

Characterization and potential of L-glutaminase enzyme from symbiotic red algae *Eucheuma spinosum* as antibacterial, anticancer, and antiviral dengue agents by in vitro

HARNINGSIH KARIM¹, ARIEF AZIS¹, ANANDA RAMADANI¹, A. TENRIUGI DAENG PINE¹, ANITA^{2,7}, AHYAR AHMAD^{2,3,*}, MUKRIANI⁴, MUH NASRUM MASSI⁵, ANDI DIAN PERMANA⁶

¹Department of Pharmacy, School of Pharmacy, Akademi Farmasi Yamasi. Jl. Mapala 2 Blok D5 No. 10, Makassar 90222, South Sulawesi, Indonesia

²Department of Chemistry, Faculty of Mathematics and Natural Science, Universitas Hasanuddin. Jl. Perintis Kemerdekaan Km. 10, Makassar 90245, South Sulawesi, Indonesia

³Research and Development Centre for Biopolymers and Bioproducts, Institute for Research and Community Service, Universitas Hasanuddin. Jl. Perintis Kemerdekaan Km. 10, Makassar 90245, South Sulawesi, Indonesia. Tel.: +62-411-587032, *email: ahyarahmad@gmail.com

⁴Graduate School of Medical Science, Medicine Faculty, Universitas Hasanuddin. Jl. Perintis Kemerdekaan Km. 10, Makassar 90245, South Sulawesi, Indonesia

⁵Department of Microbiology, Faculty of Medicine, Universitas Hasanuddin. Jl. Perintis Kemerdekaan Km. 10, Makassar 90245, South Sulawesi, Indonesia

⁶Department of Pharmacy, Faculty of Pharmacy, Universitas Hasanuddin. Jl. Perintis Kemerdekaan Km. 10, Makassar 90245, South Sulawesi, Indonesia

⁷Medical Laboratory Technology, Politeknik Muhammadiyah Makassar. Jl. Ratulangi No. 101, Makassar 90132, South Sulawesi, Indonesia

Manuscript received: 30 October 2024. Revision accepted: 20 December 2024.

Abstract. Karim H, Azis A, Ramadani A, Pine ATD, Anita, Ahmad A, Mukriani, Massi MN, Permana AD. 2024. Characterization and potential of L-glutaminase enzyme from symbiotic red algae *Eucheuma spinosum* as antibacterial, anticancer, and antiviral dengue agents by in vitro. *Biodiversitas* 25: 4939-4949. Cancer is a disease caused by the abnormal growth of body tissue cells. A new approach in cancer treatment through targeted therapy is using microbial enzymes. One enzyme that has potential for future cancer therapy is L-glutaminase. On the other hand, dengue fever is endemic in Indonesia, necessitating maximum and sustained efforts to address the dengue problem, including infection and cancer. Indonesia is an archipelagic nation, with two-thirds of its territory comprising seas, and boasts many diverse marine biota and macroalgae. One cultivated variety of marine algae is the red algae *Eucheuma* sp.. This study aimed to isolate the L-glutaminase enzyme produced by symbiotic *Cobetia marina* from red algae *Eucheuma spinosum*. The optimum activity of the L-glutaminase enzyme was found at pH 8 and 37°C. It was activated by metal ions Mg²⁺, Co²⁺, and Mn²⁺. The antibacterial activity test revealed that L-glutaminase at a concentration of 5 mg/L exhibited inhibitory effects on pathogenic bacteria, such as *Escherichia coli* and *Staphylococcus aureus*. Furthermore, the cytotoxic test demonstrated the most substantial impact in the F1 fraction at a concentration of 160 µg/mL, with a percent inhibition value of 25.65% and an IC₅₀ value of 64.26 µg/mL on MCF-7 cells. The anti-dengue activity of L-glutaminase in crude extract toward Vero cells indicates inhibition percentage and CC₅₀ value of 78% and 167.15 µg/mL, respectively. These findings suggest that the L-glutaminase enzyme fractions from symbiotic *C. marina* hold promise for antibacterial and anticancer uses and antiviral activity against dengue.

