ZULFAQAR Journal of Defence Science, Engineering & Technology
e-ISSN: 2773-5281
Vol. 6, Issue 3 (2024)
DOI: https://doi.org/10.58247/jdset-2023-0603-22
Journal homepage: https://zulfaqarjdset.upnm.edu.my

IDENTIFICATION OF MARINE IRON REDUCING BACTERIA (IRB) ISOLATED FROM PASIR PANJANG, PORT DICKSON, NEGERI SEMBILAN AND ITS ANTIMICROBIAL ACTIVITIES

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ARTICLE INFO

ARTICLE HISTORY
Received: 30-01-2023
Revised: 01-04-2023
Accepted: 30-06-2023
Published: 30-11-0000

KEYWORDS
Iron reducing bacteria (IRB)
DNA extraction
PCR amplification activity
Antibiotic susceptibility

ABSTRACT

This study focused on the identification of iron reducing bacteria (IRB) isolated from the waters off Port Dickson, Negeri Sembilan and its antimicrobial activity. Ten marine IRB isolates were successfully obtained from five different steel sample coupons immersed in seawater off Pasir Panjang, Port Dickson, Negeri Sembilan for one month. The samples were cultured in VMNI medium for 72 hours at 37°C. A total of ten pure colonies named A1, C1, etc. were obtained. Identification of the isolates was done using Gram-stain and polymerase chain reaction (PCR). Then, the antimicrobial activity of IRB isolates was investigated using the overlay method against four pathogens, namely Escherichia coli, Enterococcus faecalis, Salmonella enterica serovar Typhi and methicillin-resistant Staphylococcus aureus (MRSA). These four pathogens were selected because of their predominance in causing disease in humans and animals. The antimicrobial activity results showed inhibition zones except for isolates A1 towards E. coli and C1(2)(2) towards S. Typhi. Eight out of ten isolates of IRB strains showed antimicrobial activity towards selected pathogens. This study was able to demonstrate that there is antimicrobial activity of IRB against selected pathogens and, in the meantime, manage to identify the IRB down to the species level as well. This finding showed potential uses of IRB against pathogens that warranted further research.

1.0 INTRODUCTION

The production of secondary metabolites by microorganisms is one of their natural responses in homeostasis and to repress the settlement of ecological competitors as well. Recently, marine microorganisms have attracted much attention from investigators as sources of new biologically active compounds. This is due to the uniqueness of marine microorganisms' environments and the lack of scientific data about them [1]. Several marine bacteria represent a great deal of biotechnological interest, such as for bioremediation, the production of biosurfactants, and the biochemistry of "psychrophilic" enzymes synthesized by low-temperature-adapted microorganisms [2]. These substances have high potential in relation to biotechnological applications in the detergent and food industries, the production of chemical reagents, and medicines. In addition, many new antibiotics have been identified from marine bacteria [3].

Even though the advancement of technologies expands very well, there is still a serious threat posed by pathogenic microorganisms to the world's human population. Among those threats is the development

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of antibiotic resistance which leads to serious and growing health concerns that need to be overturned by the finding of new antibacterial drugs [4]. Their impact is significant to both developing and developed countries, as the World Health Organization (WHO), raises some awareness for future mankind and encourages searching for novel antimicrobial agents as an alternative to modern antibiotics [5]. While it is estimated that microorganisms produce more than 22,000 bioactive compounds in nature, the identification and characterization of new and useful metabolites are scarce [6]. According to Sachitra et al., to screen for new antibacterials, identification of these microorganisms needs to be done, followed by compound extraction and testing against various pathogenic bacteria [7].

The recent surge in biotechnology has significantly drive interest in obtaining new antibiotic agents derived from secondary metabolites of marine bacteria [4]. They are based on the mechanism of synthesis of major microbial metabolites, which can be categorized into different classes, non-ribosomal peptide synthetase [8] and polyketide synthase [9], which are biosynthesis pathways extensively utilized by marine microorganisms for producing antimicrobial substances [10]. The variety of environment conditions has an adverse effect on potential sources of novel pharmaceutical materials [11] as their adaptive strategies and metabolism have been set for their survival in highly diverse and challenging surroundings [12]. There are several factors that contribute to the rich taxonomic diversity of marine biota, such as oxygen concentration, variable salinity, hydrostatic pressure, and temperature conversions, which in turn provide many biologically active compounds [13].

