TRENDS IN SCIENCES 2025; 22(11): 10574
https://doi.org/10.48048/tis.2025.10574 RESEARCH ARTICLE

Novel Hydrolytic Enzymes and Protein Modelling from Marine Sedimentary Bacteria Losari Coastal, Makassar South Sulawesi, Indonesia

Anita[1,4], Hasnah Natsir[1,*], Paulina Taba[1], Ahmad Ahyar[1], Nunuk Hariani Soekamto[1], Nur Umriani Permatasari[1], Wahyudin Rauf[1], Sarlan[1], Nasrum Massi[2], Sudding[3] and Andi Fatmawati[4]

[1]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Hasanuddin University, South Sulawesi 90245, Indonesia
[2]Departement of Microbiology, Faculty of Medicine, Hasanuddin University, South Sulawesi 90245, Indonesia
[3]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Negeri Makassar, South Sulawesi 90222, Indonesia
[4]Medical Laboratory Technology, Polytechnic of Muhammadiyah Makassar, South Sulawesi 90132, Indonesia

([*]Corresponding author's e-mail: hasnahnatsir@unhas.ac.id)

Received: 7 May 2025, Revised: 9 June 2025, Accepted: 20 June 2025, Published: 5 August 2025

Abstract

Marine ecosystems are invaluable sources of biodiversity and unique chemical compounds, especially for the discovery of new natural products, including enzymes. Marine sediment bacteria from the Losari Coastal, Makassar City, Indonesia, show great potential for biotechnological applications due to their ability to produce enzymes with extraordinary properties such as salt tolerance, heat stability, and adaptability to extreme conditions. This study investigated the enzymatic capabilities of marine sediment bacteria isolated from the Losari Coastal, Makassar City, Indonesia, focusing on 3 enzymes: Amylase, lipase, and protease. Bacterial samples were collected from 4 coastal locations, and a comprehensive analysis of their morphological, physiological, and molecular characteristics was performed, combining 16S rRNA sequencing and protein modeling using SWISS-MODEL. This study yielded 15 bacterial isolates characterized by a gram-negative, rod-shaped morphology and distinctive, round, yellow colonies with serrated edges. Among these, 10 produced amylase, 9 produced lipases, and 10 showed protease activity; however, 2 isolates stood out because they could produce all three enzymes: amylase, lipase, and protease. The MB-SL 5 isolate showed 97.82% similarity to *Shewanella algae* strain DW01, and the MB-SL 6 isolate had 90.53% similarity to *Vibrio alginolyticus* NBRC 15630 strain, with predicted protein structure code A0A3M5GRJ4_1 A. These findings highlight the significant biotechnological potential of marine sediment bacteria from the Losari Coastal producing hydrolytic enzymes for industrial applications, which opens new avenues for the development of new biocatalysts.

Keywords: Marine sediment, Bacteria, Amylase, Lipase, Protease, Losari coastal

Introduction

Marine ecosystems have been identified as rich resources of biodiversity [1,2] and chemical compounds [3,4], serving as significant sources for the discovery of natural products like enzymes [5-7]. Marine bacteria play important roles in biogeochemical cycles, organic matter degradation, and nutrient dynamics in coastal environments through their evolved hydrolytic enzymes. In recent years, marine bacteria have become essential sources of enzymes with high industrial value [8-10], exhibiting various enzymatic activities and initiating various biochemical events [5]. Enzyme biotechnology from marine bacteria efficiently produces novel biocatalysts [7] with superior characteristics such as high salinity, extreme pH ranges, elevated hydrostatic

pressure, and temperature fluctuations [10], cold adaptation, and suitability for large-scale production [11,12].

Microbial hydrolytic enzymes, including amylases, proteases, and lipases, serve as essential biocatalysts in the degradation of complex organic substrates and play a central role in biogeochemical cycles in marine ecosystems [13,14]. These enzymes have attracted significant attention in biotechnological applications due to their substrate specificity, catalytic efficiency, ability to operate under diverse conditions, cost-effectiveness, and eco-friendliness as an alternative to traditional chemical processes [15]. Kocabaş *et al*. [16] reported that the global market for industrial enzymes is projected to have an annual growth rate of 10% to 15%. These applications span pharmaceuticals [17], food processing [18], starch production [19], laundry industries [20], detergent manufacturing [21], textiles [22], leather treatment [23], wood pulp processing [24], and paper production [25].

Protein modeling is a computational approach to understanding the structure and function of novel enzymes [26,27]. This computational framework supports protein engineering to improve stability, catalytic efficiency, and substrate specificity for industrial applications [28,29]. The integration of experimental characterization with computational modeling optimizes novel hydrolytic enzymes for various industrial processes [30,31].

The Losari Coastal in Makassar City, South Sulawesi, is an ecosystem with ideal environmental characteristics for isolating bacteria producing high-value industrial enzymes, where the relatively untouched coastal sediments have great potential as a source of enzyme-producing bacteria that can be applied in various industrial sectors for future biotechnological discoveries. This area has a dynamic salinity gradient due to the meeting of freshwater from surrounding rivers with seawater. This creates selective pressure that drives microorganisms to develop enzymes that are highly stable to various salt concentrations. Marine sediments are rich in organic nutrients from urban activities and natural sources, providing a variety of substrates that support the growth of bacterial communities, producing hydrolytic enzymes such as amylase, protease, and lipase [32]. The combination of sunlight exposure and tidal patterns creates substantial daily temperature variations, providing an optimal habitat for isolating bacteria producing thermally stable enzymes, a valuable trait that is important for industrial processes operating in a wide range of temperature conditions. pH variations from biogeochemical activities and anthropogenic influences also drive the adaptation of microorganisms to produce enzymes with a wide pH tolerance [33].

This study investigates the ability of coastal marine sediment bacteria in Losari Coastal to produce novel hydrolytic enzymes of industrial value, such as amylase, lipase, and protease, through isolation, characterization, molecular identification, and protein modeling. By leveraging the unique microbial diversity of the Losari Coastal and advanced protein modeling techniques, this research aims to develop new biocatalysts that can strengthen Indonesia's position in the global biotechnology market while promoting environmentally friendly and sustainable industrial practices.

Materials and methods

Study area and sample collection

Sediment samples were collected from 4 designated sites along Losari Beach, Makassar, Indonesia: Point 1 (–5.133688 N, 119.344029 E), Point 2 (–5.134539 N, 119.402609 E), Point 3 (–5.135378 N, 119.402702 E), and a Control Point (–5.123931 N, 119.344029 E), as illustrated in **Figure 1**. All samples were obtained at a depth of 5 m, approximately 2 km offshore, where water temperatures ranged from 30 to 31 °C and pH values measured between 5 - 7. The collected sediments were immediately transferred to sterile containers and preserved in a cooler box before analysis at the Bacteriology Laboratory of Polytechnic of Muhammadiyah Makassar.

Figure 1 Marine Sediment Losari Coastal, Makassar City Sampling Points.

Isolation and purification of marine bacteria

In a study by Natsir *et al*. [34] optimization of marine bacterial growth was conducted across temperature ranges of 30 - 37 °C and pH values of 5 - 7. The results revealed optimal marine bacterial growth at 37 °C with a pH of 7. Based on **Figure 2**, the methodology involved mixing sediment samples (2 mL) with sterile seawater (18 mL), followed by serial dilutions from 10^1 - 10^6. This diluted suspension was then inoculated into Brain Heart Infusion Broth (BHIB) prepared with sterile seawater and incubated at 37 °C for 48 h. Subsequently, the pour plate method isolated marine bacteria by transferring the enriched culture to nutrient agar media containing sterile seawater. Pure bacterial isolates were obtained through subculturing onto fresh nutrient agar media with sterile seawater using the streak plate method, followed by incubation at 37 °C for 48 h.

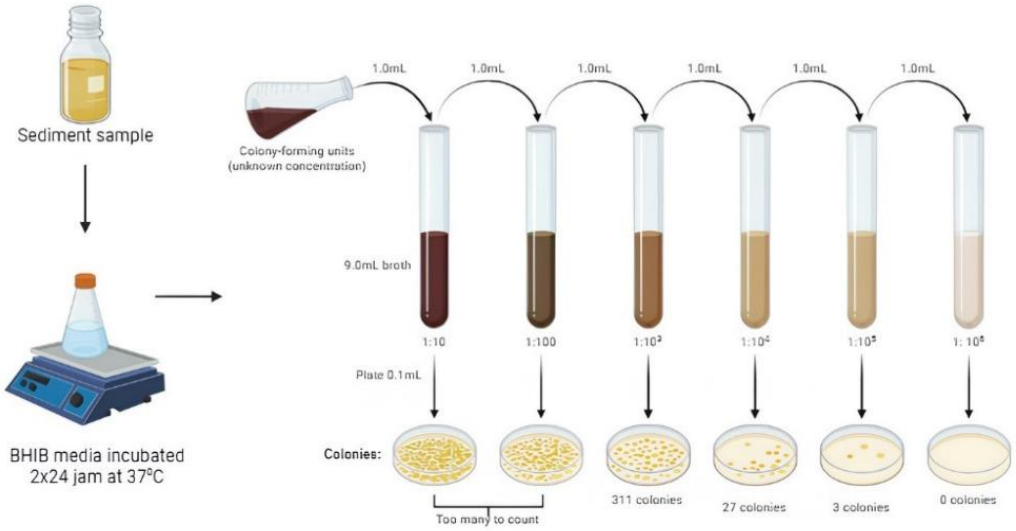


Figure 2 Schema isolation, cultivation, and purification of marine sediment bacteria.

Morphological and growth patterns of marine bacteria

Marine bacterial samples with different shapes and colors were inoculated onto nutrient agar (NA) with sterile seawater and incubated at 37 °C for 48 h. If multiple colonies grew, isolation was repeated (**Figure 2**). Once a single colony was obtained, it was purified using the streak plate method until a pure isolate was achieved. Colony morphology was then observed and recorded.

Production of extracellular enzymes

Bacteria exhibiting diverse morphologies were evaluated for their ability to produce hydrolytic enzymes, including amylase, lipase, and protease, through simple qualitative plate assays using appropriate substrates described below.