Keywords: *Cobetia marina*, L-glutaminase, red algae, symbiotic bacteria

Abbreviations: BHIB: Brain Heart Broth; BSA: Bovine Serum Albumin; CC₅₀: Cytotoxic Concentration 50%; DENV: Dengue Virus; ELISA: Enzyme-Linked Immunosorbent Assay; EMEM: Eagle's Minimum Essential Medium; IC₅₀: Inhibition Concentration 50%; MCF-7: Michigan Cancer Foundation-7; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; WHO: World Health Organization; TCA: Tri Chloro Acetate; OD: Optical Density

INTRODUCTION

Marine ecosystems represent a vast, largely unexplored frontier of scientific potential, harboring an extraordinary diversity of microorganisms and macroalgae with remarkable pharmaceutical prospects. These marine organisms, covering approximately 71% of Earth's surface, produce unique bioactive compounds demonstrating promising therapeutic characteristics, including anticancer, antimicrobial, anti-inflammatory, and antioxidant properties (Ghosh et al. 2022). The growing inclination towards reconnecting with nature has sparked public interest in the vast natural resources of Indonesia, particularly its marine biota (Ferrol-

Schulte et al. 2014; Rahman et al. 2024). Among the diverse marine biota, macroalgae is an up-and-coming resource (Biris-Dorhoi et al. 2020; Daniotti and Re 2021) with red algae *Eucheuma* sp. being a key cultivated variety in Indonesia (Rimmer et al. 2021; Wijayanto et al. 2021; Van et al. 2022). Among the *Eucheuma* species, *Eucheuma spinosum* J. Agardh has shown potential as an antibacterial agent (Safitri et al. 2018; Akbar et al. 2022; Damongilala et al. 2023). Whole extracts from *Eucheuma cottonii* Weber Bosse have been reported for enhanced antibacterial activity (Astriani and Nurjanah 2023), significant antioxidant effects (Lim et al. 2015; Wulandari et al. 2018; Teo et al.

2021), and promising anticancer potential (Tan et al. 2015; Purbosari et al. 2021; Sugrani 2021).

The red algae *Sargassum vulgare* C.Agardh has been further investigated for its antiviral potential, particularly in dengue research (Liu et al. 2021; El-Beltagi et al. 2022). Cancer remains a critical health challenge in Indonesia, with 396,914 cases and an annual death rate of 234,511 cases (Sung et al. 2021; Andinata et al. 2023; Prihantono et al. 2023), prompting innovative approaches in targeted therapy using microbial enzymes like L-glutaminase to disrupt cancer cell nutrient availability (Yang et al. 2021; Gomaa 2022). Simultaneously, dengue remains a significant health concern, with approximately 390 million infections annually, of which 96 million manifest clinically (WHO 2023; Ilic and Ilic 2024). The disease has expanded from nine countries before 1970 to being endemic in over 100 countries (Sim and Hibberd 2016; Nakase et al. 2023), with Indonesia being a particularly affected region and the number of dengue cases dramatically increasing from 2.4 million in 2015 to 4.5 million in 2022, underscoring the urgent need for comprehensive management strategy (WHO 2023; Clark et al. 2024).

Building upon these promising findings, emerging research suggests that the pharmaceutical potential of Indonesian marine ecosystems extends far beyond current applications (Atanasov et al. 2021; Zhivkoplis et al. 2024). Advanced biotechnological techniques enable researchers to isolate novel bioactive compounds with unprecedented precision, opening new frontiers in drug discovery (Teixeira et al. 2020; Ahmad et al. 2024). Interdisciplinary collaborations between marine biologists, pharmacologists, and biotechnologists are crucial in translating scientific discoveries into viable therapeutic interventions for complex diseases like cancer and infectious disorders (Hemmerling and Piel 2022; Dong et al. 2024). Indonesian government supports comprehensive marine biotechnology programs that align with global efforts to leverage marine ecosystems for human health and environmental conservation (Zhong et al. 2021; Min and Ho 2022; Kaur et al. 2023; Liu et al. 2024).