Hence, this study seeks to isolate IRB in coastal waters in Malaysia in the Straits of Malacca and identify if these local IRB demonstrate antimicrobial properties. The presence of this property in local IRB strains allows for future exploration of potential pharmaceutical and biotechnological applications of these IRBs.

2.0 SITE DESCRIPTION AND SAMPLING PROCEDURES

Sampling was conducted twice, once in December and repeated in July, in the coastal area off the Straits of Malacca, at Pasir Panjang, Port Dickson, Negeri Sembilan. This locality was chosen as it was a fishing village with a long-standing jetty in place without extensive industrialization and urbanisation which may introduce industrial and excessive human activity waste and contamination into the waterways discharging into the Straits of Malacca. The sampling to obtain colonies of IRB was conducted at the jetty at Pasir Panjang. This jetty was in a tidal zone with a tidal range of approximately two meters. The duplication of samples was to see if there was a difference in the IRBs isolated during the Northeast and Southwest Monsoon periods. The Northeast Monsoon occurs in December through February period and brings cool and dry air causing strong surface evaporation of the surface water, while the Southwest Monsoon occurs in June to August, bringing warm and dry weather [14].

The IRB was harvested by immersing steel coupons (metal plates) into the sea at the fisherman's jetty, taking care to place the coupons at a depth where they would be continuously immersed in seawater even at low tide. The coupons were left in place without disturbance for a period of one month before being retrieved for sample collection. The now-corroded steel coupons were individually processed, where each was scraped using a fresh sterile scalpel blade to remove any biofilm from the surface of the coupon before the scraping was transferred into a sterile universal bottle using a sterile moistened cotton bud (moistened with sterile normal saline). The universal bottle was then filled to overflowing with seawater from the site where the coupon was sited and then the universal bottle was capped (to ensure that there was no air space remaining in the universal bottle after capping to ensure semi anaerobic conditions).

There were five steel coupons used at each test period, thus resulting in ten samples in total. On arrival back to the laboratory (on the same day of the sample collection), the samples were then transferred into VMNI medium and then incubated for 72 hours at 37 ^{o}C and pure colonies of the ten isolates were isolated and then used for the antimicrobial activity and antibacterial susceptibility tests in this study.

2.1 Isolation of the pure cultures

Twenty percent (20 %) of the original sample (from each universal bottle) was transferred into a new, sterile universal bottle containing VMNI solution. These samples were then incubated for 24 hours at 37 ^{o}C. VMNI medium was used as an enrichment medium for the growth of isolated marine bacteria. The VMNI medium contains trace metals as described by [15] which was modified from Postgate Marine C

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medium. The IRB samples were grown anaerobically at 37 °C for three to seven days. The bacteria were then isolated onto fresh medium by streaking and pour plate techniques to obtain pure cultures that would be used for further analysis. Stock cultures were also prepared from these pure cultures, stored at 4 °C, and regularly transferred into fresh media to maintain viability.

2.2 Gram-Staining

The Gram staining was done based on the protocol proposed by [16]. First, sterile distilled water was dripped over a glass slide, and then a single colony of bacteria was spread to form a thin layer of bacteria smear. Then the smear was fixed with heat over a flame. Next, the smear was flooded with crystal violet reagent for one minute and washed by running tap water before being flooded again with iodine for 1 minute as well. After that, the smear was washed with running tap water and flooded with 70 % ethanol for 30 seconds. Lastly, the smear was flooded once again with safranin for one minute before being inspected under the light microscope with the onset of power lenses (40X) until the oil soaking lenses (100X).

2.3 DNA Extraction

DNA extraction was conducted using the chelex method. Selected colonies were inoculated in 50 to 100 µl ddH2O and 1 ml of 0.5 % saponin in PBS 1x (saved overnight). The mixture was centrifuged (12000 RPM, 10 min). Supernatant was discard. Then 100 µl ddH2O and 50 µl of 20% chelex 100 (shake up the chelex solution and ensure that some of the crystals make it into the sample) were added to a final solution, and the solution was boiled for 10 min and vortexed once after 5 min. The mixture was centrifuged (12000 RPM, ten minutes) and stored at 20 ^{o}C. The DNA concentrations were quantified and qualified by using a NanoDrop 2000 spectrophotometer (Thermo Scientific). The concentration of a 1 µl DNA sample was determined by using the NanoDrop 2000 spectrophotometer (Thermo Scientific). The 260/280 and 260/230 nm ratios were calculated by the NanoDrop spectrophotometer and used to evaluate the DNA purity as well as the concentration of DNA.

