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RESEARCH ARTICLE

Isolation and Identification of Halophilic Bacteria Isolated from Mangrove Soil in Blue Lagoon, Port Dickson, Negeri Sembilan

Siti Khadijah Azmi, Nur Zahra Zainal Fuadi, Ilyanie Hj Yaacob*

School of Biology, Universiti Teknologi MARA (UiTM) Cawangan Negeri Sembilan, Kampus Kuala Pilah, Pekan Parit Tinggi, 72000 Kuala Pilah Negeri Sembilan, Malaysia

Abstract Halophiles bacteria are capable of tolerating and surviving in extreme salinity due to the special biological structure that it possesses. Classification of halophilic bacteria includes slight, moderate, and extreme halophiles which range from low to high concentrations of salt. Biotechnological products yielded from bacteria residing in saline environment are proven to be valuable in a few industries. The isolated halophilic bacteria may yield powerful biomolecules such as enzymes which can enhance plant development, hence boosting agricultural industries. However, the insufficient data related to halophiles available in Malaysia may reduce the potential use of these bacteria in related industries. The goal of this study is to isolate and identify the potential halophilic bacteria and to determine the tolerance of halophiles in different concentrations of salt. Collection of soil samples was done at the mangrove soil in Blue Lagoon, Port Dickson, Negeri Sembilan. Isolation and purification of halophiles was done on nutrient agar media supplemented with 3% salt. Next, Gram-staining was performed on 4 colonies of bacteria with different types of single colony appearances. Then, the selected bacteria with different colony and morphological characteristics were tested in nutrient broth supplemented with 0%, 3%, 10% and 20% of salt. Molecular identification was conducted which involved DNA extraction and Polymerase Chain Reaction. All 4 isolates selected were tested to be Gram-stain positive and possessed rod-shaped. The bacteria have various salinity tolerance levels which may be due to the presence of different enzymes produced in their cells. Gene sequences and phylogenetic tree were analyzed based on 16s rRNA sequences to identify the species of the isolates. At the end of the experiment, isolate 2A1 and 2A4 were identified as *Bacillus sp.*, while isolate 2A2 and 2A3 were identified as *Cytobacillus oceanisediminis*.

Keywords: Halophiles, Halophilic bacteria, Mangrove soil, Blue Lagoon, *Bacillus sp.*, *Cytobacillus oceanisediminis*.

***For correspondence:**
yanie_yaacob@uitm.edu.my

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Introduction

Mangroves are classified as one of the halophytic plants which are members of multiple angiosperm families and possess better adaptations to an extremely harsh intertidal areas between land and sea [1]. Mangrove forests which dwell on the transitional area between land and sea are essential components of coastal defense. Mangrove forests have specialized ecosystems that can dwell in a challenging environment with limited oxygen levels in the water [2]. Mangroves spend almost all of their life period submerged in seawater, where the sea level affecting the mangroves varies depending on the tidal surges [3]. Since mangroves are capable of living in high salinity environments, microorganisms such as halophiles exist in the rhizosphere of the mangrove forests [4].

Halophiles consist of both prokaryotic and eukaryotic species having the ability to regulate osmotic potential in extreme conditions and prevent salt denaturation of its membranal structure. This

microorganism is capable of tolerating and surviving in extreme salinity due to the special biological structure that it possesses. Halophilic bacteria can tolerate a wide-ranging salinity level, (2-30% NaCl). Hence, halophilic bacteria have the adaptations to moderate and high salt concentrations where it can modify itself so they can thrive in salty environment even when tidal surges occur over an extended period along the mangrove shoreline [5].

Halophilic bacteria are considered a unique microbe as they can tolerate and live in the high salinity of the soil, and it may give a lot of benefits to Malaysian industries. Halophiles may be used in the food industry and biotechnological applications such as making solar salt from seawater and fermenting traditional foods [6]. The development of beta-carotene by the green alga *Dunaliella* and the production of ectoine, an enzyme stabilizer now utilized in cosmetic products, are two highly effective biotechnological processes employing halophiles [7]. A limited number of halophiles have been isolated from mangrove areas in Malaysia, for example, *Robertkochia* and numerous other *Flavobacteriaceae* members that were collected at Malaysia Tanjung Piai National Park [8]. Research on the halophiles isolated from mangroves in Malaysia is needed to obtain various benefits of halophilic bacteria for Malaysian industries.