The L-glutaminase enzyme emerges as a promising therapeutic agent in cancer research, with recent studies highlighting its potential to disrupt nutrient metabolism in malignant cells selectively (Vijayan et al. 2017; Cyriac and Lee 2024). This microbial enzyme demonstrates a unique mechanism of action by targeting glutamine, a critical amino acid essential for cancer cell proliferation and survival (Dhankhar et al. 2020; Vachher et al. 2021). Advanced molecular studies have revealed that L-glutaminase can effectively inhibit glutamine utilization, essentially starving cancer cells of their primary metabolic fuel without significantly impacting normal cellular functions (Edwards et al. 2021; Jin et al. 2023; Wang et al. 2024). Researchers are particularly excited about its potential for developing targeted therapeutic strategies, especially for aggressive cancer types demonstrating high glutamine dependency, such as pancreatic, lung, and certain lymphoma subtypes (Jin et al. 2023; Leng et al. 2024). The enzyme's specificity and minimal side effects compared to traditional chemotherapy make it a compelling candidate for future

cancer treatment protocols, with ongoing clinical research focusing on optimizing its delivery mechanisms and therapeutic efficacy (Anand et al. 2023; Raghani et al. 2024).

The exploration of L-glutaminase from symbiotic microorganisms associated with *E. spinosum* represents a sustainable approach to enzyme production while potentially offering unique properties due to the marine origin of these symbionts. Understanding this enzyme's characterization and biological activities through in vitro studies is crucial for developing new therapeutic strategies against bacterial infections, cancer, and viral diseases like dengue fever.

MATERIALS AND METHODS

Materials

The materials used were isolates of symbiont bacteria from the red algae *Eucheuma spinosum*, *Artemia salina* L. shrimp larvae, pure cultures of *Escherichia coli* and *Staphylococcus aureus* bacteria, MCF-7 breast cancer cells, Vero cells, DENV-2 virus were obtained from Biochemistry laboratory, Hasanuddin University collection. Nutrient agar media, Mueller-Hinton agar media, Bovine Serum Albumin (BSA), Brain Heart Broth (BHIB), L-glutamine substrate, beef extract, 2-Amino-2-(hydroxymethyl)propane-1,3-diol ((HOCH₂)₃CNH₂), Sodium Chloride (NaCl), 2-Sulfanyethan-1-ol/ β -mercaptoethanol (HOCH₂CH₂SH), 2-[4-(2,4,4-trimethylpentan-2-yl)phenoxy]ethanol/Triton X-100 (C₁₄H₂₂O(C₂H₄O)_n, Reagent Folin-Ciocalteu, Universal pH Indicator, blank disc paper (6 mm), Sodium carbonate (Na₂CO₃), Sodium Hydroxide (NaOH), Copper (II) sulfate pentahydrate (CuSO₄.5H₂O), Sodium potassium L(+)-tartrate tetrahydrate (C₄H₄KNaO₆), Ammonium Sulfate (NH₄)₂SO₄, Ammonium chloride (NH₄Cl), Nessler reagent, Trichloroacetate acid (TCA), Barium chloride dihydrate (BaCl₂.2H₂O), Dipotassium phosphat (K₂HPO₄), Sulfuric acid (H₂SO₄), Calcium sulfate (CaSO₄), Zinc sulfate (ZnSO₄), Magnesium sulfate (MgSO₄), Potassium Chloride (KCl), Cupric (II) sulfate (CuSO₄), Cobalt (II) sulfate (CoSO₄), Manganese (II) sulfate (MnSO₄) were obtained from Merck.

Rejuvenation of symbiotic bacterial isolate

The rejuvenation of the *E. spinosum* symbiont bacterial isolate was obtained from the Biochemistry laboratory at Hasanuddin University. The collection was conducted by transferring 1 dose of the symbiont bacterial isolate into a specific media using the scratch method. The media containing the bacteria were then incubated at 37°C for 24 hours (Ramadan et al. 2019; Mukriani et al. 2020; Mostafa et al. 2021).

Determination of the optimal growth time of symbiotic bacteria

Optimization of the growth of the red alga symbiont bacterial isolate *E. spinosum* was carried out by taking 2-3 loops of isolates from 24-hour old culture stock and placing them in sterile inoculum media, with a concentration of 0.5% L-glutamine. Then incubated in an incubator shaker. Next, 10% of the active inoculum was put into an Erlenmeyer

containing specific media and was then shaken in an incubator shaker at a speed of 180 rpm, a temperature of 37°C for 82 hours, and sampling was carried out every 6 hours to determine bacterial growth by measuring the optical density at a wavelength of 660 nm. Then, centrifuged at 5000 rpm, 4°C for 30 minutes, was determined for protein content using a UV-Vis spectrophotometer at a wavelength of 660 nm (Orabi et al. 2020).

