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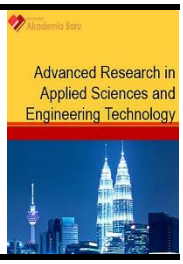

Genotypic and Phenotypic Characterisation of Isolated Marine Bacteria and its Potential to Produce Alkaline Protease

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ABSTRACT

In several industries such as food and detergent, enzymes are commonly used out of which microorganisms' proteolytic enzymes are dominant. Microorganisms from marine sources have advantageous commercial characteristics when it comes to protease production. Protease producing bacterial strains were isolated from four different stations in the South China Sea, located at Kuantan, Pahang-Malaysia. The temperature was ranging from 29 to 36.2°C; salinity varied from 36,18 to 37,9 ppt, turbidity from 10.21 to 16.4 NTU. Dissolved Oxygen had an average of 4.44 mg/mL, and pH was around 8. All the bacterial strains were screened for protease activity using Skim Milk agar plates. Out of 18 isolates, ten strains formed a clear zone on skim milk agar plates. Molecular identification by 16S rRNA results revealed that Bacillus spp was the most dominant bacteria, four isolates belong to Bacillus cereus, another 04 identified as Bacillus licheniformis in addition of one isolate was Bacillus safensis. They were followed by one isolate Staphylococcus warneri. Furthermore, a phenotypic characterization is conducted using The Biolog GENIII MicroPlate with 94 phenotypic tests. These tests consist of 71 carbon source utilization assays and 23 chemical sensitivity assays. The proteolytic activity was confirmed through a quantitative protease assay. Where the strain B. cereus-MD152 was the highest yield, followed by B. licheniformis-ABN13. On the contrary, the lowest yield was secreted by B. cereus-IIUM6 with no more than 16.5 U/ml.

Keywords:
Protease production, bacteria identification, bacteria characterisation, marine Bacillus

1. Introduction

The marine environment is considered the world's most important underwater habitat and the primary biodiversity source on the earth. The biological diversity of aquatic habitats is reckoned to be significantly greater than in tropical rain forests [15]. Caron *et al*., [16] reported that the new high-throughput technologies have demonstrated about 20,000 species per liter of seawater samples,

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with 178,000 species dropping below 34 phyla, microorganisms are considered as the foremost plentiful organisms in the oceans [34]. A significant portion of the maritime microbial population has been obscure such as the least explored naval protists [16]. Bacterial distribution, relying on variations in water salinity, temperature as well as other physicochemical parameters [2]. Marine habitats display unusual characteristics arising from the rare mixture of multiple physical factors. These habitats allow microorganisms to survive at high pressure, low temperatures in the Arctic and Antarctic cold waters, differing pHs, and salinity [12] Much of these microorganisms are used in a large variety of biotechnological applications to provide new companies with innovative bioactive compounds and biocatalysts [11,18]. The cumulative bioactive compounds discovered by marine sources account for 70 % of bacteria, whereas the rest are for fungi and other domains [29].

In comparison to chemical catalysts, microbial enzymes economically and ecologically improve bioprocess reactions by their unique characteristics, including pH resistance, temperatures, and other harsh reaction conditions [24]. *Bacillus* species create fully half of the enzymes generated on the world market, and about 60% of these are proteases [30]. Even so, there have been limited studies on the alkaline protease production from marine microorganisms [7].

Proteases are a group of enzymes, which have a catalytic function to disintegrate peptide bonds of proteins and break them down into small peptides or amino acids [13,28] produced in their exponential phase of the growth curve [25]. Proteases have applications in different manufacturers such as food detergent, pharmaceuticals, leather, and silk, and for recovery of silver from used X-ray films as well. Furthermore, they are applied in some medical applications and for meat tenderization [13]. Where, they constitute one of the three largest groups of enzymes in industry, with about two-thirds of whole enzymes utilized today [9,10]. The current study was conducted in the Southern South China Sea (SSCS) as a part of studies on bacterial diversity to add to the knowledge of Malaysian waters and is indeed part of the SSCS. That it is supposed to be the heart that links Eurasia with the Americas, as the largest shipping port in the world is situated here [3,26]. This study aims to determine the distribution of bacterial proteolytic communities that exist in the South China Sea by isolating and identifying them. As well, marine bacteria that produce alkaline protease have been characterized using Biolog GenIII.