Amylase activity

The isolates were tested for amylolytic activity by inoculating them onto starch-enriched plates (100 mL) containing starch (2 g), yeast extract (5 g), NaCl (5 g), peptone (5 g), and agar base (2 g), and incubating at 37 °C for 72 h. After incubation, the plates were stained with 2% iodine solution. A clear zone around the colony indicated amylase production [35,36].

Lipase activity

Lipolytic activity was assessed by inoculating isolates onto plates with Tween 80-enriched plates (100 mL) that included Tween 80 (2 g), yeast extract (5 g), NaCl (5 g), peptone (5 g), and agar base (2 g), and incubating at 37 °C for 72 h. A clear zone around the colonies indicated lipase production [37,38].

Protease activity

Protease production was tested by inoculating isolates onto skim milk-enriched plates consisting of skim milk (2 g), yeast extract (5 g), NaCl (5 g), peptone (5 g), and agar base (2 g) and incubating at 37 °C for 72 h. A clear zone around the colony indicated protease activity [39,40].

Quantitative enzyme activity test

The enzyme index (EI) was calculated, and the isolates with the highest enzyme index were identified. The enzyme index provides a more accurate measure of the enzyme's ability to degrade substrates. The following formula was used to determine the enzyme index [41]:

$$\text{Enzyme Index (mm)} = \frac{\text{Clear Zone Diameter (mm)}}{\text{Colony Diameter (mm)}} \quad (1)$$

Molecular identification of marine sediment bacteria isolates

Bacterial diversity from Losari Coastal sediment was analyzed by extracting genomic DNA, amplifying the 16S rRNA gene, and constructing a phylogenetic tree using the neighbor-joining method [42]. DNA was isolated using the Gen-Elute Bacterial Genomic DNA Kit (Sigma-Aldrich), and the 16S rRNA gene was amplified with primers 63F and 1387R [43]. PCR conditions included denaturation at 95 °C for 2 min, followed by 30 cycles of 95 °C for 1 min, 58 °C for 30 s, and 72 °C for 1 min [44]. Sequences were compared using the Basic Local Alignment Search Tool (BLAST) available at: https://blast.ncbi.nlm.nih.gov/Blast.cgi and visualized in a phylogenetic tree using the MEGA 11 application, constructed using the Maximum Likelihood (ML) method. Phylogenetic reconstruction using the Bootstrap 1000 method, and substitution Model with Kimura 2-parameter.

Protein modelling

The protein modeling stage using sequencing data with NCBI proteins begins with processing the sequencing data to identify open reading frames (ORFs) and translation of DNA sequences into protein sequences in FASTA format, then a homology search is carried out using BLAST on the NCBI protein database to find proteins with high similarity that have 3D structures available in PDB. NCBI provides a complete collection of protein sequences, including proteins that have been identified in the coding region of GenBank and RefSeq, as well as cross-references with SwissProt and PDB, making it easier to identify structural templates. The modeling process is carried out using a server such as SWISS-MODEL by inputting the sequenced protein sequences, where the target protein can be given as an amino acid sequence in FASTA format. The final stage is a comprehensive evaluation of the quality and reliability of the protein structure using various standard validation parameters such as MolProbity Score, Clash Score, Ramachandran analysis, and rotamer outliers, which provide a comprehensive picture of the level of confidence that can be given to each protein structure.

Results and discussion

Characteristics of marine bacterial isolates

Fifteen bacterial isolates were sourced from marine sediments at 4 locations along the Losari Coastal, Makassar, Indonesia. Each isolate was cultured on nutrient agar (NA) supplemented with sterile

seawater. Colonies were analyzed for shape, color, texture, elevation, Gram staining, and cell shape. Isolates exhibiting distinct features were further identified molecularly to assess them.

The 15 marine bacterial isolates were cultured on nutrient agar with sterile seawater. Most showed rapid growth, with microscopic examination revealing pink, gram-negative, rod-shaped colonies. Morphologically, 6 isolates had scalloped edges, 6 had raised edges, and 3 were purely round. Based on **Table 1** regarding color, 6 isolates were creamy, 4 were creamy white, and 5 were creamy yellow. Textures ranged from smooth (8 isolates) to waxy (7 isolates), and elevations mainly were convex (9 isolates), with 6 flats.

Table 1 Morphological characteristics of marine bacterial isolates.

Isolates	Shape	Color	Rough	Elevation	Cell shapes
MB-SL 1	Round with scalloped margin	Creamy white	Waxy	Flat	Negative
MB-SL 2	Round	Creamy	Smooth	Convex	Negative
MB-SL 3	Round with raised margin	Creamy yellow	Waxy	Convex	Negative
MB-SL 4	Round with raised margin	Creamy	Waxy	Flat	Negative
MB-SL 5	Round with scalloped margin	Creamy yellow	Waxy	Flat	Negative
MB-SL 6	Round with scalloped margin	Creamy yellow	Waxy	Flat	Negative
MB-SL 7	Round	Creamy yellow	Waxy	Flat	Negative
MB-SL 8	Round with raised margin	Creamy yellow	Smooth	Convex	Negative
MB-SL 9	Round with raised margin	Creamy white	Waxy	Convex	Negative
MB-SL 10	Round with scalloped margin	Creamy	Smooth	Flat	Negative
MB-SL 11	Round with scalloped margin	Creamy white	Smooth	Convex	Negative
MB-SL 12	Round with raised margin	Creamy white	Smooth	Convex	Negative
MB-SL 13	Round	Creamy	Smooth	Convex	Negative
MB-SL 14	Round with raised margin	Creamy	Smooth	Convex	Negative
MB-SL 15	Round with scalloped margin	Creamy	Smooth	Convex	Negative

Screening and quantitative hydrolytic enzymes of marine sediment bacteria

The results of **Table 2** show that amylolytic activity measurements showed significant variations among the 15 marine bacterial isolates tested. The MB-SL 6 showed the highest amylolytic activity with a clear zone diameter of 18.4 ± 1.5 mm, followed by MB-SL 5 (16.3 ± 1.0 mm) and MB-SL 3 (16.0 ± 1.2 mm). Good amylolytic activity was also shown by the MB-SL 1 (15.1 ± 0.8 mm), MB-SL 7 (14.9 ± 0.8 mm), and MB-SL 11 (14.5 ± 1.1 mm). In contrast, 5 isolates (MB-SL 8, MB-SL 10, MB-SL 12, MB-SL 14, and MB-SL 15) showed no amylolytic activity at all (0 ± 0 mm), indicating the absence of amylase enzyme production or very low enzyme activity under the test conditions, after iodine staining (A) (**Figure 3**). Marine bacteria form a clear zone in the amylase test after the addition of iodine because amylase-producing bacteria secrete enzymes that hydrolyze starch around the colony into simple sugars [45,46]. Iodine only reacts with intact starch (not yet hydrolyzed) to form a blue-black complex, while the area around the bacterial colony that has hydrolyzed starch does not react with iodine and remains clear [47]. The size of the clear zone formed indicates the level of amylase enzyme activity produced by the bacteria [48].

A **B** **C**

Figure 3 Screening of marine sediment bacterial isolates for Amylase (A), Lipase (B), and Protease (C).

The lipolytic activity profile of marine bacterial isolates exhibited a distinct pattern compared to their amylolytic activity. The MB-SL 5 showed the highest lipolytic activity with a clear zone diameter of 15.5 ± 1.3 mm, followed by MB-SL 6 (14.7 ± 1.2 mm) and MB-SL 2 (13.75 ± 1.1 mm). The MB-SL 9 and MB-SL 13 also showed quite good lipolytic activity with clear zone diameters of 13.5 ± 1.0 mm and 12.8 ± 0.9 mm, respectively. Interestingly, MB-SL 3, which showed high amylolytic activity, did not show any lipolytic activity at all (0 ± 0 mm), indicating different specificities of enzyme production between isolates. Similar to amylolytic activity, 5 isolates (MB-SL 8, MB-SL 10, MB-SL 12, MB-SL 14, and MB-SL 15) did not show lipolytic activity (**Table 2**). Lipase activity was characterized by clear zone formation on the media surface (B) (**Figure 3**). Marine bacteria isolated from sediments can release lipase into their surroundings, forming clear zones [49]. This phenomenon occurs due to the hydrolysis of Tween 80, a lipid substrate in the growth medium, which causes a clearing around the colonies [50]. Lipase activity in this approach can be detected by the appearance of a clear zone and the formation of a white precipitate of calcium monolaurate surrounding the colony [51].

Proteolytic activity was confirmed by the formation of clear zones observed on Skim Milk Agar (SKM) plates (C) (**Figure 3**). Proteolytic activity showed a more even distribution among the active isolates. The MB-SL 5 again showed the best performance with a clear zone diameter of 14.1 ± 0.9 mm, followed by MB-SL 6 (13.2 ± 0.8 mm) and MB-SL 4 (12.8 ± 0.6 mm). The MB-SL 13 also showed high proteolytic activity (12.75 ± 0.7 mm), almost equivalent to MB-SL 4. Most isolates showing proteolytic activity had clear zone diameters in the range of 11 - 13 mm, including MB-SL 1 (12.4 ± 0.7 mm), MB-SL 2 (12.1 ± 0.5 mm), MB-SL 7 (12.2 ± 0.5 mm), and MB-SL 9 (12.1 ± 0.6 mm). Consistent with the previous pattern, the same 5 isolates did not show proteolytic activity.

The process of protease enzyme production by marine bacteria in skim milk media is a biochemical phenomenon that reflects the adaptive strategy of these microorganisms. Skim milk media, with its dominant casein content, acts as a very effective trigger for proteolytic activity because it provides chemical signals that stimulate the expression of protease genes [52,53]. When marine bacteria are cultured in this medium, they recognize casein as a potential substrate and respond by activating a regulatory pathway that leads to the synthesis and secretion of protease enzymes [53,54]. This regulatory system involves molecular sensors that detect the presence of protein substrates and activate intracellular signaling cascades that ultimately result in the production of the required enzymes [55,56]. The ability of marine bacteria to produce proteases in skim milk media is manifested visually through the formation of clear zones around the colonies, indicating the degradation of casein by the enzymes produced [57,58].