L-glutaminase production from symbiotic bacteria

Following the rejuvenation, bacterial isolates (2-3 doses) were transferred into an Erlenmeyer and were then shaken in an incubator shaker for 24 hours at a speed of 180 rpm and a temperature of 37°C. The resulting symbiotic bacterial culture was inoculated into a production medium totaling 1000 mL. This culture was shaken in a shaker at 150 rpm for the optimum production time at 37°C. Subsequently, the production medium was separated by centrifugation to distinguish the filtrate and cells. The resulting filtrate is a crude extract of enzymes that will be processed further (Simay et al. 2022).

Purification of L-glutaminase enzyme from symbiotic bacteria

Purification of L-glutaminase enzyme by fractionation using ammonium sulfate at saturation thresholds of 0-40% (F1), 40-60% (F2), and 60-80% (F3). Subsequently, followed by an overnight incubation at 4°C. The resultant precipitate was separated through centrifugation at 10,000 rpm, 4°C for 30 minutes. The precipitate obtained is then dissolved in buffer B and dialyzed using a specific amount of buffer C. Dialysis continues until the buffer solution no longer forms a precipitate upon adding BaCl₂. The evaluation of this fraction is based on the protein content and activity of the L-glutaminase enzyme (Sathish et al. 2018; Mostafa et al. 2021).

L-glutaminase enzyme activity in *Eucheuma spinosum* symbiotic bacteria

Determination of L-glutaminase enzyme activity

Enzyme activity was assessed using the Nessler method. First made standard series of NH₄Cl, concentrations of 0.02, 0.04, 0.06, 0.08, 0.1, and 0.12 mg/mL, of 1 mg/mL NH₄Cl stock solution into a series of test tubes and adding distilled water up to a total volume of 1 mL. The reaction mixture for measuring enzyme activity included 0.5 mL of the enzyme, 0.5 mL of 0.04 M L-glutaminase substrate, and 0.5 mL of 0.05 M Tris-HCl buffer (pH 8), which was then incubated at 37°C for 30 minutes. Subsequently, 0.5 mL of 1.5 M TCA was added to the mixture and refrigerated for 15 minutes. A 0.1 mL aliquot of the mixture was then taken, and 3.7 mL of distilled water and 0.2 mL of Nessler's reagent were added. Enzyme activity was determined using standard NH₄Cl at λ maximum 420 nm. The calculation of enzyme activity can be performed using the formula equation (1).

$$\text{Enzyme activity (U/mL)} = \frac{y - b}{a} \times \frac{V_{\text{total}}}{V_{\text{analysis}}} \times \frac{1}{V_{\text{enzyme}} \times V_{\text{incubation}}} \times f \quad \dots(1)$$

Where: V_{total}: Volume of enzyme + substrate + buffer + TCA, V_{analysis}: Total volume analyzed, V_{enzyme}: Volume of enzyme analyzed, y: Absorbance, a: Slopes, b: Intercepts.

Determination of specific activity of L-glutaminase enzyme

The enzyme protein levels obtained are employed in determining the specific activity of enzymes using the formula provided in Equation (1). Involves the characterization of pH variation, temperature variation, time variation, and characterization with the addition of metal (Punekar 2018; Nieuwkoop et al. 2019).

L-glutaminase antibacterial activity in *Eucheuma spinosum* symbiotic bacteria

The inhibition testing of the L-glutaminase enzyme on the growth of *E. coli* and *S. aureus* bacteria was conducted through the agar diffusion method. Aseptically, sterile Mueller-Hinton agar media was poured into a 15 mL petri dish, and 0.5 mL of the tested bacterial suspension was added. Subsequently, 6 paper discs were individually immersed in each L-glutaminase enzyme fraction and ampicillin as a positive control (+). The test medium was then incubated for 24-48 hours at 37°C, followed by observation and measurement of the inhibition zone using a sliding ruler (Nindhita et al. 2022; Susanti et al. 2024).