2. Methodology

2.1 Samples collection

Seawater and sediment samples were collected in sterile containers from four different stations in the South China Sea, located at Kuantan–Pahang–Malaysia. Seawater samples have been collected from different distances (400, 700, 1500, and 2000 meters) utilizing the Van Dorn water sampler, while the sediment has been collected using the Ekman dredge bottom sampler.

2.2 Physicochemical parameters

Hydrolab DS5 DataSonde was used for field observation of physicochemical parameters such as coordinates, temperature, pH, dissolved oxygen (DO), turbidity, salinity, and conductivity.

2.3 Bacteria isolation

To isolate the bacteria from seawater and sediment samples, 10 g of marine sediment was added to 20 mL of sdH_2O (Sterile distilled water) that were vortexed till it became homogeneous. After that, the samples were incubated at 37°C for 30 min using an orbital shaker (200 rpm) and were then

centrifuged for 20 min at 5000 rpm. The supernatants were transferred to new centrifuge tubes, and from each supernatant, 1 mL was taken to conduct serial dilution. On the other hand, 10 mL of seawater was pipetted into a centrifuge tube, and just 1 ml of distilled water was transmitted into another tube containing 9 ml to perform serial dilution. Ultimately, serial dilutions (from 10-1 to 10-6) were prepared for both sediment and seawater samples [17].

2.4 Screening of proteolytic ability

The proteolytic activities of all strains were assayed using skim milk agar plates. They were incubated for 24 to 48h at 37 °C, and enzyme activity was observed. Positive colonies degraded the casein in the milk [27].

Fig. 2. Degradation of Skim Milk Agar Plates by Different Marine Bacteria. Bacterial strains producing protease indicated by the formation of clearance zones around the bacterial colonies

2.5 Bacteria identification

The isolates were sent to **1st BASE Products and Services** for Full-Length 16S rRNA Sequencing. OmniLog® Data Collection, Biolog's Microbial Identification Systems program was used to identify the bacterium from its phenotypic pattern in the GEN III MicroPlate.

2.6 Protease Enzyme Production

A preparation of 2% Casein, 1% Dextrose, 1% Peptone, 2% KH_2PO_4, 0.2% NaCl2 and 0.002% $CaCl_2$ at pH 7.0 was used as protease enzyme source. The isolates have been inoculated in nutrient broth for 24h. 1% of inoculum was transferred in 250 ml conical flasks containing 50 ml of the enzyme production medium and incubated at 37°C for three days. Afterward, the supernatant was gathered and used as a crude enzyme after centrifugation at 10,000 rpm for 10 min [28].

2.7 Enzyme Assay

Protease activity was determined by the standard assay [21]. The reaction mixture contained 2.5 ml of casein (1%w/v, prepared in 50 mM of tris buffer, pH 8), and an aliquot of 0.5 was incubated in a 37°C water bath for 10 min. After that, 2.5 ml of 0.11M trichloroacetic acid was added to the solution to stop the reaction and can remain for 45 min at room temperature. 1ml of supernatant was obtained by centrifugation at 10,000xg for 10 min at four °C and used for color development,

which was achieved by mixing 1 ml of supernatant with 2.5 ml of 0.5 M Na_2CO_3 and 0.5 ml of 0.1N Folin Ciocalteu's Phenol Reagent for 30 min. Eventually, the absorbance was read at 660nm against the blank sample. One enzyme activity unit was defined as the amount needed to release one µg of tyrosine per ml per min under assay conditions.

3. Results and Discussion

3.1 Sample Collections

Table 1 represents the conditions of the physicochemical parameters of the four different stations, which sit at different distances (400, 700, 1500, and 2000 meters) that show Fig.1.