Table 2 Measurement of clear zone diameters formed by marine bacterial isolates.

Isolate code	Diameter (mm)		
	Amylolytic index	**Lipolytic index**	**Proteolytic index**
MB-SL 1	15.1 ± 0.8	11.75 ± 0.6	12.4 ± 0.7
MB-SL 2	13.1 ± 0.9	13.75 ± 1.1	12.1 ± 0.5
MB-SL 3	16.0 ± 1.2	0 ± 0	11.1 ± 0.4
MB-SL 4	11.5 ± 0.7	12.2 ± 0.8	12.8 ± 0.6
MB-SL 5	16.3 ± 1.0	15.5 ± 1.3	14.1 ± 0.9
MB-SL 6	18.4 ± 1.5	14.7 ± 1.2	13.2 ± 0.8
MB-SL 7	14.9 ± 0.8	11.0 ± 0.7	12.2 ± 0.5
MB-SL 8	0 ± 0	0 ± 0	0 ± 0
MB-SL 9	13.0 ± 0.9	13.5 ± 1.0	12.1 ± 0.6
MB-SL10	0 ± 0	0 ± 0	0 ± 0
MB-SL11	14.5 ± 1.1	10.3 ± 0.8	11.1 ± 0.4
MB-SL12	0 ± 0	0 ± 0	0 ± 0
MB-SL13	10.2 ± 0.6	12.8 ± 0.9	12.75 ± 0.7
MB-SL14	0 ± 0	0 ± 0	0 ± 0
MB-SL15	0 ± 0	0 ± 0	0 ± 0

Of the 15 isolates tested, 10 isolates showed enzymatic activity in at least 1 type of enzyme, while 5 isolates (33.3%) did not show activity in all 3 types of enzymes tested. This indicates a high metabolic diversity among marine bacteria from the same location. Most of the active isolates showed specific enzyme production patterns, with some isolates excelling in one particular type of activity (such as MB-SL 3, which is only active in amylolytic and proteolytic) and some other isolates showing multi-enzymatic activity.

The MB-SL5 and MB-SL6 isolates were further characterized at the species level using molecular techniques. This process included extracting genomic DNA, amplifying the 16S rRNA gene via polymerase chain reaction (PCR), sequencing the 16S rRNA, and constructing a phylogenetic tree using the MEGA 11 application, using the Maximum Likelihood (ML) method.

Molecular identification of bacterial isolates

DNA was extracted from bacterial isolates with high enzyme production, and gene amplification was performed using PCR. DNA quality was assessed by the A260/A280 ratio (1.8 - 2.0) and concentration, confirming suitability for PCR [59,60]. Agarose gel electrophoresis confirmed the DNA [61]. PCR products were analysed on a 1% agarose gel, with gene bands of 1300 bp, and 16S rDNA sequences were compared to GenBank entries. The 16S rDNA sequences were determined and compared with those in GenBank. The MB-SL5 was identified with 97.82% similarity to *Shewanella algae* strain DW01 (NR_044134.1). The MB-SL6 identified 90.53% similarity to *V. alginolyticus* strain NBRC 15630 (NR_122050.1). A phylogenetic tree was constructed using MEGA 11, using the Maximum Likelihood (ML) method [62].

Based on **Figure 4**, the bootstrap value clustering analysis on the Maximum Likelihood phylogenetic tree, the MB-SL 5 in the genus *Shewanella* shows a statistical support distribution pattern that can be categorized into several groups based on the level of bootstrap strength, with this isolate itself being in the group with the highest support.

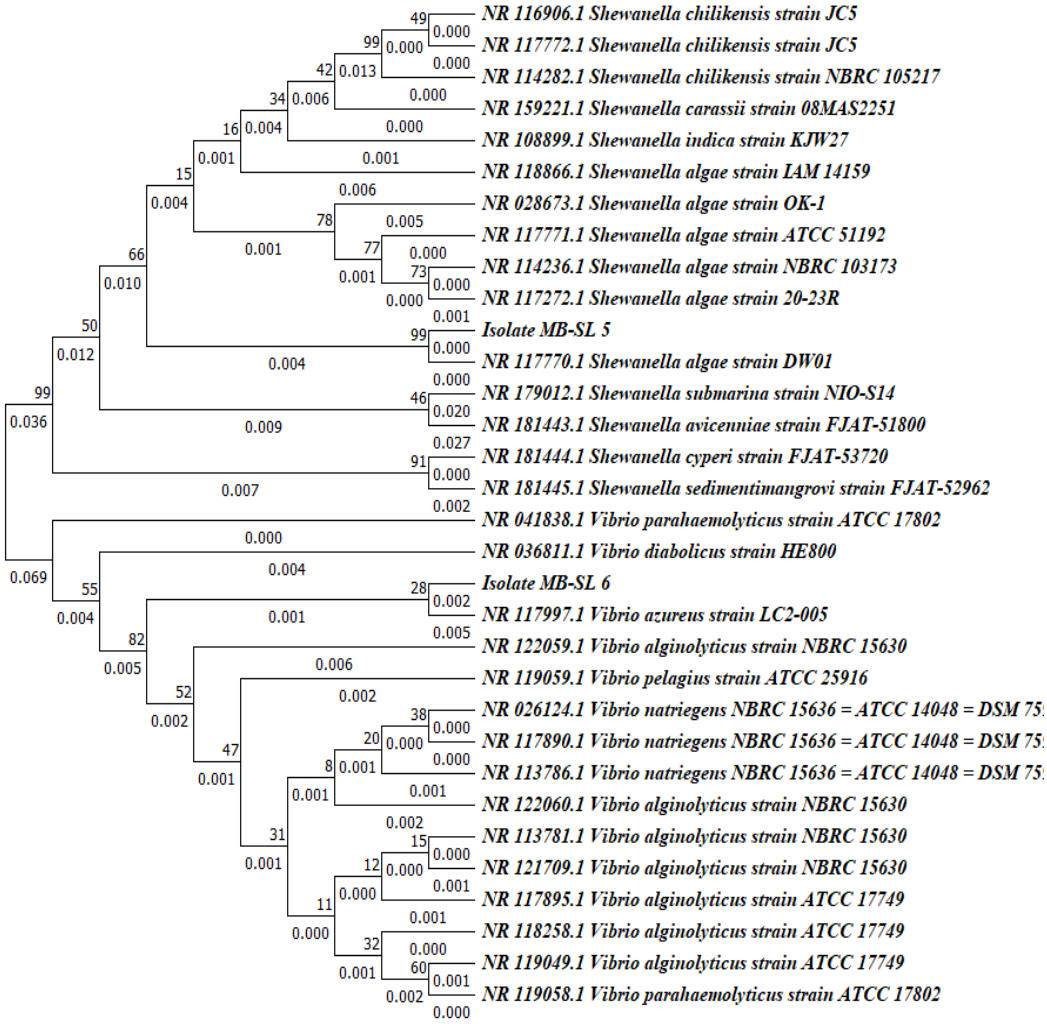


Figure 4 Phylogenetic tree of bacteria based on 16S rRNA sequences.

The MB-SL 5 is in the group with the highest bootstrap support, which is 99%, indicating an extraordinarily strong level of statistical confidence. In the genus *Shewanella*, this very high bootstrap group is also represented by several other branches, such as the *S. algae* strain DW01 group, which has a bootstrap value of 99%, and several subclusters in the *S. algae* complex, which show bootstrap support of 91%. This very high bootstrap group indicates that the species in this category have undergone clear and firm evolutionary differentiation, with the accumulation of sufficient nucleotide substitutions to provide a very strong and consistent phylogenetic signal. The position of the MB-SL 5 in this group indicates that this isolate has a very stable and well-defined taxonomic status in the genus *Shewanella*.

Within the genus *Shewanella*, several branches showed high bootstrap support, such as 82% for a particular subcluster within the *S. algae* group. Although MB-SL 5 is not directly in this group, the presence of high bootstrap groups within the same genus indicates that the genus *Shewanella* generally has good phylogenetic resolution for most of its lineages. These high bootstrap groups showed strong statistical support but were not as optimal as the very high bootstrap groups, indicating that species in this category have clear phylogenetic relationships but may still require additional confirmation for certain taxonomic aspects.

Several branches within the genus *Shewanella* showed moderate bootstrap support, such as branches with values of 78%, 77%, and 73% that support various subclusters within the *S. algae* complex. The *S. chilikensis* group showed 49% bootstrap support, which is at the lower limit of this category. Although the MB-SL 5 is not in the moderate bootstrap group, the presence of this group within the genus *Shewanella* suggests that

not all lineages within this genus have optimal phylogenetic resolution. This moderate bootstrap group indicates that although there is statistical support for the grouping, the confidence level still requires additional validation for some taxonomic aspects.

Several internal branches within the genus *Shewanella* show low bootstrap support, such as branches with values of 46%, 34%, 28%, 20%, 16%, and 15%. These low bootstrap groups are generally found on internal branches connecting larger species groups, rather than on terminal branches, such as where the MB-SL 5 is located. The presence of this low bootstrap group suggests that although the genus *Shewanella* generally has good phylogenetic resolution, there are still some aspects of evolutionary relationships that are not fully resolved, especially at deeper levels in the phylogenetic tree.

The position of MB-SL 5 in the very high bootstrap group (99%) has very significant implications for the interpretation of its taxonomic and phylogenetic status. Compared with the diverse bootstrap distribution patterns in the genus *Shewanella*, the MB-SL 5 occupies the most optimal position in terms of statistical support. This shows that the MB-SL 5 has a very clear and consistent phylogenetic signal, indicating that its taxonomic position in the genus *Shewanella* is not ambiguous and is fully reliable. This pattern is in contrast to several other isolates in the same genus that show lower bootstrap support, indicating that the MB-SL 5 has distinct and well-defined molecular characteristics.

