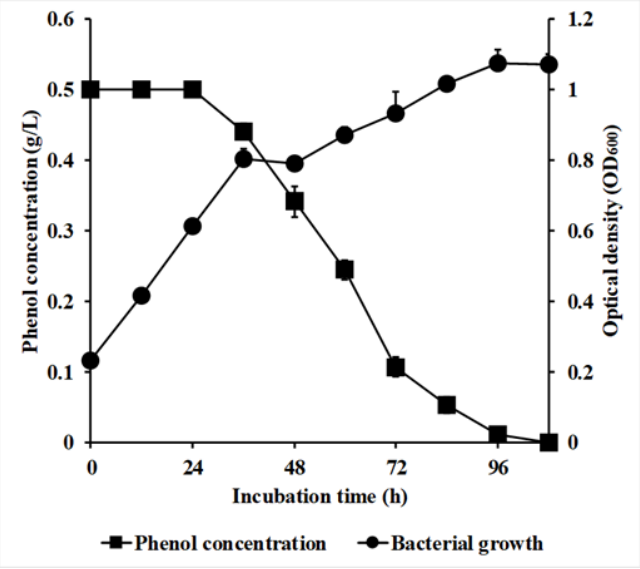

Figure 1. Degradation of 0.5 g/L phenol by isolate AQ5-15 at 10°C. The error bars represent the mean ± standard deviation for three replicates.

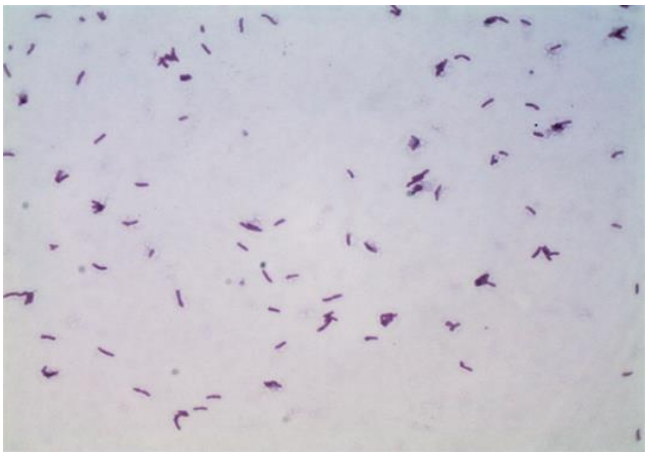

Figure 2. Gram-Staining of phenol-degrading bacterial isolate AQ5-15 under the light microscope (Olympus BX40.F4, Japan) with 1000x magnification. The strain is a Gram-positive rod shaped-bacteria (stained purple). Scale bar represent 20 µm.

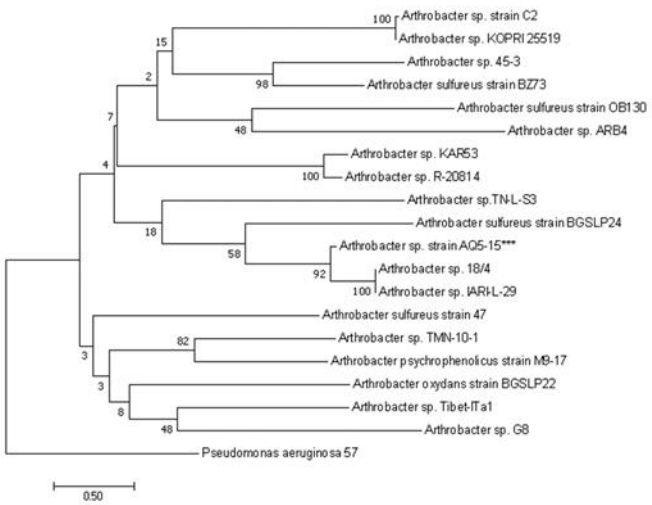

Figure 3. Neighbour-Joining phylogenetic tree of strain AQ5-15 with related type strains of *Arthrobacter* spp. imported from the GenBank database. *Pseudomonas* sp. was used as an outgroup. Bootstrap scores based on 1000 replicates shown next to the branches in which the related taxa were clustered together. This analysis was performed using MEGA 7.0.