2.4 PCR Amplification 16S rRNA Gene Sequence

All extracted DNA of IRB isolates was preceded to 16S rRNA gene amplification by PCR using universal primers 27F (5'AGAGTTTGATCMTGGCTCAG-3') and 1492 R (5'TACGGTTAACCTTGTTACGACTT-3'). The PCR mixture consisted of GoTaq®Green Master Mix Promega (25 µl), primer 27F (0,5-5 µl), primer 1492R (0,5-5 µl), DNA extract (1-5 µl), and Nuclease-Free Water (50 µl). The volume of each mixture is 50 µl and the PCR process was based on a parameter that was set for the amplification of 16S rRNA gene. The PCR parameters were as follow: 95 ^{o}C for ten minutes, 95 ^{o}C for 30 seconds, 55 ^{o}C for 30 seconds and 72 ^{o}C for ten minutes. The cyclical, plating and elongation process is 30 cycles and ends with a final elongation at 72 ^{o}C for ten minutes. The PCR product obtained was run on 1 % agarose gel at 70 volts for 90 minutes to ensure the presence of 1500 base pair PCR products. The gel is then observed under ultraviolet (UV) light using the gelCam camera (Polaroid, USA).

2.5 Antimicrobial Activity

Testing for anti-bacterial activity against pathogenic bacteria, namely *E. coli*, *E. faecalis*, S. t*yphi* and MRSA was performed using the overlay method. Antimicrobial activity was defined by the formation of clear zones around the associated bacteria [17]. This agar diffusion method was used to determine the presence of antimicrobial activity of IRB. One 100 µL aliquot from a culture of each pathogen was measured and used to prepare a standard McFarland 1.0 agar plate (MHA). Paper discs containing 30 µL of the IRB isolates were then placed on the respective prepared MHA surfaces. The MHA plates were then incubated at room temperature for 48 h. Antibacterial activity was indicated by the presence of inhibition zones around the paper disc and the diameter of the inhibition zone measured in mm.

3.0 RESULTS

3.1 Isolation of IRB Isolates

IRB morphology which includes colour, structure and shape was observed after 72 hours of incubation at 37^{o}C. Ten out of 30 isolates were successfully isolated as the results are shown in Table 1. Six isolates,

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E2B, E3B, F1S, F5S, C2(2) and C2(3) have similar characteristics: orange in colour, round shape, and sticky appearance, whereas isolates A1 and C1(2)(2) were round, pale yellow and have a sticky appearance as well (Figure 1).