Bootstrap clustering that places MB-SL 5 in the highest support category indicates that the identification of this isolate as a member of the genus *Shewanella* has a maximum level of confidence and does not require additional validation from a molecular phylogenetic perspective. The wide variation in bootstrap values (from 0% to 99%) indicates that the genus *Shewanella* has experienced a complex evolutionary history with a non-uniform diversification pattern. High bootstrap values, such as 99% for MB-SL 5 and several *S. algae* strains, indicate that these species have undergone a clear and distinct divergence, with sufficient mutation accumulation to distinguish them genetically. In contrast, low bootstrap values (below 50%) on several branches indicate rapid evolutionary radiation or recent speciation events, where the species have not had enough time to accumulate significant genetic differences. From a practical perspective, this bootstrap pattern has direct implications for the identification and characterization of new isolates such as MB-SL 5. The position of this isolate with a bootstrap of 99% provides high confidence that the identification as a member of the genus *Shewanella* is accurate.

The BLAST results showing high similarity to *S. algae* strain DW01 were consistently confirmed by the phylogenetic position of the MB-SL 5 in the Maximum Likelihood tree. In the phylogenetic tree, the MB-SL 5 is located in the same cluster as the *S. algae* group, including strain DW01, which has a bootstrap value of 99%. The consistency between the BLAST results and the phylogenetic placement indicates that both molecular analysis methods provide consistent conclusions, namely that the MB-SL 5 is a member of the *S. algae* species complex.

The 99% bootstrap value for the MB-SL 5 provides very strong statistical support for the BLAST results, indicating that the sequence similarity detected through BLAST is not a coincidence or an artifact of the analysis. This very high bootstrap support indicates that out of 1000 bootstrap replications, 99% support the grouping of the MB-SL 5 with *S. algae*, including strain DW01. This provides a very high level of confidence (99%) that the identification through BLAST is accurate and reliable for taxonomic purposes. The BLAST result relatedness with strain DW01 and the phylogenetic position supported by high bootstrap indicates that the MB-SL 5 has a very close relationship with *S. algae* strain DW01. In the phylogenetic tree, strain DW01 itself has 99% bootstrap support, indicating that this strain has a stable and well-defined taxonomic position. The phylogenetic closeness between the MB-SL 5 and strain DW01 indicates that the 2 may be different strains of the same species or very closely related species within the *S. algae* complex.

Based on the bootstrap value clustering analysis on the Maximum Likelihood phylogenetic tree in **Figure 4**, the MB-SL 6, which is in the genus *Vibrio*, shows a statistical support distribution pattern that can be categorized into several distinct groups based on the level of bootstrap strength. In the genus *Vibrio*, the group with high bootstrap support is represented by the *V. azureus* subcluster strain LC2-005, which has a bootstrap value of 82%. This group shows a clear

separation and is strongly supported statistically, indicating that *V. azureus* has a stable and well-defined taxonomic position within the genus *Vibrio*. Although MB-SL 6 is not directly in this subcluster, the existence of a group with high bootstrap within the same genus indicates that several *Vibrio* lineages have reached a sufficient level of divergence to provide a strong phylogenetic signal.

The MB-SL 6 is in the context of a group that includes several branches with moderate bootstrap support. The *V. alginolyticus* group, consisting of multiple strains (NBRC 15630, ATCC 25916), formed a cluster with 52% bootstrap support, indicating moderate but acceptable statistical support for identification purposes. Other subclusters within the genus *Vibrio* also showed bootstrap values in this range, such as a branch with a value of 55% supporting the formation of a particular group. This moderate bootstrap group indicates that although there is statistical support for the grouping, the level of confidence is not as optimal as the group with a high bootstrap. Most of the internal branches within the genus *Vibrio* relevant to the position of the MB-SL 6 showed low bootstrap support, with values such as 47, 38, and even very low values such as 28%, 20%, 15%, 12%, 11%, and 8%. This low bootstrap group includes many branches connecting different *Vibrio* species, including the area where the MB-SL 6 is located. These low bootstrap values indicate high uncertainty in phylogenetic relationships at this level, suggesting that the available sequence data do not provide a strong enough signal to resolve evolutionary relationships with high confidence.

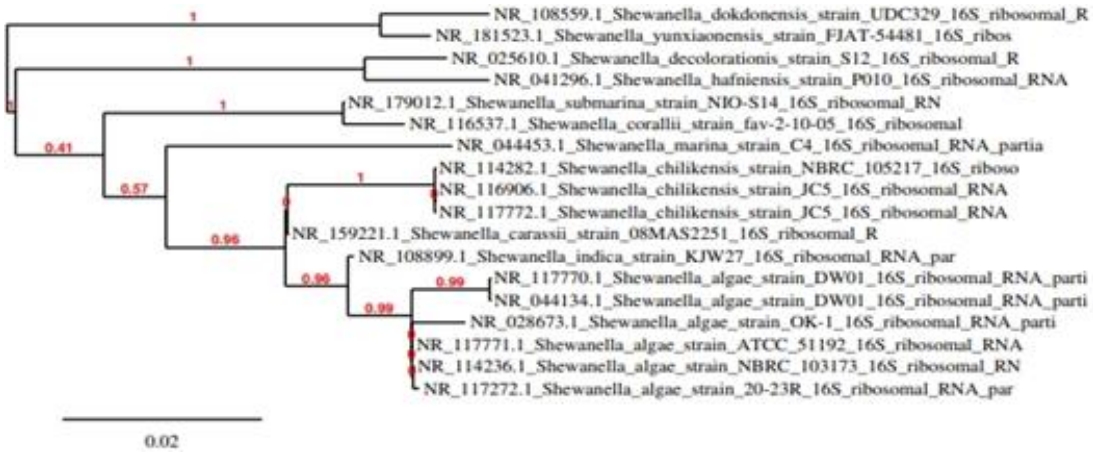
The significance of the diverse clustering patterns of bootstrap values for the MB-SL 6 within the genus *Vibrio* reflects the evolutionary complexity and taxonomic challenges faced in classifying these marine bacteria. The dominance of low-to-moderate bootstrap groups within the genus *Vibrio*, in contrast to several *Shewanella* groups that show high bootstraps, indicates that the genus *Vibrio* has undergone a fundamentally different evolutionary history. This pattern suggests that *Vibrio* species may have undergone rapid adaptive radiation over a relatively short period of time, during which diverse environmental selection pressures in marine ecosystems have driven rapid diversification but have not allowed sufficient time for the accumulation of sufficient mutations to produce a strong and consistent phylogenetic signal.

The methodological significance of these bootstrap patterns is that traditional single-gene (e.g., 16S rRNA)-based phylogenetic approaches may not be optimal for taxonomic resolution within the genus *Vibrio*. The dominance of low bootstrap values indicates that a single genetic marker does not provide enough information to resolve the complex phylogenetic relationships in this genus. This suggests the need to adopt more comprehensive genomic approaches, such as multilocus sequence analysis (MLSA), core genome phylogeny, or even whole genome sequencing, to achieve adequate phylogenetic resolution. For isolating MB-SL 6 in particular, this means that accurate and reliable identification may require investment in more advanced sequencing technologies and more sophisticated bioinformatics analyses. Ecologically and evolutionarily, the significance of this bootstrap pattern suggests that the genus *Vibrio* is likely a highly plastic and adaptive group of bacteria to variations in marine environments. The rapid speciation indicated by the low bootstrap may be an evolutionary response to the high heterogeneity of marine habitats, ranging from coastal to deep sea, from tropical to polar zones, and from aerobic to anaerobic conditions. The MB-SL, as a part of this actively diversifying genus, likely has high adaptive potential and may have unique characteristics that have not been fully characterized. This condition suggests that the genus *Vibrio*, including the MB-SL 6, may harbor an as-yet-unexplored metabolic and functional diversity, which may have important biotechnological applications or ecological roles in marine ecosystems that still need to be further investigated through multidisciplinary and integrative research approaches.

Protein modelling

Protein modeling from sequencing results involves a series of systematic steps that convert amino acid sequence information into a predictable 3-dimensional structure. The 1st step is sequence analysis and quality control, where the sequenced results need to be cleaned of contaminants, verified for quality, and translated from DNA into a protein sequence using the appropriate genetic code. This step also involves identifying the correct open reading frame (ORF) and

confirming that the sequence does not contain internal stop codons or frameshifts that could interfere with the accuracy of the structure prediction.



>lcl|ORF1
MWFNSMQREEPYLLLTSTESGRDTSVPSGTVRQVLHGCRQLVL
>lcl|ORF4
MWFNSMQREEPYLLLTSRELSRDGLVPSGTLRQVLHGCRQLVL

Figure 5 (A) The amino acid sequence of *Shewanella algae* strain DW01 and (B) The amino acid sequence of *V. alginolyticus* strain NBRC 1563.

Comparative amino acid sequence analysis between ORF1 of *S. algae* strain DW01 (**Figure 5(A)**) and ORF4 of *V. alginolyticus* strain NBRC 15630 (**Figure 5(B)**) revealed an extraordinarily high level of conservation, with sequence identity reaching approximately 92.7%. Both proteins are identical in length, i.e., 41 amino acid residues, indicating that they are likely to be orthologous proteins with the same or very similar biological functions in these 2 different bacterial species of the genus. This very high level of similarity indicates that these proteins have very important functional roles and are evolutionarily conserved among Gram-negative bacteria.

Three specific amino acid substitutions distinguish the 2 sequences, all concentrated in the central region of the protein (positions 19 - 29). The 1st substitution at position 19 represents a change from Threonine (T) in *S. algae* to Arginine (R) in *V. alginolyticus*, which is a transition from a neutral polar amino acid to a positively charged amino acid with a larger side chain. The 2nd substitution at position 22 shows a change from Glutamic acid (E) to Leucine (L), which is a dramatic substitution from a negatively charged amino acid to a neutral hydrophobic amino acid. The 3rd substitution at position 29 shows a conservative change from Valine (V) to Leucine (L), both of which are hydrophobic amino acids with similar branched chain structures.

This pattern of substitution distribution is significant because it shows that the N-terminal (residues 1 - 18) and C-terminal (residues 30 - 41) regions are highly conserved across species, indicating that these regions may contain active sites, binding domains, or structural motifs that are critical to protein function. In contrast, the variability in the central region likely reflects species-specific adaptations that do not interfere with the primary function of the protein but may be related to optimization for different environmental conditions. The presence of nearly identical N-terminal MWFNSMQREEPYLLL and C-terminal PSGT[VL]RQVLHGCRQLVL motifs strengthens the hypothesis that these 2 proteins are functional homologs that have undergone minimal evolutionary divergence, possibly playing roles in basic metabolism or essential cellular functions that require

high structural conservation to maintain their biological activity.