Many halophiles are unique in their ability to withstand hypersaline conditions as well as various heavy metals and metalloids. These microbes are very useful for biotechnological applications such as bioremediation and biomineralization due to their high-stress tolerance capacity [9]. Because of their ability to catalyze under high salinity, halophilic microorganisms are an ideal source for producing salt-stable enzymes. The high salinity environments required by halophilic microorganisms may suppress the growth of other organisms, significantly reducing sterilization costs. Halotolerant proteases are used to treat burns and purulent wounds for therapeutic purposes. It also functions as a thrombolytic agent due to its fibrinolytic activity [10]. The halotolerant proteases act as plasminogen activators or thrombolytic drugs that able to change the plasminogen zymogen into the active enzyme plasmin. It will break down the fibrin. Halophilic bacteria are incredibly widely known as spoiling agents for sun-salted fish and meat, and certain types of halophilic bacteria also have even been used to ferment food that is high in protein [11].

Even though halophilic bacteria have been studied by many researchers up until now, there is very limited study on the isolation of halophilic bacteria from mangrove soil in Malaysia. The benefits of halophiles may not be recognized and discovered due to a lack of study that may help to improve the Malaysian industry such as the biotechnological and medicine industries. Malaysian industries may be advanced if they found an enzyme that can withstand high concentrations of salt that only can be found in halophiles. Despite the widespread use of remote sensing to describe and monitor mangrove change over a variety of spatial and temporal scales [12]. Malaysian industry may be more advanced and improved with the help of halophilic bacteria with their various benefits. Halophilic bacteria may provide salt-tolerant genes to plants. As a result, plants can absorb and store salt as a nutrient, yielding a high yield all year [13]. This study also may give insight into the microbial diversity found in mangrove ecosystems [14]. As a result, it supports human development in two ways, which are by inhibiting the growth of pathogenic bacteria and by indirectly benefiting human welfare due to the possible favorable impact on the environment [15]. Consequently, research on these halophilic bacteria is needed. This study aims to isolate and identify the halophilic bacteria that is collected from mangrove soil using Polymerase Chain Reaction (PCR) and to determine the tolerance of halophiles in different concentrations of salt.

Materials and Methods

Collection of Soil Sample

Soil sampling was carried out from mangrove forest located in Blue Lagoon, Port Dickson, Negeri Sembilan at 2.41480˚N, 101.85321˚E and 2.41480˚N, 101.85321˚E. The sample was transported to UiTM, Negeri Sembilan branch, Kuala Pilah Campus for storage. The soil sample then was kept in refrigerator at 4˚C until further use.

Preparation of Halophilic Agar Media

Six grams of nutrient agar powder was weighed and added into a Schott bottle. 3 g of salt was diluted in 100 mL of distilled water, and the solution was poured into the same Schott bottle. Next, the Schott bottle was placed onto a hotplate to allow the powder to dissolve. The Schott bottle was autoclaved for 15 minutes at 121°C. The nutrient agar solution was poured into petri dish, and the media plates were stored in refrigerator.

Isolation and Purification of Halophilic Bacteria

The isolation and purification were done following Rahman *et al*. [13] with slight modifications. Seven test tubes were prepared and 9ml of distilled water was added into each of the test tubes. The test tubes were labelled as 10^{-1} up until 10^{-7}. Then, 10g of the soil sample was added in the first tube and it was mixed thoroughly by using vortex. Next, 1ml of solution from the first tube was pipetted and transferred into the second test tube and the dilution is marked as 10^{-2}. This step was repeated until the 10^{-7} tube was reached. Then, 1ml from 10^{-5}, 10^{-6} and 10^{-7} dilution was inoculated onto a petri dish, respectively. The sample was spread over the top of the media by using a glass spreader. Then, the petri dishes were incubated at 30˚C for 24 hours or up to 48 hours. The purification of the bacteria was done multiple times by streaking the visible colonies onto the new nutrient agar media that was supplemented with 3% salt concentration.

Gram Staining

An inoculation loop was used to transfer a drop of suspended culture to the microscope slide. The slide was heat-dried over a gentle flame. The heat will promote cell adhesion to the glass slide and prevent significant culture loss during rinsing.

Gram-staining method was performed. A crystal violet stain was applied to the fixed culture. The stain was poured off after 60 seconds, and the excess stain was rinsed with water. Then, the iodine solution was applied to the smear for 60 seconds to fix the dye. The iodine solution was drained, and the slide was rinsed under running water. Water from the surface was shaken off. After that, a few drops of decolorizer were added to the slide. In 5 seconds, the slide was rinsed with water. The addition of decolorizer will be stopped as soon as the solvent is not colored as it flows over the slide to avoid excess decolorization in Gram-positive cells [16]. For 40 to 60 seconds, the smear was counterstained with Safranin solution. Water was used to wash away the Safranin solution, and excess water was blotted with a tissue. The excess water was shaken off to dry off the slide. The slide was examined under a microscope with oil immersion for 100x magnification to determine the shape and color of the halophilic bacteria.