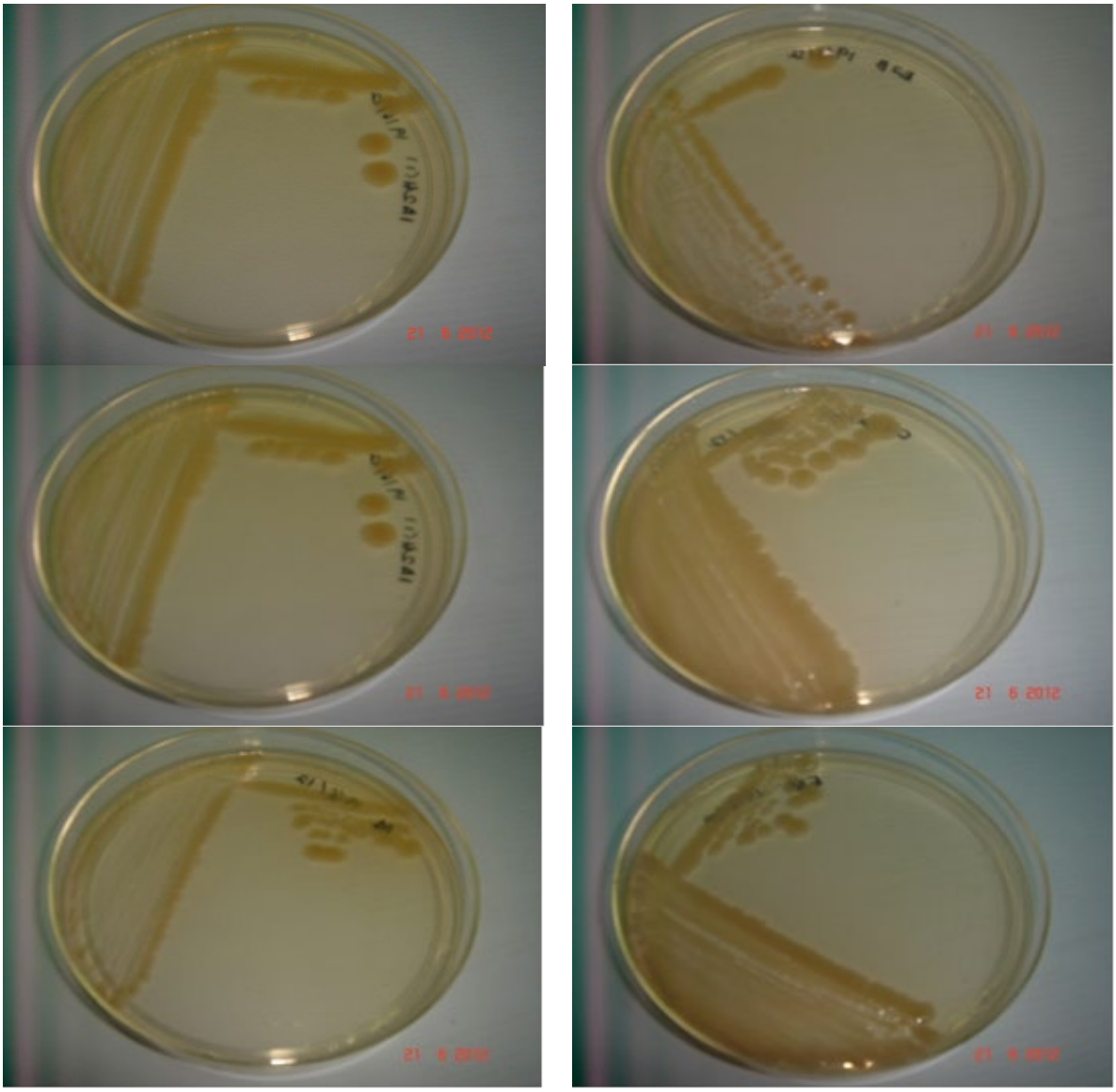

Figure 1. IRB isolates from the waters of Port Dickson, Negeri Sembilan on VMNI agar

Table 1. Colony morphology for IRB isolates from the surface of mild steel immersed in waters of Port Dickson, Negeri Sembilan

Isolates	Colony Morphology		
	Colour	Shape	Structure
A1	Pale yellow	Round	Sticky
C1(2)(2)	Pale Yellow	Round	Sticky
E2B	Orange	Round	Sticky
E3B	Orange	Round	Sticky
F1S	Orange	Round	Sticky
F5S	Orange	Round	Sticky
1A2H (1)	Brown	Round	Sticky
1A2H (2)	Brown	Round	Sticky
C2(2)	Orange	Round	Sticky
C2(3)	Orange	Round	Sticky

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3.2 Gram-staining

Gram-staining and microscopic observation were conducted to observe the bacteriological characteristics of IRB based on Gram reaction and cell shape. Observation by using a light microscope at 1000X scale revealed that the 10 IRB isolates, A1, C1(2)(2), E2B, E3B, F1S, F5S, 1A2H(1), 1A2H(2), C2(2) and C2(3), have similar characteristics such as pink Gram reactions and rod shapes. The isolates were Gram-negative bacteria as shows in Figure 2 and Table 2.