Figure 6(A) shows the results of 3-dimensional protein structure modeling for ORF1 from *S. algae* strain DW01 bacteria using the SWISS-MODEL platform accessed via a web browser. This protein model was successfully showed quite good modeling quality with a GMQE (Global Model Quality Estimation) score of 0.64 on a scale of 0 - 1, indicating that the model's confidence level is in the moderate to good category.

The template used for homology modeling is the A0A3M5GRJ4_1. A protein which is the Putative ORF58e from the AlphaFold database, with the source organism *Pseudomonas savastanoi* (*Pseudomonas syringae* pv savastanoi). Although the template comes from a different bacterial genus from the target (*Pseudomonas* vs *Shewanella*), the high level of sequence identity reaching 76.19% with good coverage provides a solid basis for structural modeling. This protein is predicted to function as a monomer, meaning it does not form a multi-subunit complex under physiological conditions.

The resulting 3-dimensional structure shows a compact protein architecture with a combination of secondary structure elements in the form of α-helices and β-sheets. Visualization using a rainbow coloring scheme from the N-terminus (red) to the C-terminus (blue/purple) shows a well-organized protein fold with several well-defined structural domains. The presence of loops and turn regions connecting the secondary structure elements indicates structural flexibility that may be important for protein function.

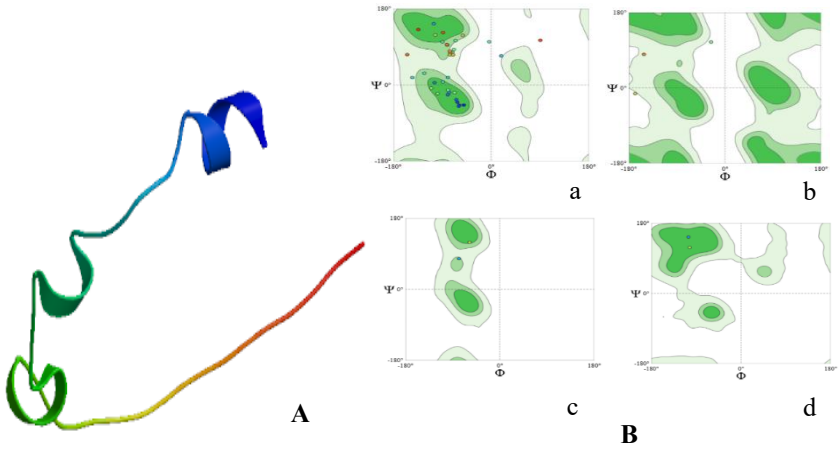


Figure 6 (A) Structural protein prediction of *Shewanella algae* strain DW01; (B) Ramachandran plots (a) General, (b) Glycine, (c) Proline, and (d) Pro-proline.

Analysis of Ramachandran plots specific to *S. algae* strain DW01 (**Figure 6(B)**) proteins provides insight into the structural characteristics of proteins of this pathogenic marine bacterium with remarkable environmental adaptability. The general plot (a) shows a typical backbone conformational distribution for facultative anaerobic gram-negative bacterial proteins, with the green areas reflecting conformational regions optimized for function under varying marine environmental conditions. The concentration of dots in the alpha-helix and beta-sheet regions indicates that *S. algae* strain DW01 proteins maintain stable secondary structures as a foundation for enzymatic activity and complex protein-protein interactions, particularly in the anaerobic respiration and alternative energy metabolism systems characteristic of the S*hewanella* genus.

The glycine plot (b) for *S. algae* strain DW01 displays a very broad distribution, reflecting the critical need for conformational flexibility in proteins that interact with the outer membrane and the electron transport system. High flexibility of glycine residues is essential for proteins such as cytochrome c and components of the external electron transport system that enable *S. algae* strain DW01 to respire over a variety of electron acceptors, including metal oxides and complex organic compounds. This widespread distribution also indicates structural adaptation of the protein to the varying salinity and osmotic pressures

experienced by this bacterium in its marine and estuarine habitats, where protein conformational changes must accommodate fluctuating physicochemical conditions.

The proline plot (c) shows very tight conformational restrictions, reflecting the critical role of proline residues in maintaining rigid structures in key *S. algae* strain DW01 proteins involved in pathogenesis and virulence. This restricted conformation is particularly important for proteins interacting with siderophores and the iron ion binding system, where structural precision is required for optimal binding affinity and specificity. The cyclic structure of proline provides the thermal and chemical stability required for *S. algae* strain DW01 proteins to maintain function under conditions of oxidative stress and pH variations frequently encountered in host tissues during infection.

The pro-proline plot (d) reveals extreme conformational restrictions, indicating that *S. algae* uses the pro-proline structural motif to create highly specific turns and loops in proteins involved in adhesion, invasion, and evasion of the host immune system. This highly restricted region indicates that the pro-proline sequence in *S. algae* proteins serves as a rigid structural link between functional domains, ensuring precise spatial orientation for complex molecular interactions. This characteristic is particularly relevant for cell surface proteins and virulence factors that require highly precise conformations to interact with host cell receptors and evade detection by the adaptive immune system, reflecting the molecular evolution of *S. algae* strain DW01 that has successfully adapted to both the marine environment and vertebrate hosts.

The significance of Ramachandran's modeling results for *S. algae* strain DW01 reveals a highly sophisticated evolutionary strategy by this marine bacterium to optimize its protein architecture for a dual life as an environmental organism. The conformational distributions shown in the 4 plots collectively reflect how *S. algae* strain DW01 has evolved a molecular toolkit that allows it to survive in the harsh marine habitat while also being able to infect vertebrate hosts when the opportunity arises. The overall plots show that *S. algae* strain DW01 proteins maintain the basic structural stability required for essential metabolic functions, but with additional flexibility that allows rapid adaptation to drastically changing environmental conditions.

The clinical and ecological significance of this modeling lies in the understanding that the high conformational flexibility at glycine residues allows *S. algae* to modulate its surface proteins and secretion systems during the transition from the marine environment to host tissues. The ability of proteins to adopt these diverse conformations explains why *S. algae* strain DW01 can rapidly adapt from anaerobic respiratory metabolism in marine sediments to aerobic metabolism in host tissues, as well as changing its antigenic profile to evade immune responses. Meanwhile, the tight conformational restrictions on proline residues and pro-proline motifs indicate that this bacterium has evolved to maintain key protein structures critical for virulence and pathogenesis in a highly stable form that is resistant to selective pressures from the host immune system.

From a biotechnological and therapeutic perspective, this model provides a blueprint for the development of antimicrobial strategies that target specific conformational regions of the *S. algae* strain DW01 proteins. Inhibitory molecules can be designed to disrupt the conformational flexibility required for environmental adaptation or to disrupt the rigid conformation required for virulence function. Understanding the Ramachandran distribution also opens up opportunities for the development of *S. algae* strain DW01 protein-based biosensors that can exploit natural conformational flexibility for environmental change detection, as well as vaccines that target epitopes in regions with the most stable and conservative conformations.

The most profound evolutionary implication of this model is that *S. algae* strain DW01 represents a successful example of convergent evolution between extreme environmental adaptation and opportunistic pathogenesis. This unique Ramachandran pattern suggests that selective pressures from the marine environment and host-pathogen interactions have shaped a protein conformational landscape that allows these bacteria to function as molecular generalists able to optimize protein structures for a wide range of functions depending on the environmental context, while maintaining a coherent genomic and proteomic identity as a single species.

Figure 7(A) shows the results of 3-dimensional protein structure modeling for ORF4 from *V. alginolyticus* strain NBRC 15630 bacteria using the SWISS-MODEL platform accessed via a web browser. This page displays the modeling results for model 01 with the status Oligo-State Monomer and a GMQE (Global Model Quality Estimation) value of 0.64, indicating a fairly good model quality.

The template used is A0A3M5GRJ4_1 A Putative oRF58e, which is an AlphaFold DB model of the A0A3M5GRJ4_PSESS protein with the *Pseudomonas savastanoi* organism. The data shows a sequence identity of 88.10% with high coverage, indicating a good template suitability for homology modeling. Model-Template Alignment information is also available for further analysis.

Visualization of the 3D structure of the protein showing a ribbon representation with a gradient color from red to purple. This structure shows the characteristics of a protein with several folded domains, including complex loops and turns. There is also a control panel at the bottom for manipulating the structure display, including options for carbon arrangements and other visualization features. This interface allows researchers to perform interactive protein structure analysis and download results for further analysis.

Figure 7(B) shows the Ramachandran plot analysis for the protein of *V. alginolyticus* strain NBRC 15630, consisting of 4 different panels that each analyze the distribution of phi (φ) and psi (ψ) dihedral angles for a different category of amino acid residues. Panel (a) shows a general Ramachandran plot that includes all amino acid residues in the protein structure. The distribution of green dots indicates stereochemical allowed conformations, with a high concentration in the alpha helix (lower left quadrant) and beta sheet (upper left quadrant). The distribution of residues in allowed regions indicates good quality of the protein structure.

Panel (b) specifically analyzes the glycine residue, which has the highest conformational flexibility because its side chain consists of only hydrogen atoms. The wider distribution seen in this plot indicates that glycine can adopt a variety of conformations that are inaccessible to other amino acids, including regions that are usually forbidden to other residues.

Panels (c) and (d) analyze proline and pre-proline residues (the residue immediately before proline in the sequence). Proline has a cyclic structure that limits its flexibility, so its distribution is more restricted to certain regions in the Ramachandran plot. This restricted distribution pattern reflects the unique geometric constraints of proline due to the covalent bond between the side chain and the protein backbone, which results in a relatively fixed phi angle of about 60°.