Optimisation using conventional OFAT approach

The physiological factors have a significant effect on the metabolism of bacteria; thus, optimising these parameters is a key step in many bioprocesses especially bioremediation. In most cases, these factors act as one of the detrimental aspects limiting the success of biodegradation at field-scale [48]. In concordance, the effects of selected parameters including nitrogen source and concentration, NaCl concentration, pH, and temperature were initially assessed using the 'one-factor-at-a-time' approach as demonstrated in **Figures 4** to **9**.

Effect of nitrogen source and its concentration

Figure 4 exhibits the phenol degradation and bacterial growth of this strain using different nitrogen-consisting compound as its sole nitrogen source. Strain AQ5-15 showed the highest growth when ammonium sulphate, ammonium nitrate or serine were used as the nitrogen source with no significant difference between them ($p>0.05$). Glycine was the following most effective source, while the use of leucine, sodium nitrate or ammonium chloride led to no significant difference in growth compared to the control. Phenol degradation by this strain followed a similar pattern with maximum phenol degradation observed using ammonium sulphate, ammonium nitrate and serine with no significant difference ($p>0.05$). Of all, ammonium sulphate was chosen for the following procedures due to its extensive usage as a cheap source of nitrogen for bioremediation [49, 50]. Several bacterial species have been reported to show optimum growth and degradation of hydrocarbon when they are supplemented with ammonium sulphate as the sole nitrogen source [49-51]. Optimisation of ammonium sulphate concentrations showed that the optimal concentration for bacterial growth was at 0.05% (w/v) with no significant difference between the concentrations from 0.03% and 0.04% (w/v). Meanwhile, optimum phenol degradation was observed at 0.05% (w/v) with significant difference ($p<0.05$) to any other concentrations tested (**Figure 5**). Singling out the best nitrogen source and its optimum concentration for growth and degradation would benefit the designation of effective bioremediation strategy for hydrocarbon contamination [52].

Effect of salinity

Salinity portrays osmotic and special ion effects on microorganisms by affecting their metabolic production and biomass [53]. The salt tolerance of strain AQ5-15 with phenol as sole carbon source is exhibited in **Figure 6** over a salinity range of 0% to 0.03% (w/v) NaCl. This strain showed maximum growth and degradation at 0.01% (w/v) NaCl and when the concentration was increased to 0.015% (w/v), the growth and degradation rate gradually decreased until 0.03% (w/v). The phenol degrading ability of this strain was minimal at the absence of salt. Hence, the presence of salt is essential for enhancing bacterial growth and phenol degradation in line with those of several other studies [51, 53, 54]. This strain requires minimum salt content to function efficiently as reported by other studies examining optimum phenol degradation by phenol-degrading bacteria at low salinity [25, 29]. Salt content above tolerance level can adversely affect microbial biomass and their activity due to the osmotic stress, which results in lysis and dying of cells [55, 56].

Effects of pH

The effects of pH on bacterial growth and phenol degradation by strain AQ5-15 was identified using overlapping buffer system as shown in **Figure 7**. Maximum growth was observed from pH 7.5 (phosphate buffer) with no significant difference ($p>0.05$) with pH 7 to 8. At pH less than 7 and more than 8, the growth of bacteria was considerably decreased. The rate of phenol degradation was optimum at pH 7 (phosphate buffer) with significant difference ($p<0.05$) to any other pH tested. At pH 5 to 5.5 (acetate buffer), the phenol degradation was inhibited and decreased at pH more than 7. These data showed that phenol degradation by strain AQ5-15 was significantly sensitive to pH compared to bacterial growth. From the observation, it can be deduced that this strain requires a neutral or near neutral condition for both bacterial growth and phenol degradation. Both acidic and alkaline condition minimise the degradation efficiency of this strain since the H^+ and OH^- ions may have detrimental effect on the cell membrane. Similar result has been reported for phenol degradation in several studies [57-59].