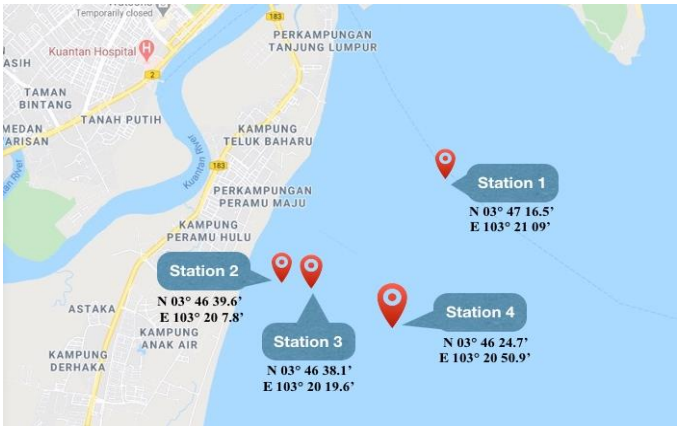

Fig. 1. South China Sea and its designated sampling area

Table 1
Physicochemical Parameters Conditions of the different four stations

Stations	Coordinates	Temperature (C°)	conductivity (mS/cm)	pH	Salinity (ppt)	Turbidity (NTU)	DO (mg/L)	Distance (Meter)
Station (1)	N 03° 47 16.5 E 103° 21 09	29	56.6	8.1	37.9	14.2	4.53	1500
Station (2)	N 03°46 39.6 E 103° 20 7.8	36.2	56.4	8.04	37.78	10.21	4.05	400
Station (3)	N 03° 46 38.1 E 103° 20 19.6	32.6	55.3	8.05	37.24	12.32	4.33	700
Station (4)	N 03° 46 24.7 E 103° 20 50.9	30.04	55	8.07	36.18	16.4	4.86	2000

The highest value of salinity was 37.9 ppt that located in the station (3), whereas the lowest value was placed in the station (4), with 36.18 ppt. Changes in salinity levels can influence sea organisms. Salinity is one of the essential environmental components that affect the reproduction, growth, and dissemination of a variety of marine life forms [19]. The aquatic water quality parameters such as temperature, dissolved oxygen, and conductivity can be affected by the salinity levels within the water [23]. The range of temperature at all stations was between 29 to 36.2°C. The ocean water temperature varies from 28°C to 32°C [23], while Bartram *et al.*, [5] indicated that the body of water retains temperature variations. Conductivity showed the lowest value (55.3 mS/cm) in the station (3) and the highest value (56.6 mS/cm) in the station (1). pH values of the four stations were almost similar and ranged from 8.05 to 8.1. The lowest value of dissolved oxygen (DO) was 4.05 mg/L in the

station (2), which sited at 400 meters, while the highest value was in the station (4), with 4.86 mg/L. Indeed, the amount of dissolved oxygen in seawater is affected by many factors such as the atmospheric pressure, temperature, ion activity, the number of organisms utilizing oxygen for respiration, and the volume and velocity of the seawater [17]. Among the sampling station areas, station (2) was the most polluted. Perhaps, a high number of microorganism communities have existed at this station. Therefore, they have been used more dissolved oxygen compared to other stations. The presence of *Staphylococcus* at this station can also be explained that the domestic sewage run-off pathogenic bacteria present in huge quantities [17]. This was owing to dissolved oxygen (DO) necessary for the marine systems, growth, and existence of many marine organisms. UNESCO WHC* Reported that Seawater requires at least four mg/L of oxygen and, preferably, five mg/L of oxygen for a biological environment that functions at optimum levels for aquatic. Turbidity represents the combination of sand and xenobiotic compounds that have accumulated over decades. The highest turbidity value (16.4 NTU) was recorded in the station (4) at 2000m, while the lowest value (10.21 NTU) was in the station (2) at 400m from shore. Much of the time, this event would result in harmful effects on aquatic sediment.

These results demonstrate that the physicochemical parameters of coastal waters of the Southern South China Sea in the four different stations were insignificant, which revealed that the sea was well mixed. Similar to the finding recorded by Schmidt *et al*., [31]. A significant association between salinity and conductivity for all stations is primarily caused by variations in temperature, dissolved oxygen, and conductivity. There was a connection between temperature and salinity. DO increases when the temperature and salinity decrease except for station (1). Those results were in line with Sverdrup *et al*., [33].

3.2 Isolation of Bacteria and Screening of its Ability to Produce Protease

In the present study, eighteen strains were isolated from eight different samples (sediments and seawater) from the South China Sea. However, only ten strains were found able to produce the protease enzyme by forming a transparent hydrolytic zone on skim milk agar, which could be owing to casein hydrolysis. This finding is similar to Asha and Palaniswamy [4], Cui *et al*., [8] and Fulzele *et al*., [13] . Simple and quick plate test used for primary inspection of protease production was very effective and successful; it enables a significant number of proteolytic colonies to be screened for a very short time [32]. The colony morphology and Gram staining of the ten isolates have been examined. The cultural morphological and physiological characteristics of selected bacterial isolates are presented in Table (2) below.