Toxicity properties test using the Brine Shrimp Lethality Test (BSLT)

Method Toxicity tests are performed as modified previously (CLSI 2019). Enzyme fractions were prepared at concentrations of 100 ppm, 10 ppm, and 1 ppm in triplicate. To each tube, sterile seawater containing 10 shrimp larvae was added until the final volume was 5 mL, and the mixture was incubated for 24 hours at room temperature under a 50-watt incandescent lamp. Subsequently, the number of dead and live larvae was counted, and the LC₅₀ value (µg/mL) was determined using the Bliss Method program. The number of dead larvae in the test and control samples was calculated, and the % mortality was determined using the formula provided in equation (2).

$$\% \text{Death} = \frac{\sum \text{dead test larvae} - \sum \text{dead control larvae}}{\sum \text{test larvae}} \times 100\% \quad \dots(2)$$

L-glutaminase enzyme cytotoxic test against MCF-7 breast cancer cells with the MTT method

The initial step involved preparing the extract at various concentrations, namely 5, 10, 20, 40, 80, and 160 µg/mL, in Eagle's minimum essential medium (EMEM). Michigan Cancer Foundation (MCF)-7 cell preparations, with a density of 8000 cells/well, were distributed into 96-well plates and incubated for 48 hours. Next, the L-glutaminase enzyme was added at concentrations of 0, 10, 20, 40, 80, and 160 µg/mL, followed by another 48-hour incubation period. 100 µL of EMEM culture medium containing MTT at a concentration of 5 mg/mL in the mixture was incubated for 3 hours at 37°C. The process was halted using a stopper reagent, sodium dodecyl sulfate (SDS) at 10%, dissolved in 0.01N HCl. Absorbance was measured using an ELISA reader (Maharem et al. 2020; Gomaa 2022).

Anti-dengue test

For the preliminary test, toxicity assays were conducted using the Brine Shrimp Lethality Test (BSLT) method, as previously reported by Zakaria et al. (2024). Cytotoxicity tests were carried out following the modified methods described by (Ahmad et al. 2021; 2022). Vero cells with a density of 1×10^4 cells/well were cultured in well plates containing deck glass coated with poly-L-lysine to facilitate cell adhesion. The well plates were used for 1 hour and 2 days incubation groups. The experiment was conducted on the following groups: (i) a group infected with DENV-2 virus without treatment with L-Glutaminase fraction and incubated at 37°C for 1 hour; (ii) a group infected with DENV-2 virus was treated with 10 µg/mL L-glutaminase fraction and incubated at 37°C for 1 hour; (iii) positive control (Vero cells infected with DENV-2 and incubated at 37°C for 1 hour) and negative control (Vero cells not infected with DENV-2 and incubated at 37°C for 1 hour). The grouping for cells infected with DENV-2 and incubated for 2 days followed the same setup as the groups incubated for 1 hour. At the end of the treatment, the supernatant from each well was measured using the Enzyme-Linked Immunosorbent Assay (ELISA) method. The percentage of cell viability (%) and the CC₅₀ value (µg/mL) were calculated based on the % inhibition using probit analysis as Fouada et al. 2022 explained.

RESULTS AND DISCUSSION

The screening of L-glutaminase-producing bacterial isolates from *E. spinosum*

The identification of symbiont bacteria isolates producing L-glutaminase enzyme from *E. spinosum* is determined by developing a pink color on selective media, which is modified with the inclusion of L-glutamine substrate. The bacterial isolates were cultured on selective media, incorporating the L-glutamine amino acid as a substrate. Following an incubation period at a temperature of 37°C for 3 days, an observable alteration in the color of the media was noted. Figure 1.A shows an isolates ES 1.1 of L-glutaminase-producing bacteria from *E. spinosum*. The biochemical and microbiological identification results of isolates ES 1.1 (data not shown), confirmed through BLASTN and molecular analysis of 16S rRNA, were processed using a phylogenetic tree program, resulting in the data presented in Figures 1.B and 1.C, respectively. These results indicate that the symbiont bacteria isolates ES 1.1 from *E. spinosum* has a high 16S rRNA similarity (99% similarity) with *C. marina* strains HNS035 and UC15. It was therefore concluded that the symbiont bacteria isolate ES 1.1 belongs to the species *C. marina*.