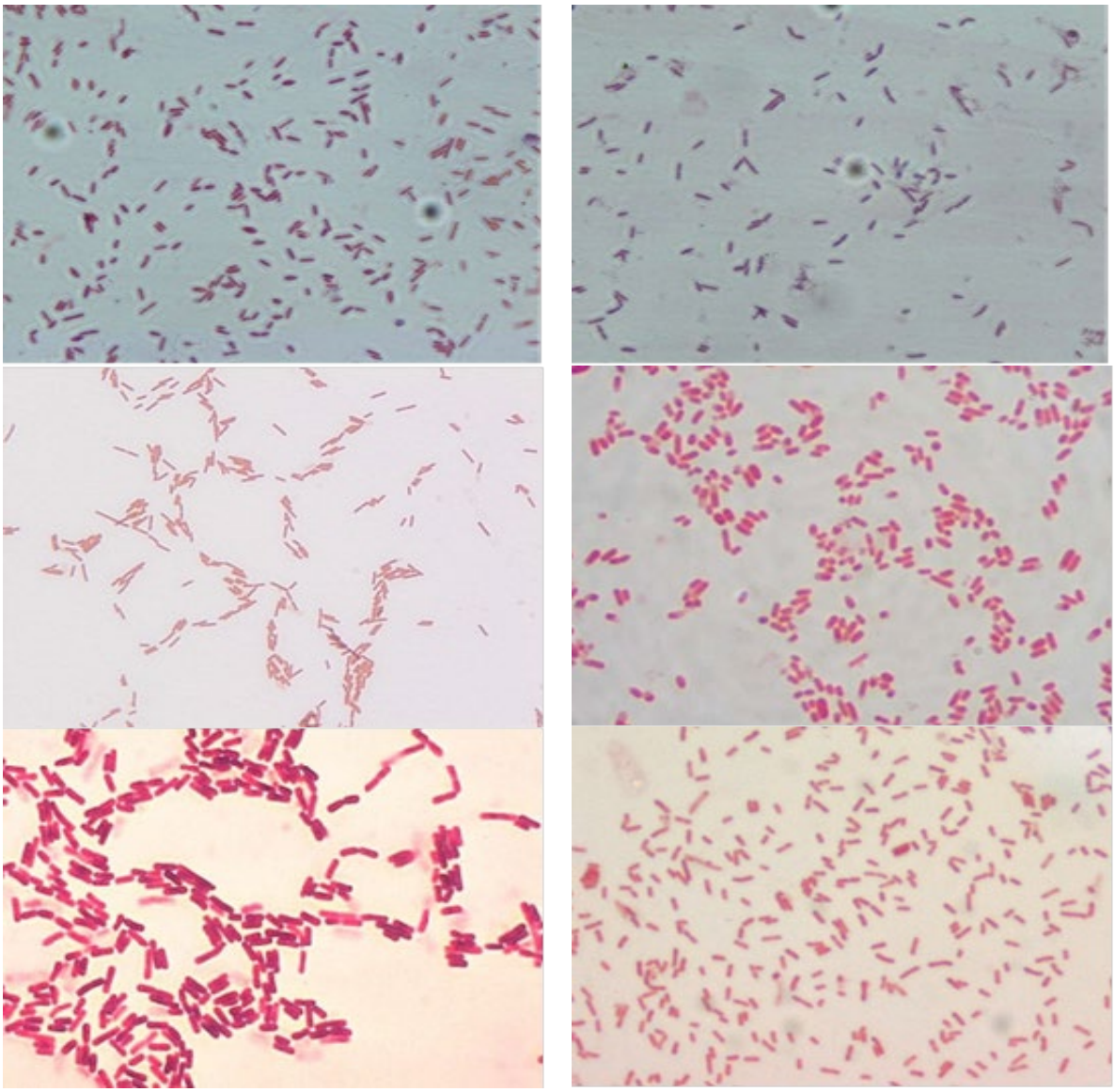

Figure 2. Gram-staining results of IRB isolates from the waters of Port Dickson, Negeri Sembilan (1000X scale)

Table 2. Microscopic characteristics of IRB isolates from the surface of mild steel immersed in the waters of Port Dickson, Negeri Sembilan

Isolates	Colony Morphology		
	Colour	Shape	Structure
A1	Pink	Rod	Negative
C1(2)(2)	Pink	Rod	Negative
E2B	Pink	Rod	Negative
E3B	Pink	Rod	Negative
F1S	Pink	Rod	Negative
F5S	Pink	Rod	Negative
1A2H (1)	Pink	Rod	Negative