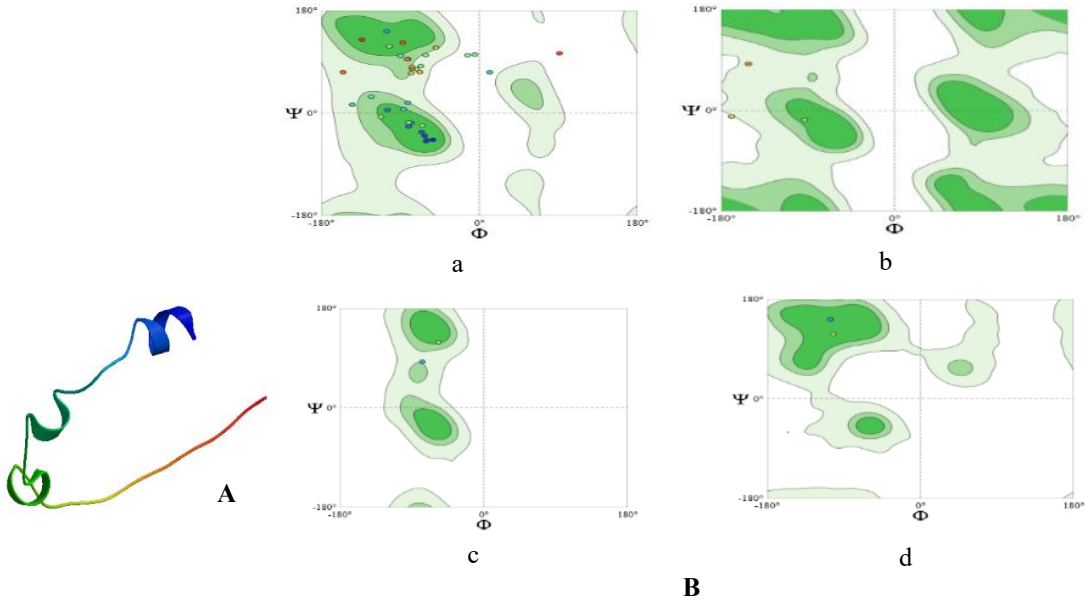


Figure 7 (A) Structural protein prediction of *V. alginolyticus* strain NBRC 15630 by SWISS-MODEL; (B) Ramachandran plots (a) General, (b) Glycine, (c) Proline, and (d) Pro-proline.

The Ramachandran plot results for the *V. alginolyticus* strain NBRC 15630 protein have very important significance in validating the quality of the protein structure and its implications for biological function. The distribution of residues concentrated in stereochemically permitted regions in all 4 panels indicates that the analyzed protein structure has very good quality and is biophysically realistic, which is a crucial indicator that there are no significant problems in the structure determination or modeling process carried out.

The distribution patterns that are in accordance with theoretical expectations for each category of amino acid residues confirm the normal structural characteristics of this protein. The high flexibility shown by glycine residues, the typical conformational constraints of proline, and the general distribution of residues in the classical alpha helix and beta sheet regions all indicate that this protein has a stable and functional molecular architecture. This agreement is very important because it indicates that the protein is likely to be able to perform its biological function optimally, considering that a proper structure is a prerequisite for catalytic activity, binding specificity, and protein stability in a complex cellular environment.

From the perspective of applied research, this good Ramachandran validation provides a high level of confidence that the protein structure can be used for further analysis, such as drug design, protein engineering, or molecular interaction studies. A structure with solid validation becomes a reliable foundation for the development of biotechnological applications, especially in the context of molecular characterization of strain NBRC 15630. These results not only provide insight into the quality of the protein produced by this organism but are also relevant for a deeper understanding of the pathogenicity mechanism of *V. alginolyticus* and its potential applications in marine and medical biotechnology.

The structure validation comparison **Table 3** using Mol-Probity Results data aims to conduct a comprehensive evaluation of the quality and reliability of protein structures from 2 different bacterial species, namely *S. algae* and *V. alginolyticus.* This table serves as a systematic quality control benchmark to ensure that the protein structure that has been determined or modeled has acceptable geometric and stereochemical accuracy before being used for further biological analysis. By using various standard validation parameters such as MolProbity Score, Clash Score, Ramachandran analysis, and rotamer outliers, this table provides a comprehensive overview of the level of confidence that can be given to each protein structure. In addition, this table aims to identify specific structural differences between homologous proteins from both bacterial species, which can provide insight into the molecular evolution and phylogenetic relationship between *S. algae* and *V. alginolyticus*. Direct comparison allows researchers to understand how amino acid sequence variations at certain positions can affect local structural stability, protein flexibility, and potential functional implications. This information is invaluable for comparative genomics studies and can help in understanding possible structural adaptations related to the different ecological niches of the 2 bacterial species.

From a practical application perspective, this table also serves as a guide to determine which structure is more suitable for a particular downstream application, such as drug design, protein engineering, or molecular interaction studies. By identifying specific outlier residues and problematic regions, researchers can make informed decisions about whether the structure is accurate enough for their research purposes or requires further refinement before being used in computational experiments or molecular design.

Table 3 Mol-probity results.

Categorical	**Value**	
	Shewanella algae	***Vibrio alginolyticus***
Mol-Probity Score	1.92	1.92
Clash Score	0.00	0.00
Ramachandran Favoured	65%	65.00%

Categorical	Value	
	Shewanella algae	***Vibrio alginolyticus***
Ramachandran Outliers	12.50% A40 GLN, A29 GLY, A12 TYR, A21 GLY, A18 THR	12.50% A40 GLN, A29 GLY, A21 SER, A18 ARG, A12 TYR
Rotamer Outliers	5.13% A16 THR, A35 LEU	5.13% A16 THR, A35 LEU
C-Beta Deviation	0	0
Bad Bonds	0/342	0/350
Bad Angels	2/463	3/3742
Twisted Non-Proline	(A17 SER-A18 THR), A41 LEU 2/39 (A39 ARG-A40 GLN), (A40 GLN-A41 LEU)	(A11 PRO-A12 TYR), (A26 VAL-A27 PRO), (A17 SER-A18 ARG) 2/39 (A39 ARG-A40 GLN), (A40 GLN-A41 LEU)

Comparative analysis of protein structure validation between *S. algae* and *V. alginolyticus* shows very similar structural quality characteristics but with some significant specific differences. Both proteins have an identical Mol-Probity Score of 1.92, indicating acceptable structural quality, with a Clash Score of 0.00 indicating no steric conflict between atoms in both structures. The percentage of residues located in Ramachandran favored regions is also identical at 65%, indicating that most of the protein backbone conformations are in the energetically favored range.

A striking difference is seen in the identification of Ramachandran outliers, where both proteins have the same percentage (12.50%) but with different specific residues. *S. algae* showed outliers at residues A40 GLN, A29 GLY, A12 TYR, A21 GLY, and A18 THR, while *V. alginolyticus* had outliers at A40 GLN, A29 GLY, A21 SER, A18 ARG, and A12 TYR. These differences indicate variations in local structural flexibility between the 2 proteins, where amino acid substitutions at certain positions (GLY → SER at position 21 and THR → ARG at position 18) can significantly affect the backbone conformation.

Rotamer outliers analysis showed identical patterns in both proteins (5.13%) with the same residues A16 THR and A35 LEU, indicating that side chain conformation problems occur at conservative positions. Other validation parameters, such as C-Beta deviation and bad bonds, showed very good results for both proteins (value 0), but there were differences in bad angles, where *S. algae* had 2/463 while *V. alginolyticus* had 3/474. Twisted non-proline analysis revealed conformational problems in different segments, with *S. algae* showing twisted backbone at (A17 SER-A18 THR) and A41 LEU, while *V. alginolyticus* had problems in several segments including (A11 PRO-A12 TYR), (A26 VAL-A27 PRO), and (A17 SER-A18 ARG), indicating that sequence differences may affect flexibility and local strain in the protein structure.

Shewanella is an ecologically crucial bacterial genus whose members are widely distributed in freshwater, seawater, sediment, and deep oceans [55-57]. *Shewanella* comprises Gram-negative, facultatively anaerobic, oxidase-positive, and motile bacteria [64]. Due to its unique physiological and respiratory versatility, *Shewanella* spp. can survive in a wide range of ecological niches (for example, suboptimal environmental conditions with extreme salinity and high barometric pressure, spoiled foods, and clinical specimens) and has been applied in environmental protection and industrial development. Particular species of *Shewanella* spp produce chitinase, lipase, protease, elastin, and alkyl sulfate enzymes [63]. *Shewanella indica* KJW27, isolated from the coastal sediments of the Arabian Sea, was used as the host bacterium to isolate its phages from the Yellow Sea, China [65].

V. alginolyticus is a Gram-negative bacterium found in marine and estuarine environments [66,67] and discovered a novel multifunctional enzyme, Amy63,

produced by the marine bacterium *V. alginolyticus* 63. Remarkably, Amy63 possesses amylase activities. *V. alginolyticus* Jme3-20 produced a multitude of extracellular enzymes in isolated mangrove sediments of Pantai Gading, Secanggang District, Langkat Regency, North Sumatra, Indonesia [67]. *V. alginolyticus* produces some of the most promising bacterial proteases (including collagenases), which have attracted interest for a long time and are routinely used for biomedical and pharmaceutical applications [68]. Biosynthesis of gold nanoparticles using the marine microbe *V. alginolyticus* can be cost-effective and environmentally friendly, with anti-inflammatory and anti-cancer effects against colon cancer [69].

Conclusions

This study yielded 15 bacterial isolates characterized by a gram-negative, rod-shaped morphology and distinctive, round, yellow colonies with serrated edges. Among these, 10 produced amylase, 9 produced lipases, and 10 showed protease activity; however, 2 isolates stood out because they could produce all three enzymes: amylase, lipase, and protease. The MB-SL 5 isolate showed 97.82% similarity to *S. algae* strain DW01, and the MB-SL 6 isolate had 90.53% similarity to *V. alginolyticus* NBRC 15630 strain, with predicted protein structure code A0A3M5GRJ4_1 A. These findings highlight the significant biotechnological potential of marine sediment bacteria from the Losari Coastal producing hydrolytic enzymes for industrial applications, which opens new avenues for the development of new biocatalysts.