Effects of temperature

The effects of temperature on the growth of strain AQ5-15 in 0.5 g/L of phenol were observed using different incubation temperatures ranging from 5ºC to 30ºC as shown in **Figure 8**. The bacterial growth was minimal at 5ºC and dramatically increased to maximum at 20ºC to 25ºC and inhibited at 30ºC. Slightly different to the growth, phenol degradation by this strain was optimum at 20ºC with significant difference ($p<0.05$) to any other temperatures tested and lose its ability to degrade phenol at 30ºC. From the result obtained, this strain can be classified as a psychrotolerant according to the definition by Morita [60], which exhibits a maximum growth above 20ºC but not more than 30ºC. Several studies have reported the ability of different bacterial species to degrade phenol in lower temperature. A study reported that that *Arthrobacter* sp. strain AG31 isolated from an Alpine ice

cave in Austria has the ability to degrade phenol at 10ºC [61]. Another study reported that *Arthrobacter* spp. strain AQ5-5 and AQ5-6 and *Rhodococcus* sp. strain AQ5-7 which isolated from Antarctica can degrade phenol at 10ºC to 15ºC [18]. Of all other parameters, temperature is commonly considered as one of the most significant parameters especially in polar region as degradation ought to obey the Arrhenius law. According to the Arrhenius relationship, the degradation of a pollutant is highly dependent on temperature in where an increase or decrease in temperature can affect the rate of reaction [62-64]. Although it has been assumed that extremely low temperature of Antarctic continent is not suitable for efficient biodegradation, the metabolism of native bacteria is adapted to function optimally at such condition [65-67].

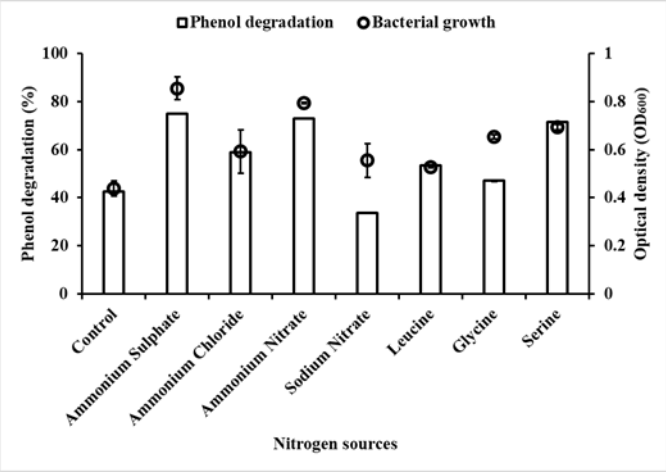

Figure 4. The effects of different nitrogen sources on the growth and phenol degradation of strain AQ5-15. The error bars represent the mean ± standard deviation for three replicates.

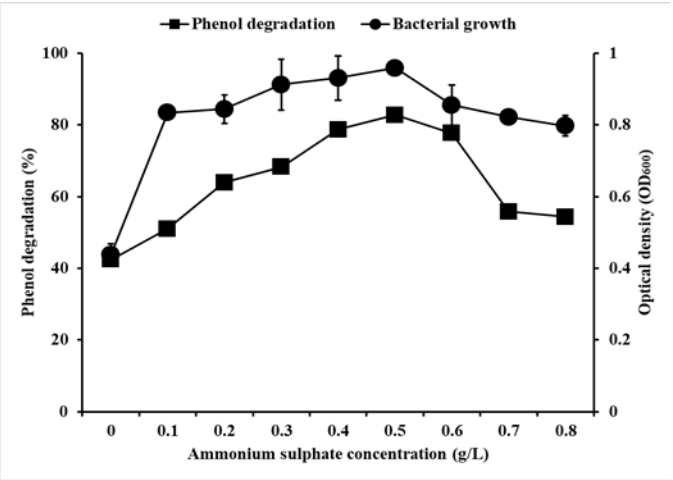

Figure 5. The effects of different concentration of ammonium sulphate on the growth and phenol degradation of strain AQ5-15. The error bars represent the mean ± standard deviation for three replicates.