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Isolates	Colony Morphology		
	Colour	Shape	Structure
1A2H (2)	Pink	Rod	Negative
C2(2)	Pink	Rod	Negative
C2(3)	Pink	Rod	Negative

3.3 Identification of IRB Isolates

All IRB isolates DNA was successfully extracted based on the protocol proposed [18]. The 16s RNA gene of the isolates was amplified by polymerase chain reaction (PCR). A total of ten 16S rRNA gene amplification products with a size of 1500 base pairs were successfully obtained, as shown in Figure 3. A 1kb DNA ladder marker was positioned at the end of the gel as a marker to facilitate the identification of the band that formed. The PCR products were then purified and sent for sequencing (First Base Sdn. Bhd).

The sequencing data obtained were then compared and matched with the data available in GenBank by using BLAST software (http://www.ncbi.nlm.nih.gov). The results in Table 3 and Figure 3 show that IRB isolates from Port Dickson waters belong to the *Shewanellacea* family and *Shewanella* genus, where two isolates (E2B) and A1 were managed to be identified until their species level, which were *Shewanella haliotis* and *Shewanella hanedai*.

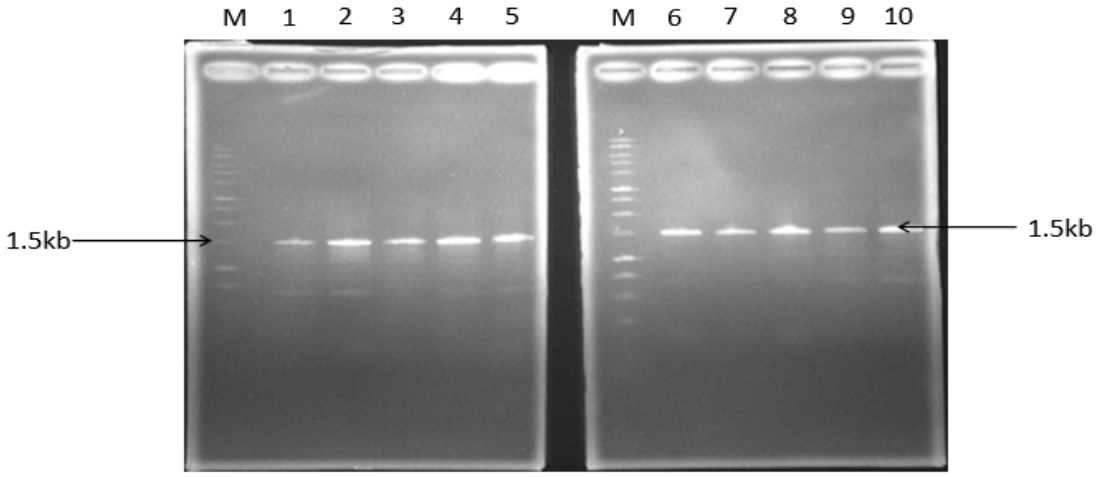

Figure 3. 16sRNA PCR products of IRB isolates from the waters of Port Dickson, Negeri Sembilan. M: 1kb marker; 1: isolate C1(2)(2); 2: isolate E2B; 3: isolate E3B; 4: isolate C2(2); 5: isolate C2(3); 6: isolate 1A2H(2); 7: isolate 1A2H(2); 8: isolate F2(1); 9: isolate F1S; 10: isolate F5S

Table 3. Comparison between IRB isolate sequences isolated from the waters of Port Dickson, Negeri Sembilan, with reference sequences in GenBank

Isolates	Species from GenBank	Accession Number	Similarity (%)	E. value
C2(2)	*Shewanella* sp.	KF500918.1	96	0.0
A1	*Shewanella haliotis*	FN997635	99	0.0
E2B	*Shewanella upenei*	KP090164.1	98	5e-176
E3B	*Shewanella* sp.	JX429809.1	97	0.0
F1S	*Uncultured Shewanella* sp.	KC337207.1	97	0.0
F5S	*Shewanella algae*	AB205579.1	98	0.0
1A2H(2)	*Shewanella* sp.	JF431412	96	0.0
1A2H(1)	*Shewanella* sp.	AF513471	97	0.0
C1(2)(2)	*Shewanella* sp.	KF500918.1	97	0.0
C2(3)	*Shewanella* sp.	KF500918.1	97	0.0