3.3 Molecular characterization

The proteolytic strains were identified employing 16S rRNA sequencing. The sequences of seven isolates were reported in the GenBank database under accession numbers shown in Table 2 and then it has been matched with those available in the NCBI database. On the other hand, the sequencing findings indicate that samples 06 and 09 are mixed isolate samples. Therefore, the results cannot be used in the BLAST analysis. The Phylogenetic test revealed that *Bacillus genus* was found predominantly, the strains (ABN13, AD242, TB212, NID706) were *B. licheniformis, (those findings are similar to the results reported by Vivi Mardina and Yusof [22] ,* other two strains (MD152 and ISD488) were identified as *Bacillus cereus (Abou-Elela, Ibrahim, Hassan, Abd-Elnaby, & El-Toukhy, 2011).* While, KRF402 displayed a similarity to *Bacillus safensis* [20] with a similarity of 99% to strain (NBRC100820). The strain DK131 was identified as *Staphylococcus warneri, which was* found at St2,

400 meters from the shore. *This can be referred to* as the domestic waste discharges and the food industry by the coast [14,17].

Table 2
Morphological Characteristics, Accession Numbers and Names of Identified Isolates

Bacteria	N°	Strain name	Accession number	Source	Station	Protease activity U/ml	Morphological characteristics
Bacillus cereus	01	MD152	**MT642947**	S	St3	33.82	Gram-positive bacteria with creamy white colonies, glistening, translucent, small, circular, raised. Some are irregular.
	06	IIUM6	---	S	St2	23.09	
	09	IIUM9	---	S	St1	16.31	
	10	ISD488	---	S	St4	13.50	
Bacillus licheniformis	04	ABN13	**MT642943**	W	St3	29.96	Gram-positive bacteria Circular white small colonies, dull and glistening. Earlier, the colonies got bigger, dry, dull, and filamentous. They are firmly adhered to the agar.
	05	AD242	**MT642944**	S	St4	23.2	
	07	TB212	**MT642945**	W	St1	17.01	
	08	NID706	**MT642946**	S	St1	16.73	
Bacillus safensis	02	KRF402	**MT642941**	S	St4	21.14	Gram-positive, raised colonies. with irregular margins
Staphylococcus warneri	03	DK131	**MT642942**	W	St2	19.30	Gram-positive bacteria. Very small circular beige to yellowish colonies, glistening and raised.

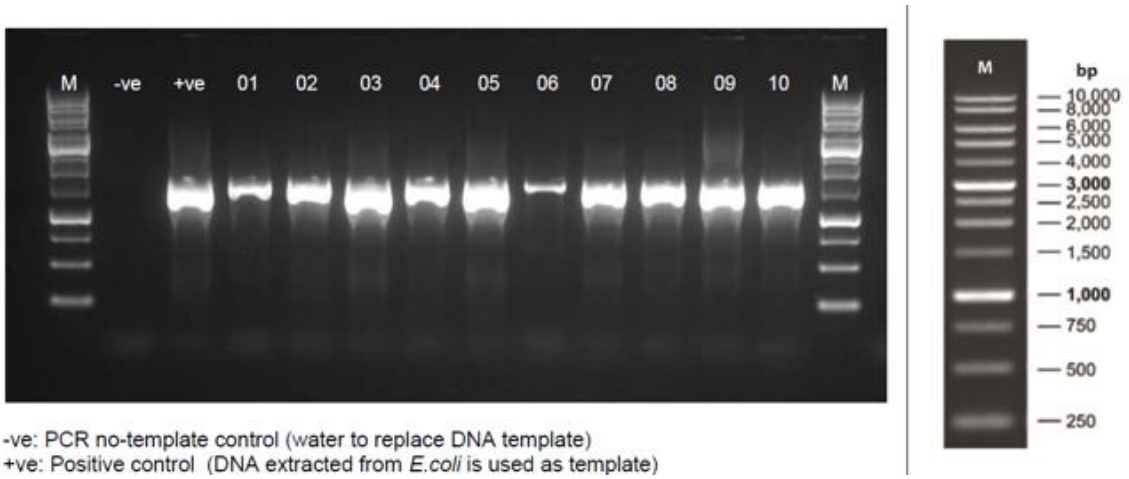

Fig. 3. Agarose Gel Electrophoresis for 10 Isolates Producing Protease

3.4 Biolog GEN III MicroPlate Characterisation

In the biochemical test conducted by **Biolog GENIII MicroPlate,** positive reactions are indicated by forming a purple color due to the tetrazolium redox dye reduction.