Salinity Tolerances of Halophiles

The screening for salt tolerance followed the methods by Ji *et al*. [17] with slight modifications. To prepare the overnight culture, 5mL of nutrient broth with 3% salt concentration was poured into four autoclaved universal bottles. 4 samples of isolated halophilic bacteria colonies from media plates were selected and carefully picked using a sterile inoculation loop. The mixture was mixed homogenously before it was incubated in a shaking incubator at 30˚C and at the speed of 170 rpm. The growth of the culture was indicated by the cloudy appearance of broth and was standardized to 10^8 cfu/mL.

25µL of overnight culture sample was put into the test tube that contains 0% salt concentration by using micropipette. The step was repeated by using test tubes that contain 3%, 10% and 20% salt concentrations. The test tubes were mixed homogenously. After that, the test tubes were incubated at 30˚C and at the speed of 170 rpm. After 24 hours, the absorbance of the bacterial culture was observed by using UV-Vis at 600 nm. The tolerance of halophilic bacteria towards the different concentrations of salt was determined by comparing the absorbance of bacteria with the control, which is the bacteria that contains 3% salt concentration.

Molecular Identification of Halophilic Bacteria By PCR

The purpose of performing PCR was to amplify and identify the isolated halophiles species. Several steps were taken which include DNA extraction, amplification of bacterial DNA segment through PCR and gel electrophoresis. Identification of the isolated rod-shaped strain was performed by 16S rRNA sequence analysis where 27F primer with sequence (5`→ 3`) AGAGTTTGATYMTGGCTCAG and 1492R primer with sequence (5`→ 3`) GGTTACCTTGTTACGACTT were used, which can amplify 16S under wide range of bacterial taxa [18].

Genomic DNA was extracted from the bacterial cells using PrimeWay Genome (1st BASE Biochemicals) DNA extraction kit. The DNA extraction was done based on the protocol supplied by the manufacturer. The samples (1.5mL) from overnight-grown culture were transferred into Eppendorf tube and harvested by centrifugation for 1 minute at 12,000 rpm. After centrifugation, the supernatant was removed. 100µL of autoclaved distilled water was added into the Eppendorf tubes and bacterial pellet was resuspended by pipetting up and down. Then, 100µL of Tissue Lysis Buffer was added, thoroughly vortexed and incubated at 37˚C for 10 minutes. Next, 10µL of Proteinase K was added into the tubes, briefly vortexed and incubated at 56˚C for 1 hour in a thermal mixer. After 1 hour, 3µL of RNase A was added into the lysate, briefly vortexed and incubated at 56˚C for 5 minutes.

Subsequently, 400µL of gDNA Binding Buffer was added to the samples and were mixed thoroughly by pulse-vortexing for 5 seconds. The lysates were transferred to a gDNA Purification Column that was inserted into a collection tube. The lid of the column was closed and centrifuged for 5 minutes at 12,000 rpm. The collection tubes and the flow through were removed. Next, the column was transferred to a new collection tube and 500µL of gDNA Wash Buffer was added into the column. The lid of the column was closed and was inverted a few times. Immediately, the tubes were centrifuged at 12,000 rpm for 1 minute and the flow through was removed. The column was inserted into a new collection tube and 500µL of gDNA Wash Buffer was added before centrifuging at 12,000 rpm for 1 minute. The collection tube and the flow through was discarded. Lastly, 70µL of preheated gDNA Elution Buffer was added into the samples. The cap of the Eppendorf tube was closed, incubated at room temperature for 1 minute. The DNA was eluted by centrifuging at 12,000 rpm for 1 minute.

PCR Master Mix was thawed at room temperature and was kept on ice after thawing. The following reaction mix was prepared with volume of 25µL in a sterile PCR tube (Table 1).

Table 1. Components of PCR reaction mix

Components	Volume (µL)	Final Volume (µL)
PCR Master Mix	12.5	75
Forward primer	1	6
Reverse primer	1	6
DNA template	3	18
Autoclaved distilled water	7.5	45

The lid of the PCR tubes was closed, and the mixture was briefly vortexed. Next, the PCR tubes were placed in a thermal cycler. The PCR amplification was performed for 35 cycles (Table 2).