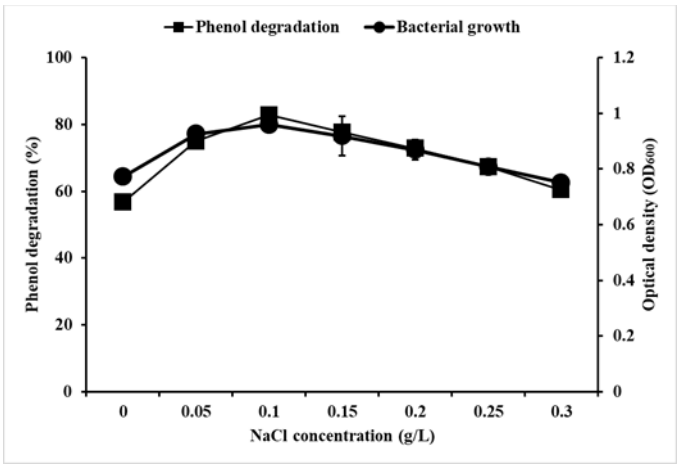

Figure 6. The effects of different concentration of sodium chloride on the growth and phenol degradation of strain AQ5-15. The error bars represent the mean ± standard deviation for three replicates.

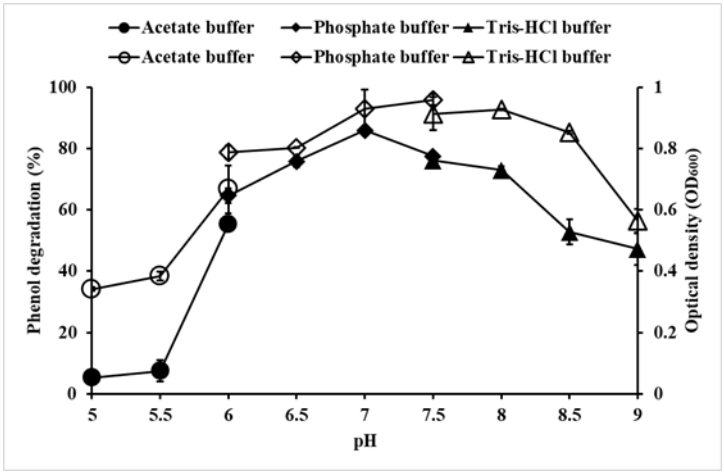

Figure 7. The effects of pH on the growth (non-filled marker) and phenol degradation (filled marker) of strain AQ5-15. The error bars represent the mean ± standard deviation for three replicates.

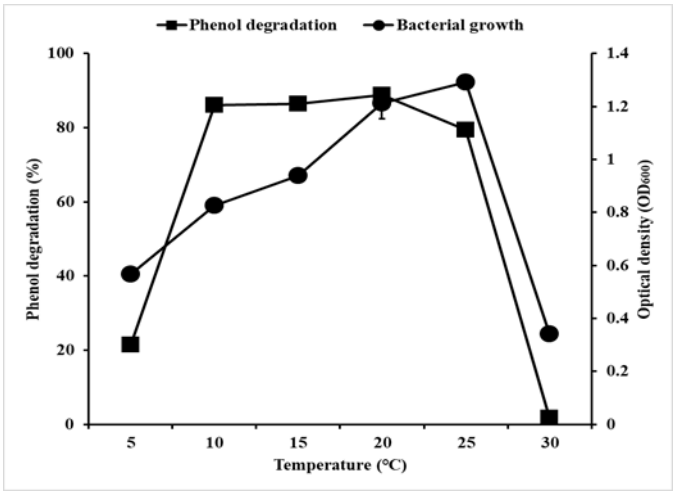

Figure 8. The effects of temperature on the growth and phenol degradation of strain AQ5-15. The error bars represent the mean ± standard deviation for three replicates.

CONCLUSION

In summary, a cold-adapted bacterial strain capable of degrading phenol up to 0.5 g/L within 108 h at 10ºC has been isolated from Antarctic soil and identified as *Arthrobacter* sp. strain AQ5-15 based on the 16S rRNA sequence and the phylogenetic analysis. The optimal condition for phenol degradation was at 20ºC, with growth optimum growth at 25ºC, indicating its psychrotolerant status. This strain prefers neutral or near neutral condition to work efficiently on phenol degradation. Other physicochemical factors including nitrogen source and its concentration as well as salt concentration were observed to have a significant influence on the enhancement of phenol-degrading activity in this strain. Regarding that the metabolism of native bacteria is more adaptive in cold environment, their preponderance can be an advantage to bioremediation of phenol-polluted regions. The ability of this strain can contribute to the bioremediation of phenol in Antarctica as an initiative to retain the pristine environment of the continent as well as in other cold-climate regions. Current research is being conducted to identify the enzyme-cleavage pathway for phenol degradation and the underlying genomic process.

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