Optimization and production of symbiont bacterial growth

The effect of fermentation time on the growth of optical density (OD) bacteria and the production of L-glutaminase enzyme from symbiont bacteria isolate can be seen in Figure 2. As shown in Figure 2, fluctuation in the growth phase of bacterial cells over the period of 18 to 54 hours,

accompanied by an increase in protein levels. The curve depicts a bacterial cell with an optical density (OD) value of 1.41, showcasing the capacity to produce protein at a concentration of 1.11 mg/mL by the 54th hour, marking the peak of enzyme activity, indicating the highest level achieved. Subsequently, from the 60th to the 72nd hour, enzyme activity experiences a decline, signaling the death phase within the stationary phase of bacterial cells. This decrease is attributed to reduced nutrient absorption, leading to a decline in the cell's ability to produce proteins.

Isolation, purification, and enzymatic activity of L-glutaminase enzyme from symbiont bacteria

The L-glutaminase enzyme was produced by cultivating the symbiotic *C. marina* ES 1.1 isolate in 250 mL of inoculum medium in a 1000 mL Erlenmeyer flask. In this study, the symbiotic bacterial isolate *C. marina* ES 1.1 was fermented in a production medium with a substrate concentration of 1.5% for 54 hours (optimum time). The obtained filtrate is the crude enzyme extract, which was subsequently analyzed for protein content and tested for L-glutaminase enzyme activity. The crude extract's enzyme protein content was 22.0 mg/mL (Table 1).

The purification stages of the L-glutaminase enzyme involved fractionation using the ammonium sulfate fractionation method at different saturation levels for each enzyme fraction (i) F1 (0-40%); (ii) F2 (40-60%); and (iii) F3 (60-80%) saturation level. This fractionation process aimed to separate proteins based on their solubility in water. Each fractionated precipitate obtained from ammonium sulfate fractionation underwent additional purification through dialysis. Apart from the undialyzed crude extract fraction, the F1 fraction had the highest protein content at 9.9 mg/mL, followed by F3 and F2, with protein contents of 2.1 and 1.4 mg/mL, respectively (Table 1). Determining L-glutaminase enzyme activity involves the enzyme's reaction with the appropriate substrate. The calculated enzyme activity results for L-glutaminase in each fraction are presented in Table 1. According to Table 1, the highest activity was observed in F3, with 13.3 U/mg, followed by F2 and F1, with enzyme activities of 11.2 and 1.9 U/mg protein, respectively. These findings align with the research, indicating that F3 exhibited the highest enzyme activity at 14.9 U/mL, followed by F2 and F1 with 13.1 and 9.2 U/mL, respectively.

Characterization of L-glutaminase enzyme from symbiont bacteria isolates

Effect of pH variation on enzyme activity

The highest L-glutaminase enzyme activity was achieved in the fermentation medium at the optimal pH of 8, with an enzyme activity of 2.2 U/mL (Figure 3.A). Previous studies have indicated that the optimum pH for L-glutaminase is pH 7, displaying the highest activity at 16.1 U/mL. Conversely, at pH 6, enzyme activity is low due to the suboptimal acidity for bacterial growth, as reported by Nindhita et al. (2022) and Susanti et al. (2024). It's important to note that excessively low or high pH conditions can lead to a denaturation process, decreasing enzyme activity. The production of L-glutaminase by *Achromobacter xylosoxidans*

RSHG1 showed optimal results at a pH of 9. Additionally, *Vibrio azureus* JK-79, isolated from a marine environment, exhibited maximum L-glutaminase production at pH 8, as

indicated by a separate study conducted by Jambulingam et al. (2018), yielding results are consistent with those found in this study.