Table 2. The PCR amplification steps

No.	Step	Temperature	Time	Cycle
1	Initial Denaturation	95˚C	1 minute	1x
2	Denaturation	95˚C	30 seconds	34x
3	Annealing	54.2˚C	30 seconds	
4	Extension	72˚C	1 minute	
5	Final Extension	72˚C	5 minutes	1x
6	Soak	12˚C	∞	

Gel Electrophoresis

Gel was prepared using the procedure by Lee et. al [19], with slight modifications where 0.8g of agarose gel powder was weighed using analytical balance and was poured into an Erlenmeyer flask. 100mL of 1X TBE buffer was measured, subsequently transferred into the Erlenmeyer flask that contained agarose powder and the content was swirled. The mixture was heated on a hotplate. After that, the flask was removed from the hotplate and the content was allowed to be swirled at intervals of 30 seconds. This step was repeated until the agarose mixture appeared clear, which indicates the content has completely dissolved. After that, 2µL of GelStain was added into the mixture to allow the visibility of DNA bands. The agarose was removed from the hotplate and allowed to cool on the benchtop. Next, both open edges of a gel tray were taped by using masking tape before agarose solution was poured. A suitable comb was inserted onto the gel tray to create wells to load samples. The molten, and cooled agarose solution was poured into the gel mold. The agarose was allowed to harden at room temperature for 1 hour. After 1 hour, the comb was removed, and the solidified agarose was placed into the buffer tank.

Approximately 800mL of 1X TBE Buffer was poured until the solidified agarose gel has completely submerged. 6 dots of loading dye were made on a parafilm. Using the procedure by Koonzt, L. [20] with slight alterations, 1kb of DNA ladder (positive control) was mixed with loading dye and was carefully loaded into a well. 3µL of DNA sample was mixed with loading dye. The stained DNA was loaded into a well. Distilled water (negative control) was mixed with loading dye and loaded into a well. Next, the gel tank was connected to the power supply. The black wire (cathode) and red wire (anode) were connected to a correct slot of power supply. Since DNA is a negatively charged molecule, the DNA will move from the negative cathode to the positive anode once the electrical field is present. The power supply was turned on and the desired voltage was set up to 80 volts (V) for 90 minutes.

Sequencing, BLAST Analysis and Phylogenetic Tree

The PCR products were sent to Apical Scientific Sdn. Bhd for sequencing purposes. After the sequencing results were obtained, the bacterial nucleotide sequence was blasted on National Center for Biotechnology Information (NCBI). The index similarity of the isolate's nucleotide sequences was compared with the existing sequence in the NCBI Blast database [21] by analyzing the query cover percentage and per identity to determine the species of the halophiles. Next, phylogenetic tree was constructed by using MEGA 11 software.

Results and Discussion

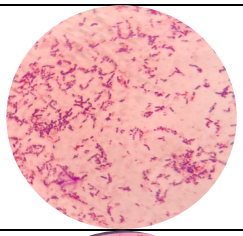
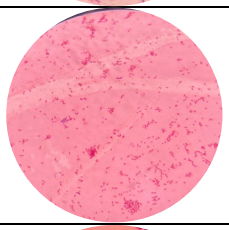
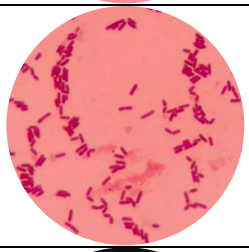
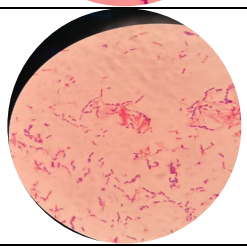
Isolation of Halophiles

The morphological characterization of the isolated halophiles was performed on nutrient agar media that was added with 3% salt concentration. Most of the isolated colonies were circular, smooth, and convex in shape. Only a few transparent and translucent colonies have been identified. Pure isolates were obtained and were observed under the microscope. Four isolated bacteria were selected based on their morphology, shape, colour and appearance.

Microscopic Characterization of Halophilic Bacteria by Gram-Staining

Table 3 showed the colonies that were observed under microscope after having been incubated for 24 hours. From the result below, all the halophiles were rod-shaped and were Gram-positive bacteria.