Acknowledgements

The authors gratefully acknowledge the financial support from the Indonesian Education Scholarship, Center for Higher Education Funding and Assessment, and Indonesian Endowment Fund for Education with No. 011479/PPAPT.1.2/BPI.06/02/2025.

Declaration of Generative AI in Scientific Writing

The author hereby declares that QuillBot AI has been used during the writing of this article to assist in correcting grammar. The grammar starting from Abstract, background, methods, discussion, and conclusion has been compiled with the help of AI. Still, all content has been critically reviewed, verified for correctness, and revised by the author. The author acknowledges the limitations of current AI technology and has verified all data and information presented. The author asserts full responsibility for the content and views expressed in this work.

Credit Author Statement

1. Anita

Marine Microbiology Specialist (Marine sediment sample collection from Losari Coastal area, bacterial isolation and identification protocols, microbial community analysis and characterization, maintenance of bacterial culture collections, and environmental parameter assessment and documentation)

2. Hasnah Natsitr

Biochemistry and Enzyme Analysis Lead and Project Coordinator (Overall project management and supervision, enzyme extraction and purification protocols, hydrolytic enzyme activity assays and characterization, biochemical property analysis (pH, temperature, substrate specificity), enzyme kinetics studies and optimization, and quality control of biochemical analyses)

3. Paulina Taba

Research design and methodology development (Coordination between team members and institutions, manuscript preparation and publication oversight, budget management and resource allocation, and progress monitoring and quality control.

4. Ahmad Ahyar

Molecular Biology and Genetics Specialist (DNA extraction and genomic analysis, gene sequencing and annotation, phylogenetic analysis of bacterial isolates, molecular identification and taxonomic classification, and genetic diversity assessment)

5. Nunuk Hariani Soekamto

Natural Products Chemistry Expert (Chemical characterization of enzyme products, structure-activity relationship analysis, and integration of chemistry and biology findings)

6. Nur Umriani Permatasari

Protein Biochemistry and Structure Analysis (Protein purification and characterization, protein concentration and stability studies, and protein-substrate interaction studies)

7. Wahyudin Rauf

Computational Biology and Bioinformatics (Protein structure prediction and modeling, sequence analysis and homology modeling)

8. Sarlan

Analytical Chemistry and Instrumentation (Instrumental analysis setup and operation, method validation and standardization, technical troubleshooting and maintenance, data quality assurance and validation)

9. Nasrum Massi

Microbiology Consultant (Environmental sampling strategy design, ecological context and environmental factors analysis, microbial ecology interpretation, environmental impact assessment, field work coordination, and safety protocols)

10. Sudding

Microbiology Consultant and Laboratory Management (Sample preparation and processing, microbial ecology interpretation, and environmental impact assessment)

11. Andi Fatmawati

Data Analysis (Data visualization and interpretation, Report preparation and documentation, and Quality control of data analysis procedures)

References

[1] AD Rogers, W Appeltans, J Assis, LT Ballance, P Cury, C Duarte, F Favoretto, LA Hynes, JA Kumagai, CE Lovelock, P Miloslavich, A Niamir, D Obura, BC O'Leary, E Ramirez-Llodra, G Reygondeau, C Roberts, Y Sadov, O Steeds, T Sutton, …, O Aburto-Oropeza. Discovering marine biodiversity in the 21st century. *Advances in Marine Biology* 2022; **93**, 23-115.

[2] A Karthikeyan, A Joseph and BG Nair. Promising bioactive compounds from the marine environment and their potential effects on various diseases. *Journal of Genetic Engineering and Biotechnology* 2022; **20(1)**, 14.

[3] DL Dayanidhi, BC Thomas, JS Osterberg, M Vuong, G Vargas, SK Kwartler, E Schmaltz, MM Dunphy-Daly, TF Schultz, D Rittschof, WC Eward, C Roy and JA Somarelli. Exploring the diversity of the marine environment for new anti-cancer compounds. *Frontiers in Marine Science* 2021; **7**, 614766.

[4] F Ameen, S AlNadhari and AA Al-Homaidan. Marine microorganisms as an untapped source of bioactive compounds. *Saudi Journal of Biological Sciences* 2021; **28(1)**, 224-231.

[5] S Ghattavi and A Homaei. Marine enzymes: Classification and application in various industries. *International Journal of Biological Macromolecules* 2023; **230**, 123136.

[6] AH Banday, NU Azha, R Farooq, SA Sheikh, MA Ganie, MN Parray, H Mushtaq, I Hameed and MA Lone. Exploring the potential of marine natural products in drug development: A comprehensive review. *Phytochemistry Letters* 2024; **59**, 124-135.

[7] J Zhang, L Jiang, X Chen, K Lv, M Basiony, G Zhu, L Karthik, L Ouyang, L Zhang and X Liu. Recent advances in biotechnology for marine enzymes and molecules. *Current Opinion in Biotechnology* 2021; **69**, 308-315.

[8] S Bhandari, DK Poudel, R Marahatha, S Dawadi, K Khadayat, S Phuyal, S Shrestha, S Gaire, K Basnet, U Khadka and N Parajuli. Microbial enzymes used in bioremediation. *Journal of Chemistry* 2021; **2021(1)**, 8849512.

[9] S Raveendran, B Parameswaran, SB Ummalyma, A Abraham, AK Mathew, A Madhavan, S Rebello and A Pandey. Applications of microbial enzymes in food industry. *Food Technology and Biotechnology* 2018; **56(1)**, 16-30.

[10] N Kochhar, IK Kavya, S Shrivastava, A Ghosh, VS Rawat, KK Sodhi and M Kumar. Perspectives on the microorganisms of extreme environments and their applications. *Current Research in Microbial Sciences* 2022; **3**, 100134.

[11] A Trincone. Enzymatic processes in marine biotechnology. *Marine Drugs* 2017; **15(4)**, 93.

[12] K Maldonado-Ruiz, R Pedroza-Islas and L Pedraza-Segura. Blue biotechnology: Marine bacteria bioproducts. *Microorganisms* 2024; **12(4)**, 697.

[13] E Nikolaivits, M Dimarogona, N Fokialakis and E Topakas. Marine-derived biocatalysts: Importance, accessing, and application in aromatic pollutant bioremediation. *Frontiers in Microbiology* 2017; **8**, 265.

[14] P Solanki, C Putatunda, A Kumar, R Bhatia and A Walia. Microbial proteases: Ubiquitous enzymes

with innumerable uses. *3 Biotech* 2022; **11(10)**, 428.

[15] A Kumar, S Dhiman, B Krishan, M Samtiya, A Kumari, N Pathak, A Kumari, RE Aluko and T Dhewa. Microbial enzymes and major applications in the food industry: A concise review. *Food Production, Processing and Nutrition* 2024; **6**, 85.

[16] DS Kocabaş, J Lyne and Z Ustunol. Hydrolytic enzymes in the dairy industry: Applications, market and future perspectives. *Trends in Food Science and Technology* 2022; **119**, 467-475.

[17] A Fasim, VS More and SS More. Large-scale production of enzymes for biotechnology uses. *Current Opinion in Biotechnology* 2021; **69**, 68-76.

[18] W Yang, F Lu and Y Liu. Recent advances of enzymes in the food industry. *Foods* 2023; **12(24)**, 4506.

[19] J John. *Biochemical and biotechnological aspects of microbial amylases*. *In*: R Goldbeck and P Poletto (Eds.). Polysaccharide-degrading biocatalysts. Academic Press, Cambridge, 2023, p. 191-204.

[20] R Seth and A Meena. Enzyme-based nanomaterial synthesis: An eco-friendly and green synthesis approach. *Clean Technologies and Environmental Policy* 2024. https://doi.org/10.1007/s10098-024-02854-7.

[21] AA Al-Ghanayem and B Joseph. Current prospects in using cold-active enzymes as eco-friendly detergent additives. *Applied Microbiology and Biotechnology* 2020; **104(7)**, 2871-2882.

[22] D Kumar, R Bhardwaj, S Jassal, T Goyal, A Khullar and N Gupta. Application of enzymes for an eco-friendly approach to textile processing. *Environmental Science and Pollution Research International* 2023; **30(28)**, 71838-71848.

[23] Y Khambhaty. Applications of enzymes in leather processing. *Environmental Chemistry Letters* 2020; **18(3)**, 747-769.

[24] GK Gupta, M Dixit, RK Kapoor and P Shukla. Xylanolytic enzymes in pulp and paper industry: New technologies and perspectives. *Molecular Biotechnology* 2022; **64(2)**, 130-143.

[25] KKR Shah, S Devanshi, GB Patel and VD Patel. *Application of microbial enzymes: Biodegradation of paper and pulp waste, in innovations in environmental biotechnology*. *In*: S Arora, A Kumar, S Ogita and YY Yau (Eds.). Springer Nature Singapore, Singapore, 2022, p. 283-304.

[26] N Soni and MS Madhusudhan. Computational modeling of protein assemblies. *Currnt Opinion in Structural Biology* 2017; **44**, 179-189.

[27] Y Kumar, H Ramesh, K Dhabade, M Shahare and B Kalra. Structural study and molecular docking insights into laccase-mediated dye degradation. *Sustainable Chemistry for the Environment* 2024; **8**, 100175.

[28] K Nam, Y Shao, DT Major and M Wolf-Watz. Perspectives on computational enzyme modeling: From mechanisms to design and drug development. *ACS Omega* 2024; **9(7)**, 7393-7412.

[29] K Grigorakis, C Ferousi and E Topakas. Protein engineering for industrial biocatalysis: Principles, approaches, and lessons from engineered PETases. *Catalysts* 2025; **15(2)**, 147.

[30] S Mao, J Jiang, K Xiong, Y Chen, Y Yao, L Liu, H Liu and X Li. Enzyme engineering: Performance optimization, novel sources and applications in the food industry. *Foods* 2024; **13(23)**, 3846.

[31] L Lin, D Xiao, W Song and W Lu. A breakthrough computational strategy for efficient enzymatic digestion of walnut protein to prepare antioxidant peptides. *Food Chemistry* 2025; **476**, 143311.