The four *Bacillus cereus* strains are also positive to the following Amino-acids: Gelatine and L-Histidine, similar to IIUM6 and which are positive even to L-Glutamic Acid. Unlike sugar sources, these strains' positivity to Hexose-Acids is likely less, with all *B. cereus* strains positive only to D-Galacturonic Acid and to D-Gluconic Acid. The reaction with L-Lactic Acid, Acetoacetic Acid was positive for the four strains. Also, IIUM6 was positive for Methyl Pyruvate, where IIUM9 was positive for Methyl Pyruvate, Formic Acid, and acetic acid, the same as MD152. As an exception, ISD488 and

IIUM6 were negative to Acetic Acid. The tolerance level of *Bacillus cereus* strains to pH was at pH6. All strains showed salt tolerance at 1 and 4% NaCl and 1% Sodium Lactate concentrations. They were positive to salt compounds D-Serine -except strain IIUM9-, Guanidine HCl, Lithium Chloride, Potassium Tellurite (except MD152), Aztreonam, and Sodium Butyrate. Additionally, MD152 had a higher salt tolerance concentration at 8% NaCl. However, IIUM9 and MD152 had no tolerance for D-Serine and Potassium Tellurite, respectively.

B.licheniformis -NID706 could assimilate Dextrin and D-trehalose only while *B. safensis*- KRF402 and B3 reacted to each of Dextrin, D-maltose, and sucrose in addition to D-turanose by *B. safensis*-KRF402 also. All *B.licheniformis* strains catabolized D-salicin and N-acetyl-D-glucosamine, where *B. safensis*-KRF402, *B.licheniformis*-ABN13, *B. licheniformis- AD242*, and *B. licheniformis* -NID706 showed positive reaction only with D-salicin, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, and β-methyl-D-glucoside, respectively. Both *B. safensis*- KRF402 and *B. licheniformis- AD242* reacted positively to D-serine and D-fructose. Additionally, *B. licheniformis- AD242* was also positive for D-fucose and inosine with *B. licheniformis* ABN13. However, *B. safensis*- KRF402 was alone capable of disassembling D-galactose and *B. licheniformis*-ABN13 to L-fucose. Furthermore, the results showed that bacteria *B. safensis*- KRF402 and B3 were able to break down D-sorbitol, D-mannitol, D-arabitol, Myo-inositol, glycerol, D-glucose-6-PO4, and D-fructose-6-PO4, except for *B.licheniformis*-ABN13, which was significant with D-serine, same as *B. licheniformis- AD242*, but ineffective with D-sorbitol. *B.licheniformis* -NID706, otherwise, reacted positively to D-glucose-6-PO4 and D-fructose-6-PO4 only. Moreover, gelatin, L-alanine, L-aspartic-acid, L-glutamic acid, and L-serine were catabolized by all strains when *B.licheniformis*-ABN13 was found positive with L-pyro-glutamic acid together with *B. safensis*- KRF402 and *B.licheniformis* -NID706, which, in turn, responded to L-histidine. D-saccharic acid, exceptionally, is consumed by *B. safensis*- KRF402, *B.licheniformis*-ABN13, and *B.licheniformis*-NID706. However, all strains are positive for pectin. *B.licheniformis*-ABN13 and *B.licheniformis*-NID706 responded positively to methyl pyruvate, citric acid, α-keto-glutaric acid, D-malic acid, L-malic acid, and Bromo-succinic acid. *B. licheniformis- AD242* was also positive but to L-lactic acid, α-keto-glutaric acid, and L-malic acid only.