Table 3. Gram-staining results from isolated bacteria sample (1000 x magnification using immersion oil)

Sample	Result	Colour	Shape	Gram (-/+)
2A1		Purple	Rod-shaped	Gram-positive
2A2		Purple	Rod-shaped	Gram-positive
2A3		Purple	Rod-shaped	Gram-positive
2A4		Purple	Rod-shaped	Gram-positive

Tolerance of Halophilic Bacteria on Different Concentrations of Salt

Salinity tolerance determination of each halophilic bacteria was evaluated following the method of Halder *et al.* [9] with some modifications. The bacteria were transferred into nutrient broth containing 0%, 3%, 10% and 20% of salt concentrations, respectively. The nutrient broth containing 3% salt concentration was served as control because the 3% salt concentration is the optimal environment of salt for the halophilic bacteria to grow. The tolerance of strain 2A1 towards different concentrations of salt is recorded in Figure 1. The salinity tolerance of strain 2A1 does not follow the concentration gradient. Strain 2A1 can grow best at 0% salt concentration as the growth of the bacteria is slightly lower than the control. The growth strain 2A1 in the concentration of 10% and 20% salt is lower than the control. The percentage of tolerance was significantly lower at the concentration of 20% salt than 0% and 3% salt concentrations ($p<0.05$). The result is consistent with the findings from Pallavi *et al.* [22] that done a screening of halophiles on salt stress tolerance with salt concentrations of 10%, and 20%, they found that the growth of the isolates that could withstand more salt than 10% is higher than the growth of the isolates that could tolerate up to 15% salt. It shows that the higher the concentration of salt is, the lesser the survival rate of the halophilic bacteria. Strain 2A1 can be classified as an extreme halophile as can survive at 20% salt concentration, which is an extremely salinity environment.

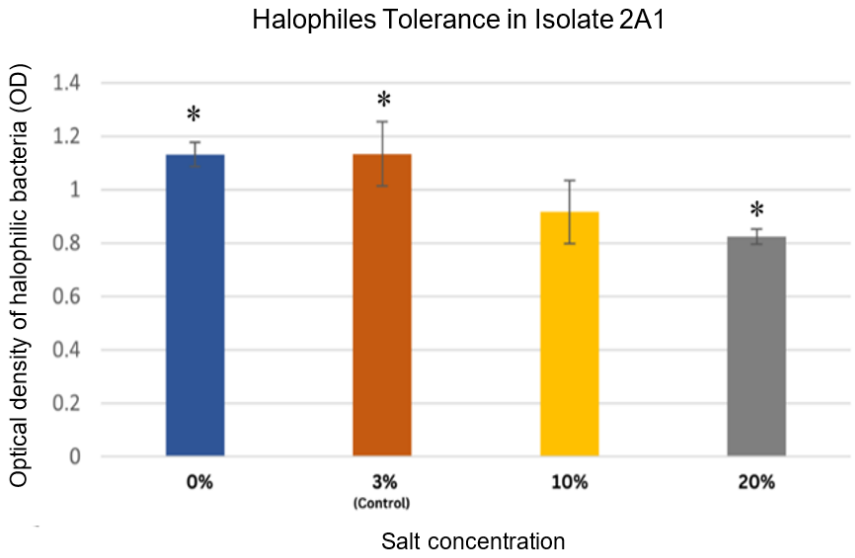


Figure 1. Effect of different salinity on survival of isolate 2A1 after incubation at 30°C. The asterisk (*) shows the percentage of tolerance significantly different ($p<0.05$) among the salt concentrations

The tolerance of strain 2A2 towards different concentrations of salt is recorded in Figure 2. The salinity tolerance of strain 2A2 does not follow the concentration gradient. Strain 2A2 grew best at 0% but survived less in 10% and 20% salt concentrations. The percentage of tolerance was significantly higher at 0% salt concentration compared to 10% and 20% salt concentrations. It may be because of the different enzymes' presence in their body. Halophiles' stress tolerance system may also differ due to the different production of enzymes such as trihalose, glutamates, or prolines to overcome the various stress conditions during harsh environments [23]. Strain 2A2 may have enzymes that can tolerate best in extreme environments but do not tolerate well in moderate halophilic environment.