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3.4 Antimicrobial Activity

The antimicrobial activities differed among the 10 isolates and are shown in Table 4. Eight isolates [C2(2), C2(3), E2B, E3B, F1S, F5S, 1A2H(1) and 1A2H(2)] exhibited antimicrobial activity against the four pathogenic bacteria. Only two isolates showed no antibacterial activity at all which were isolate A1 against *E. coli* and isolate C1(2)(2) against *S.* Typhi. It was found that the highest activity was exhibited by the isolates C1(2)(2), which had an inhibition zone of 23 mm against *E. coli*. The images of the inhibition zones produced by IRB isolates against pathogenic bacteria are shown in Figure 4.

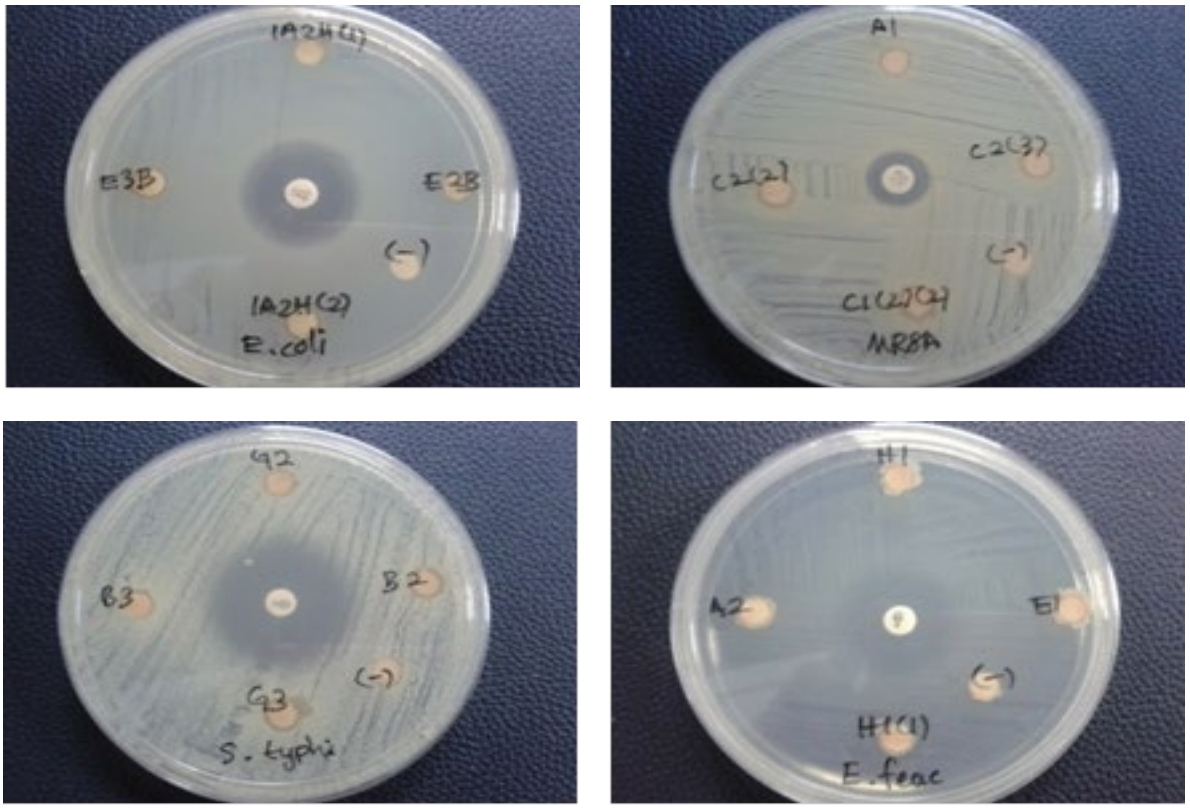

Figure 4. Inhibition zone produced by the IRB strain towards pathogenic bacteria

Table 4. Diameter of inhibition zone produced by the IRB strain towards pathogenic bacteria