3.5 Quantitative estimation of protease activity using crude cell

Figure 4 showed that with more than 33 U/ml, *B. cereus*-MD152 had the highest protease activity, followed by *B. licheniformis*-ABN13 with 29 U/ml. Both were in St1. In comparison, with no more than 13,5 U/ml isolated from St2, the lowest output was reported for *B.cereus- ISD488.* When *B. safensis*-KF402 showed a yield of 21 U/ml. Singh *et al*., [32] stated that *B. safensis* potentially secretes protease in its stationary phase.

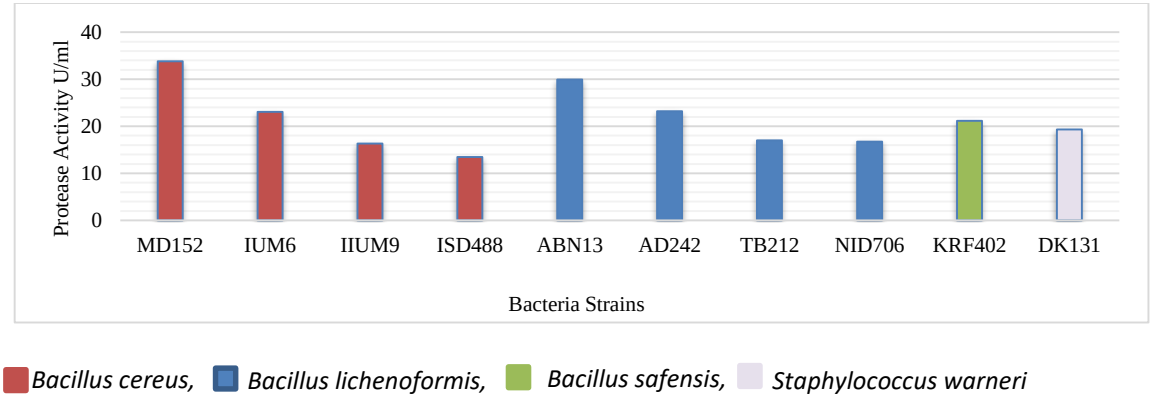

Fig. 4. Protease Production for 10 isolates

4. Conclusion

In this study, the results show a negligible difference in the physicochemical parameters of seawater between the four different stations of the South China Sea, which have shown that the sea is well blended. Ten strains out of 18 isolates were able to produce protease enzyme to *Bacillus cereus, B. safensis, B. lichenoformis,* and *Staphylococcus warneri* based on the molecular identification of 16S rRNA gene and Biolog GEN III Microplate. Isolates of MD152 and ABN13 *B. cereus* and *B. lichenoformis*, respectively, were found to be the most promising proteolytic producers based on their quantitative enzyme production results.

The purpose of this research was to explain the connections between the physicochemical parameters and the bacterial communities that flourish in the Southern South China Sea. The knowledge of bacterial diversity gives us a profound impression of the potential behavior of the microbe. However, the results do not reveal any signs of serious pollution.

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References

[1] Abou-Elela, Gehan M., Hassan AH Ibrahim, Sahar W. Hassan, Hanan Abd-Elnaby, and Nabil MK El-Toukhy. "Alkaline protease production by alkaliphilic marine bacteria isolated from Marsa-Matrouh (Egypt) with special emphasis on Bacillus cereus purified protease." *African Journal of Biotechnology* 10, no. 22 (2011): 4631-4642.

[2] Alavandi, S. V. "Relationship between heterotrophic bacteria and suspended particulate matter in the Arabian Sea." *Indian Journal of Microbiology* 30, no. 1 (1990): 89-92.

[3] Arai, Takaomi. "Diversity and conservation of coral reef fishes in the Malaysian South China Sea." *Reviews in Fish Biology and Fisheries* 25, no. 1 (2015): 85-101. https://doi.org/10.1007/s11160-014-9371-9

[4] Asha, B., and M. Palaniswamy. "Optimization of alkaline protease production by Bacillus cereus FT 1 isolated from soil." *J App Pharm Sci* 8, no. 02 (2018): 119-127.