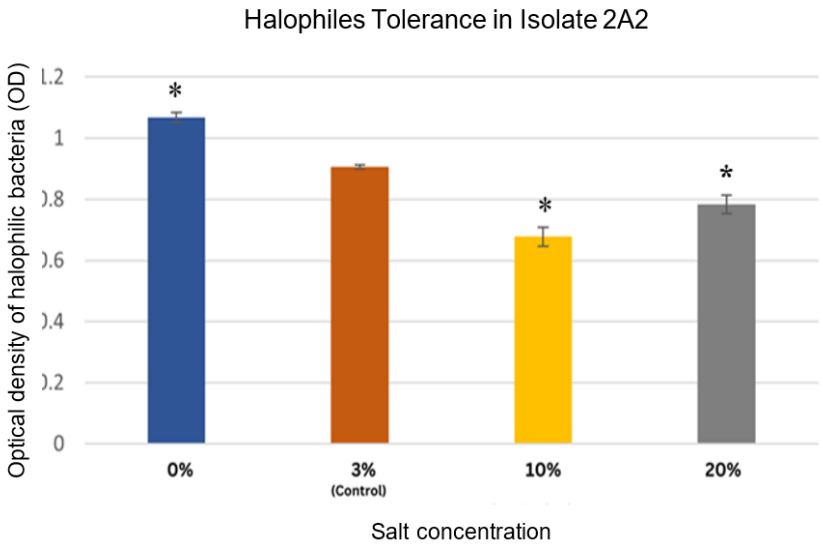


Figure 2. Effect of different salinity on survival of isolate 2A2 after incubation at 30°C.The asterisk (*) shows the percentage of tolerance significantly different ($p<0.05$) among the salt concentrations

From the result displayed in Figure 3, isolate 2A3 can grow best in 0% and 10% of salinity where it recorded the highest growth level compared to 3% and 20%. The isolate was growing well in the environment without salt, but the growth was slightly reduced when observed in 3% of salt content. This result indicates that the bacteria can survive well in 0% of salt and 10% of salt. The significant difference can be observed at salt concentration of 3% and 20%. Based on the result as shown in Figure 3.3, the isolate showed a remarkable growth level in 0% salt, declining in 3% but it survived better in 10% of salt concentration. This result was comparable to research that was performed by Remonsellez *et al.* [24] where the bacteria can be categorized as halotolerant due to the ability to grow in the absence of salt and thrived well in 10% of salt. Another study showed that halophilic bacteria is capable of alleviating salt levels in sediment, hence, promoting plant growth under stressful conditions of salinity while reducing salinity of the sediments [25]. Enzymes that are produced by halophiles are closely related to the adaptation of bacteria towards high salinity. As supported by research done by Edbeib *et al*. [26], isolate 2A3 may yield α-Amylase enzyme since it showed the highest growth in 10% salt concentration.

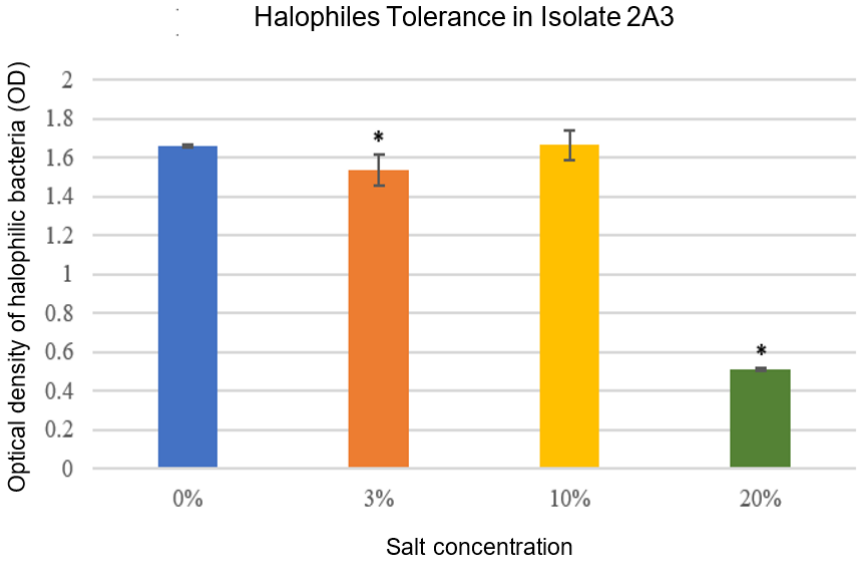


Figure 3. Effect of different salinity on survival of isolate 2A3 after incubation at 30°C. The asterisk (*) shows the percentage tolerance significantly different ($p < 0.05$) among the salt concentrations

The tolerance of strain 2A4 towards different concentrations of salt is recorded in Figure 4. The salinity tolerance of strain 2A4 does follow the concentration gradient. Strain 2A4 grew best at 0% salt concentration but less survived at 10% and 20% salt concentrations. The percentage of tolerance was significantly higher at 0% salt concentration compared to 10% and 20% salt concentrations while the percentage of tolerance at 20% salt concentration was significantly lower compared to the remaining. It can be concluded that the halophilic bacterial diversity decreased significantly as media salt content increased. These findings are related to the findings from Irshad *et al.* [27] who found that saline soil produced mostly moderately halophilic and halotolerant bacteria rather than severely halophilic microorganisms. These kinds of occurrences are mostly caused by the high salt concentration of the coastline soil, which inhibits the growth of extremely halophilic bacteria. Strain 2A4 is considered as moderate halophiles as it can tolerate and able to survive in moderately salinity environment, which is 10% salt concentration.