[32] R Ruginescu, P Lavin, L Iancu, S Menabit and C Purcarea. Bioprospecting for novel bacterial sources of hydrolytic enzymes and antimicrobials in the romanian littoral zone of the black sea. *Microorganisms* 2022; **10(12)**, 12-26.

[33] SV Jagannathan, EM Manemann, SE Rowe, MC Callender and W Soto. Marine actinomycetes, new sources of biotechnological products. *Marine Drugs* 2021; **19(7)**, 365.

[34] H Natsir, A Ahmad, N Massi, P Taba, Anita and W Rauf. Isolation, production of protease, and antimicrobial activities from marine sediment gamma - proteobacteria of MBS-L3 isolate. *Research Journal of Pharmacy and Technology* 2024; **17(6)**, 2855-2862.

[35] SA Fasiku, OF Ogunsola, A Fakunle and AA Olanbiwoninu. Isolation of bacteria with potential

of producing extracellular enzymes (amylase, cellulase and protease) from soil samples. *Journal of Advances in Microbiology* 2020; **20(3)**, 21-26.

[36] P Gómez-Villegas, J Vigara, L Romero, C Gotor, S Raposo, B Gonçalves and R Léon. Biochemical characterization of the amylase activity from the new *Haloarchaea* strain *Haloarcula sp.* HS isolated in the odiel marshlands. *Biology* 2021; **10(4)**, 337.

[37] J Carrazco-Palafox, BE Rivera-Chavira, N Ramírez-Baca, LI Manzanares-Papayanopoulos and GV Nevárez-Moorillón. Improved method for qualitative screening of lipolytic bacterial strains. *MethodsX* 2018; **5**, 68-74.

[38] OI Ilesanmi, AE Adekunle, JA Omolaiye, EM Olorode and AL Ogunkanmi. Isolation, optimization and molecular characterization of lipase-producing bacteria from contaminated soil. *Scientific African* 2020; **8**, e00279.

[39] SK Marathe, MA Vashistht, A Prashanth, N Parveen, S Chakraborty and SS Nair. Isolation, partial purification, biochemical characterization and detergent compatibility of alkaline protease produced by *Bacillus subtilis*, *Alcaligenes faecalis* and *Pseudomonas aeruginosa* obtained from sea water samples. *Journal of Genetic Engineering and Biotechnology* 2018; **16(1)**, 39-46.

[40] C Masi, G Gemechu and M Tafesse. Isolation, screening, characterization, and identification of alkaline protease-producing bacteria from leather industry effluent. *Annals Microbiology* 2021; **71**, 24.

[41] E Rosa, CN Ekowati, TT Handayani, A Ikhsanudin, F Apriliani and A Arifiyanto, Characterization of entomopathogenic fungi as a natural biological control of American cockroaches (*Periplaneta americana*). *Biodiversitas* 2020; **21(11)**, 5276-5282.

[42] DL Church, L Cerutti, A Gürtler, T Griener, A Zelazny and S Emler. Performance and application of 16S rRNA gene cycle sequencing for routine identification of bacteria in the clinical microbiology laboratory. *Clinical Microbiology Reviews* 2020; **33(4)**, e00053-19.

[43] R Srinivasan, U Karaoz, M Volegova, J MacKichan, M Kato-Maeda, S Miller, R Nadarajan, EL Brodie and SV Lynch. Use of 16S rRNA gene for identification of a broad range of clinically relevant bacterial pathogens. *PLoS One* 2015; **10(2)**, e0117617.

[44] AK Farha, TR Thasneem, A Purushothaman, JA Salam and AM Hatha. Phylogenetic diversity and biotechnological potentials of marine bacteria from the continental slope of eastern Arabian Sea. *Journal, Genetic Engineering and Biotechnology* 2018; **16(2)**, 253-258.

[45] EH Sanjaya, S Suharti, M Alvionita, I Telussa, S Febriana and H Clevanota. Isolation and characterization of amylase enzyme produced by indigenous bacteria from sugar factory waste. *The Open Biotechnology Journal* 2024; **18**, e18740707296261.

[46] PT Nnaji, E Adukwu, HR Morse and RU Chidugu-Ogborigbo. Amylase production from marine sponge Hymeniacidon perlevis; potential sustainability benefits. *PLoS One* 2023; **18(12)**, e0294931.

[47] S Pesek and R Silaghi-Dumitrescu. The iodine/iodide/starch supramolecular complex. *Molecules* 2024; **29(3)**, 641.

[48] A Budiharjo, D Wulandari, J Shabrina, RA Mawarni, AR Maulana, Nurhayati, W Wijanarka, L Hartajanie and Lindayani. Bioprospecting and molecular identification of amylase and cellulase producing thermophilic bacteria from sediment of Nglimut Hot Springs, Kendal Regency. *Journal of Tropical Biodiversity and Biotechnology* 2024; **9(3)**, jtbb86756.

[49] P Chandra, Enespa, R Singh and PK Arora. Microbial lipases and their industrial applications: A comprehensive review. *Microbial Cell Factories* 2020; **19**, 169.

[50] D Bharathi and G Rajalakshmi. Microbial lipases: An overview of screening, production and purification. *Biocatalysis and Agricultural Biotechnology* 2019; **22**, 101368.

[51] S Lanka and JNL Latha. A short review on various screening methods to isolate potential lipase producers: Lipases-the present and future enzymes of biotech industry. *International Journal of Biological Chemistry* 2015; **9(5)**, 207-219.

[52] YA Lemenh, TG Biru, AZ Chernet and FB Lema. Isolation and identification of protease-producing bacteria from sludge and sediment soil around

Adama, Ethiopia. *Indonesian Journal Biotechnology* 2025; **26(4)**, 159.

[53] P Song, X Zhang, S Wang, W Xu, F Wang, R Fu and F Wei. Microbial proteases and their applications. *Frontiers in Microbiology* 2023; **14(9)**, 1236368.

[54] DE Cruz-Casas, CN Aguilar, JA Ascacio-Valdés, R Rodríguez-Herrera, ML Chávez-González and AC Flores-Gallegos. Enzymatic hydrolysis and microbial fermentation: The most favorable biotechnological methods for the release of bioactive peptides. *Food Chemistry: Molecular Sciences* 2021; **3**, 100047.

[55] J Su, Y Song, Z Zhu, X Huang, J Fan, J Qiao and F Mao. Cell-cell communication: New insights and clinical implications. *Signal Transduction and Targeted Therapy* 2024; **9**, 196.

[56] Y Hong, A Boiti, D Vallone and NS Foulkes. Reactive oxygen species signaling and oxidative stress: Transcriptional regulation and evolution. *Antioxidants* 2024; **13(3)**, 164-176.

[57] N Prihatiningsih, A Asnani and HA Djatmiko. Extracellular protease from *Bacillus subtilis* B315 with antagonistic activity against bacterial wilt pathogen (*Ralstonia solanacearum*) of chili. *Biodiversitas* 2022; **22(3)**, 1291-1295.

[58] M Dehimi, F Yusof, RA Raus, NF Hadry and R Nedjai. Genotypic and phenotypic characterisation of isolated marine bacteria and their potential to produce alkaline protease. *Journal of Advanced Research in Applied Sciences and Engineering Technology* 2021; **22(1)**, 16-25.

[59] A Abdel-Latif and G Osman. Comparison of three genomic DNA extraction methods to obtain high DNA quality from maize. *Plant Methods* 2017; **13**, 1.

[60] W Qamar, MR Khan and A Arafah. Optimization of conditions to extract high-quality DNA for PCR analysis from whole blood using SDS-proteinase K method. *Saudi Journal of Biological Sciences* 2017; **24(7)**, 1465-1469.

[61] HR Dash, P Shrivastava and S Das. *Quantification of DNA by using agarose gel electrophoresis technique*. *In*: HR Dash, P Shrivastava and S Das (Eds.). Principles and practices of DNA analysis: A laboratory manual for forensic DNA typing. Springer Protocols Handbooks, Humana, New York, 2020, p. 119-125.

[62] R Veneziano, TR Shepherd, S Ratanalert, L Bellou, C Tao and M Bathe. *In vitro* synthesis of gene-length single-stranded DNA. *Scientific Reports* 2018; **8(1)**, 6548.

[63] J Philips, L Procopio and IPG Marshall. Insights into the various mechanisms by which *Shewanella* spp. induce and inhibit steel corrosion. *NPJ Materials Degradation* 2023; **7**, 95.

[64] Z Wang, J Zhao, L Wang, C Li, J Liu, L Zhang and Y Zhang. A novel benthic phage infecting shewanella with strong replication ability. *Viruses* 2019; **11(11)**, 1081.

[65] HI Sheikh, NII Alhamadin, HJ Liew, A Fadhlina, MEA Wahid, N Musa and KCA Jalal. Virulence factors of the zoonotic pathogen *Vibrio alginolyticus*: A review and bibliometric analysis. *Applied Biochemistry and Microbiology* 2024; **60**, 514-531.

[66] KMJ Slifka, AE Newton and BE Mahon. *Vibrio alginolyticus* infections in the USA, 1988 - 2012. *Epidemiology and Infection* 2017; **45(7)**, 1491-1499.

[67] J Mamangkey, D Suryanto, E Munir, AZ Mustopa, MT Sibero, LW Mendes, A Hartanto, S Taniwan, MJ Ek-Ramos, A Harahap, A Verma, E Trihatmoko, WS Putranto, L Pardosi and LOAP Rudia. Isolation and enzyme bioprospection of bacteria associated with *Bruguiera cylindrica*, a mangrove plant of North Sumatra, Indonesia. *Biotechnology Reports* 2021; **30**, e00617.

[68] M Salamone, A Nicosia, G Ghersi and M Tagliavia. *Vibrio* proteases for biomedical applications: Modulating the proteolytic secretome of *V. alginolyticus* and *V. parahaemolyticus* for improved enzymes production. *Microorganisms* 2019; **7(10)**, 387.

[69] R Shunmugam, SR Balusamy, V Kumar, S Menon, T Lakshmi and H Perumalsamy. Biosynthesis of gold nanoparticles using marine microbe (*Vibrio alginolyticus*) and its anticancer and antioxidant analysis. *Journal of King Saud University - Science* 2021; **33(1)**, 101260.