[5] Bartram, Andrea K., Michael DJ Lynch, Jennifer C. Stearns, Gabriel Moreno-Hagelsieb, and Josh D. Neufeld. "Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end Illumina reads." *Applied and environmental microbiology* 77, no. 11 (2011): 3846-3852. https://doi.org/10.1128/AEM.02772-10

[6] Caron, David A., Alexandra Z. Worden, Peter D. Countway, Elif Demir, and Karla B. Heidelberg. "Protists are microbes too: a perspective." *The ISME journal* 3, no. 1 (2009): 4-12. https://doi.org/10.1038/ismej.2008.101

[7] Zhenming, Chi, Liu Zhiqiang, Gao Lingmei, Gong Fang, M. A. Chunling, Wang Xianghong, and L. I. Haifeng. "Marine yeasts and their applications in mariculture." *Journal of Ocean University of China* 5, no. 3 (2006): 251-256. https://doi.org/10.1007/s11802-006-0010-5

[8] Cui, Hongxia, Liping Wang, and Yang Yu. "Production and characterization of alkaline protease from a high yielding and moderately halophilic strain of SD11 marine bacteria." *Journal of Chemistry* 2015 (2015). https://doi.org/10.1155/2015/798304

[9] Cui, Hongxia, Muyang Yang, Liping Wang, and Cory J. Xian. "Identification of a new marine bacterial strain SD8 and optimization of its culture conditions for producing alkaline protease." *PloS one* 10, no. 12 (2015): e0146067. https://doi.org/10.1371/journal.pone.0146067

[10] Cui, Jian Dong, and Shi Ru Jia. "Optimization protocols and improved strategies of cross-linked enzyme aggregates technology: current development and future challenges." *Critical reviews in biotechnology* 35, no. 1 (2015): 15-28. https://doi.org/10.3109/07388551.2013.795516

[11] de Pascale, Donatella, Concetta De Santi, Juan Fu, and Bjarne Landfald. "The microbial diversity of Polar environments is a fertile ground for bioprospecting." *Marine Genomics* 8 (2012): 15-22. https://doi.org/10.1016/j.margen.2012.04.004

[12] Faulkner, D. John. "Marine natural products." *Natural Product Reports* 5, no. 6 (1988): 613-663. https://doi.org/10.1039/np9880500613

[13] Fulzele, Rachana, Elisha DeSa, Amit Yadav, Yogesh Shouche, and Rama Bhadekar. "Characterization of novel extracellular protease produced by marine bacterial isolate from the Indian Ocean." *Brazilian Journal of Microbiology* 42, no. 4 (2011): 1364-1373. https://doi.org/10.1590/S1517-83822011000400018

[14] Gunn, B. A., J. F. Keiser, and R. R. Colwell. "Numerical taxonomy of staphylococci isolated from clinical sources." *International Journal of Systematic and Evolutionary Microbiology* 33, no. 4 (1983): 738-750. https://doi.org/10.1099/00207713-33-4-738

[15] Haefner, Burkhard. "Drugs from the deep: marine natural products as drug candidates." *Drug discovery today* 8, no. 12 (2003): 536-544. https://doi.org/10.1016/S1359-6446(03)02713-2

[16] Hahn, Martin W. "The microbial diversity of inland waters." *Current opinion in biotechnology* 17, no. 3 (2006): 256-261. https://doi.org/10.1016/j.copbio.2006.05.006

[17] Jalal, K. C. A., Y. Kamaruzzaman, A. Fairuz, B. Akbar, S. Shahbudin, and Y. Faridah. "Bacterial communities in Kuantan estuary of Pahang Malaysia." *Journal of Applied Sciences* 10, no. 8 (2010): 652-657. https://doi.org/10.3923/jas.2010.652.657

[18] Kennedy, J., N. D. O'leary, G. S. Kiran, J. P. Morrissey, Fergal O'Gara, J. Selvin, and A. D. W. Dobson. "Functional metagenomic strategies for the discovery of novel enzymes and biosurfactants with biotechnological applications from marine ecosystems." *Journal of Applied Microbiology* 111, no. 4 (2011): 787-799. https://doi.org/10.1111/j.1365-2672.2011.05106.x

[19] Kumlu, M., and D. A. Jones. "Salinity tolerance of hatchery-reared postlarvae of Penaeus indicus H. Milne Edwards originating from India." *Aquaculture* 130, no. 2-3 (1995): 287-296. https://doi.org/10.1016/0044-8486(94)00319-J

[20] Liu, Yang, Qiliang Lai, Chunming Dong, Fengqin Sun, Liping Wang, Guangyu Li, and Zongze Shao. "Phylogenetic diversity of the Bacillus pumilus group and the marine ecotype revealed by multilocus sequence analysis." *PloS one* 8, no. 11 (2013): e80097. https://doi.org/10.1371/journal.pone.0080097

[21] Mardina, V., F. Yusof, and Md Z. Alam. "Statistical optimization of physichochemical factors for protease production by Bacillus licheniformis on skim latex serum fortified media." *Journal of Engineering Science and Technology* 6, no. 1 (2015): 42-52.