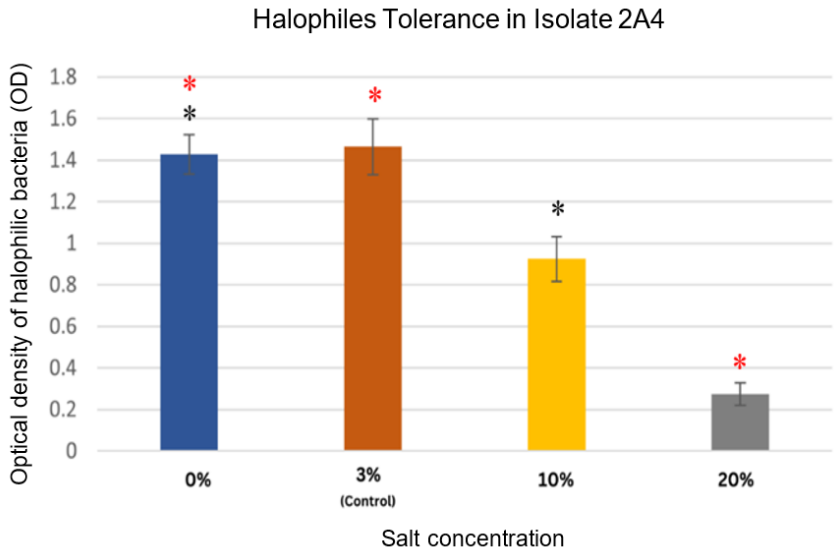


Figure 4. Effect of different salinity on survival of isolate 2A4 after incubation at 30°C. The asterisk (*) shows the percentage of tolerance significantly different ($p<0.05$) among the salt concentrations

Each isolate showed different salinity tolerance towards multiple salt concentration environment. The ability of the isolates to grow at different salinity may indicate that it can yield different types of biomolecules such as enzymes, composition of carbohydrates and amino acids that made up the cell wall structure of the bacteria, hence, allowing the bacteria to survive in distinct salt environments. Previous studies showed that halotolerant organisms may produce enzymes that work better under harsh environments [28]. The presence of these bacteria could also enhance the nutrient gain under salinity pressure [29].

Polymerase Chain Reaction (PCR)

PCR is a highly sensitive tool for rapidly replicating or amplifying a specific DNA segment [30]. The PCR product was successfully amplified, and the size of the PCR product was determined. The band patterns of all strains were shown in Figures 5, 6 and 7. The band size of all strains using 27F and 1492R primers corresponded to the expected size of the 16S rRNA gene, which is approximately 1600 bp. The DNA Ladder that was used as reference to the size of the DNA is HyperLadder 1kb.

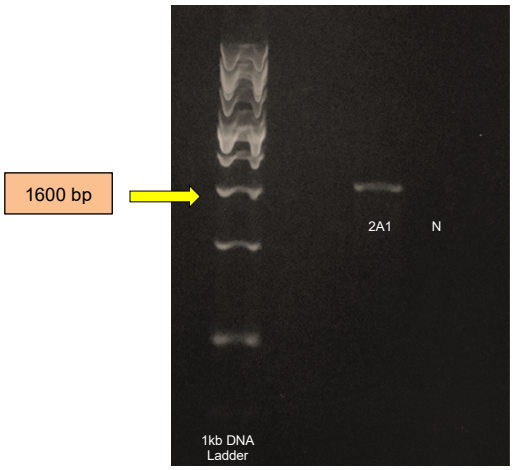


Figure 5. Agarose Gel Electrophoresis Analysis of PCR Amplification of the 16S rRNA Gene of isolate 2A1