Isolate	Pathogenic Bacteria			
	Escherichia coli (mm)	*Salmonella enterica serovar Typhi* (mm)	MRSA (mm)	*Enterococcus faecalis* (mm)
A1	-	8	12	11
C1(2)(2)	23	-	14	9
C2(2)	12	8	8	10
C2(3)	8	9	7	11
E2B	11	7	11	9
E3B	11	9	11	8
F1(S)	15	12	10	11
F5(S)	10	11	11	10
1A2H (1)	11	7	12	12
1A2H (2)	12	8	15	13
Negative control (-)	-	-	-	-
Positive control (+)	19	29	14	19

4.0 DISCUSSION

The colour of IRB isolates on VMNI agar, most of which were orange and pale yellow, produced some kind of rotten egg smell. It is because of the presence of the Fe(II) ion after the Fe(III) was reduced by the IRB during the process of metabolism and respiration [19]. The production of hydrogen sulfide during IRB

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growth caused the bad smell, where hydrogen gas was produced during the reduction of Fe(III) ions by *Alteromonas putrefaciens* combined with sulfur ions to produce hydrogen sulfide gas. According to an investigation, the purpose of using 16S rRNA in polymerase chain reaction (PCR) was to figure out the connection between microorganisms in GenBank and the unidentified bacteria [20]. *Shewanella* belongs to the *Vibrionaceae* family, apart from the other four main genera in the same family, which are *Aeromonas*, *Photobacterium*, *Plesiomonas* and *Vibrio*. *Shewanella* also were grouped under the class Gammaproteobacteria. The *Shewanellaceae* family was originally expanded from the marine bacterial group *Alteromonas* due to the lack of association with any genus under the same family and an overly deep phylogenetic approach. There is only one genus in the family of *Shewanellaceae*, which is *Shewanella*. Based on studies, *Shewanella* was isolated from different sources, including the marine environment and sediment. It was supported by findings from this research, where mild steel was immersed in seawater in Port Dickson, Negeri Sembilan.

For antimicrobial activity, most IRB strains tested with pathogenic *E. coli* produced a large inhibition zone compared to the inhibition zone produced by IRB strains tested with *S.* Typhi, while the inhibition zones produced by most IRB strains when tested with *E. faecalis* were smaller compared to the zone of inhibition produced by IRB strains tested with MRSA. This is because some marine bacteria can produce a unique peptide compound that can be modified and be useful to the bacterial enzyme. The use of gentamicin as a positive control for antimicrobial screening tests coincides with a study which states that gentamicin, piperacillin and cefotaxin are effective antimicrobial agents against *S. putrefaciens* and *S. algae,* which belongs to the IRB group. However, Vogel et. al., proved that there is a difference in the ability of both species to develop antimicrobial resistance to some antimicrobial agents, including penicillin, ampicillin and tetracycline. Pathogenicity level for each species is different; there are strains that gave a small and large reading of inhibition zone, and this is because of the possibility of hemolytic factor and exotoxin shown by *S. algae* than *S. putrefaciens* [19, 21].

From Table 2, the IRB strains that did not produce zones of inhibition when tested with bacterial pathogens, were strains A1 against *E. coli* and C1 (2) (2) against *S.* Typhi. This occurs because the bacterial pathogens used were Gram-negative bacteria, as proved by [16], in which low-income antimicrobial compounds produced against Gram-negative bacteria are caused by the presence of an outer layer that does not exist in Gram-positive bacteria, which in turn prevents the occurrence of any antibiotic molecule penetration. Besides, the outer layers also contain enzymes that are capable of solving the foreign molecule. Debbab et. al., also state that the antimicrobials produced by marine microorganisms are more often effective against Gram-positive bacteria than Gram-negative bacteria [12].

5.0 CONCLUSION

This study was able to demonstrate that there is antimicrobial activity of IRB against selected pathogens and manage to identify the IRB until the species level as well. This finding showed potential uses of IRB against pathogens that warranted further research.

6.0 ACKNOWLEDGEMENT

This research is fully supported by GPJP, UPNM/2019/GPJP/2. The authors fully acknowledge National Defence University Malaysia for the approved fund which makes this important research viable and effective.

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