[22] Mardina, Vivi, and Faridah Yusof. "Purification and characterization of surfactant-stable protease from Bacillus licheniformis: A potential additive for laundry detergent." *International Journal of Advanced Biotechnology and Research* 7, no. 2 (2016): 634.

[23] Hamzah, F. Mohamad, S. N. S. Mohammed, SM Syed Abdullah, KN Abdul Maulud, SF Mohd Razali, and M. K. A. Kamarudin. "The effect of marine parameters on salinity via statistical approaches." In *Journal of Physics: Conference Series*, vol. 1489, no. 1, p. 012035. IOP Publishing, 2020. https://doi.org/10.1088/1742-6596/1489/1/012035

[24] Nigam, Poonam Singh. "Microbial enzymes with special characteristics for biotechnological applications." *Biomolecules* 3, no. 3 (2013): 597-611. https://doi.org/10.3390/biom3030597

[25] Nurullah, Akcan. "High Level Production of Extracellular Protease from Bacillus licheniformis ATCC 12759 in Submerged Fermentation." *RESEARCH JOURNAL OF BIOTECHNOLOGY* 7, no. 3 (2012): 46-51. https://doi.org/10.5897/AJB11.2183

[26] Onuoha, M. D. U., I. Ismail, A. S. Ismail, and M. F. Mansor. "Experimental determination of flow patterns and water holdup of low viscosity oil-water system in horizontal pipes." *Sains Malaysiana* 45, no. 11 (2016): 1635-1640.

[27] Padmapriya, Balakrishnan, Thamaraichelvan Rajeswari, Rajendran Nandita, and Flanet Raj. "Production and purification of alkaline serine protease from marine Bacillus species and its application in detergent industry." *Eur J Appl Sci* 4, no. 1 (2012): 21-26.

[28] El-Hassayeb, H. E. A., and S. M. Z. Abdel Aziz. "Screening, production and industrial application of protease enzyme from marine bacteria." *Int. J. Curr. Microbiol. Appl. Sci* 5 (2016). https://doi.org/10.20546/ijcmas.2016.507.099

[29] Prabavathy, Vaiyapuri R., Narayanasamy Mathivanan, and Kandasamy Murugesan. "Control of blast and sheath blight diseases of rice using antifungal metabolites produced by Streptomyces sp. PM5." *Biological Control* 39, no. 3 (2006): 313-319. https://doi.org/10.1016/j.biocontrol.2006.07.011

[30] Reddy, L. V. A., Young-Jung Wee, Jong-Sun Yun, and Hwa-Won Ryu. "Optimization of alkaline protease production by batch culture of Bacillus sp. RKY3 through Plackett–Burman and response surface methodological

approaches." *Bioresource technology* 99, no. 7 (2008): 2242-2249.
[31] Schmidt, Hannes, Steffen Seitz, Egon Hassel, and Henning Wolf. "The density–salinity relation of standard seawater." *Ocean Science* 14, no. 1 (2018): 15-40. https://doi.org/10.5194/os-14-15-2018
[32] Singh, Ram, Rupinder Singh, and Mukesh Yadav. "Molecular and biochemical characterization of a new endoinulinase producing bacterial strain of Bacillus safensis AS-08." *Biologia* 68, no. 6 (2013): 1028-1033. https://doi.org/10.2478/s11756-013-0259-2
[33] Sverdrup, H. U., Johnson, M. W., & Fleming, R. H. (1942). "The Oceans". 1087 pp. Prentice-Hall Inc., New York.
[34] UEPA (United States Environmental Protection Agency) (2006). Marine ecosystems. http:// www.epa.gov/bioiweb1/aquatic/marine.html.
[35] UNESCO WHC*: Anon UNESCO World Heritage Centre - New Inscribed Properties (1978)