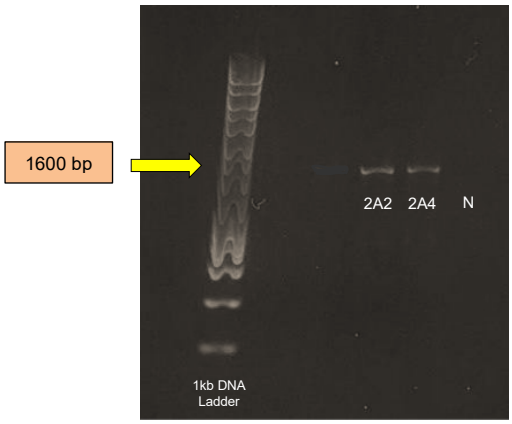


Figure 6. Agarose Gel Electrophoresis Analysis of PCR Amplification of the 16S rRNA Gene of isolate 2A2 and 2A4

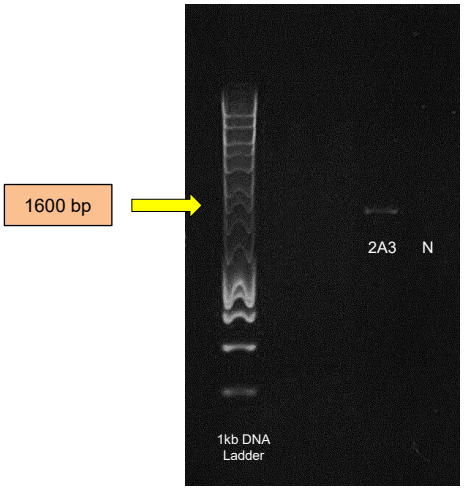


Figure 7. Agarose Gel Electrophoresis Analysis of PCR Amplification of the 16S rRNA Gene of isolate 2A3

Sequencing of PCR Product

The PCR product was sent for sequencing at Apical Scientific Sdn. Bhd. After that, the sequences were exported into the National Center of Biotechnology Information (NCBI) website to identify the species of the isolated strains. The 16S rRNA sequences of isolate 2A2 and 2A3 matched those of *Cytobacillus oceanisediminis,* while isolate 2A1 and 2A4 matched those of *Bacillus sp* (Figure 8). All sequences were consequently deposited into the GenBank database with accession numbers of OR247787 (2A1), OR247788 (2A2), OR247790 (2A3) and OR247789 (2A4).

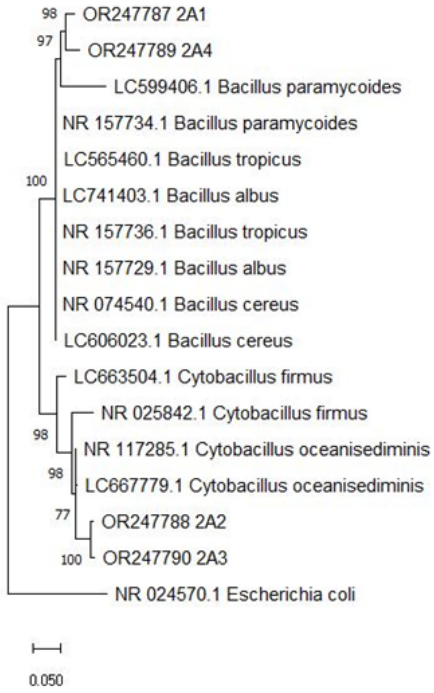


Figure 8. Maximum-likelihood Phylogenetic tree of isolate and reference strains based on 16S rRNA gene sequence obtained from NCBI GenBank database. *Escherichia coli* was selected as the outgroup

Conclusions

In conclusion, a total of four halophilic bacteria were successfully isolated from the mangrove sediment. All the bacteria were rod-shaped and Gram-positive. The selected halophilic bacteria showed distinct growth patterns when cultured on different salt concentrations. It can be concluded that various halophilic bacteria species have various salinity tolerance levels, and some can endure higher salt concentrations than others. Different tolerance of halophilic bacteria may be caused by the presence of different enzymes produced in their bodies. Phylogenetic tree presented that isolate 2A2 and 2A3 belong to genus *Cytobacillus* since it was positioned in the same branch as other *Cytobacillus* sp. and it is identified as *Cytobacillus oceanisediminis*. On the other hand, isolate 2A1 and 2A4 belong to genus *Bacillus* and it is identified as *Bacillus sp*. For future recommendations, isolation of halophile's enzyme must be conducted to study about the necessity of the enzyme which could provide various benefits for agricultural purpose such as enhancing and accelerating the growth of mangroves' plant. Halophilic bacteria have a variety of biotechnological applications, and their enzymes, stabilizers, and chemicals may offer advantages in the development of biotechnological manufacturing processes. Hence, the percentage of mangroves resides in Malaysia can be increased which in turn can protect Malaysia's coastline from bad storm surges.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

Acknowledgement

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