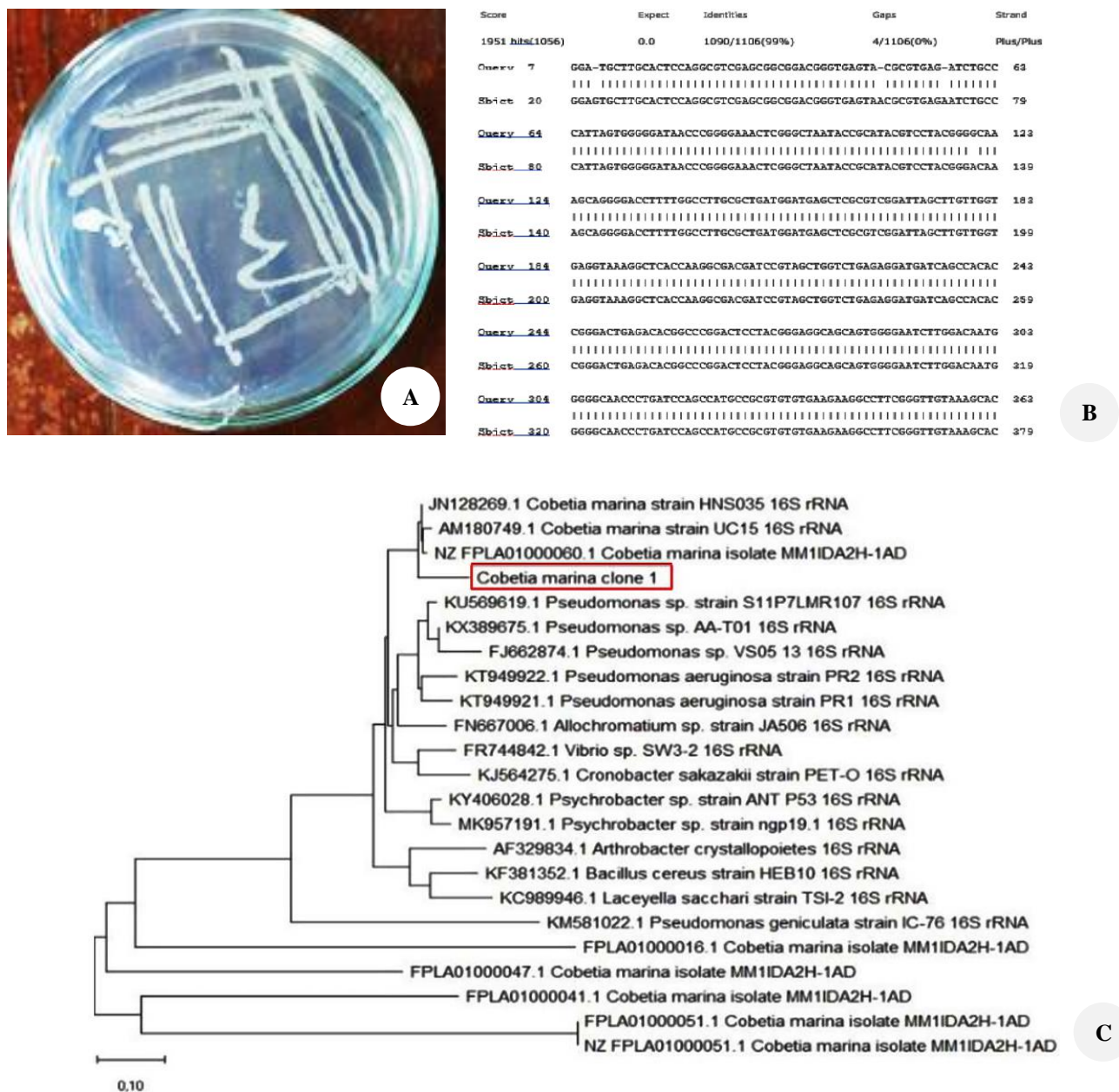


Figure 1. A. Isolates ES 1.1 of L-glutaminase-producing bacteria from *Eucheuma spinosum*; B. The BLASTN results and nucleotide sequence of ES 1.1 isolate showed a high similarity to the bacterial species *Cobetia marina*; C. The phylogenetic tree is based on the 16S rRNA encoding DNA sequence from the symbiotic bacterial ES 1.1 isolate

Table 1. L-glutaminase enzyme activity in each enzyme dialysate

Enzyme dialysate	Protein content (mg/mL)	Enzyme activity (U/mL)	Specific activity (U/mg protein)
Crude extract (F0)	22.0	11.1	0.5
0-40% (F1)	9.9	19.1	1.9
40-60% (F2)	1.4	15.1	11.2
60-80% (F3)	2.1	27.9	13.3

Effect of temperature variation on enzyme activity

The enzyme activity in response to temperature variation is illustrated in Figure 3.B. According to the data, the highest activity of the L-glutaminase enzyme was observed at an incubation temperature of 37°C, reaching 1.0 U/mL. This finding is consistent with the research conducted by Nindhita et al. (2022) and Susanti et al. (2024), which reported the highest L-glutaminase enzyme activity at an incubation temperature of 37°C, measuring 1.06 U/mL. Another scientist documented that *A. xylooxidans* RSHG1-produced L-glutaminase reached its peak activity at 40°C. Additionally, *Streptomyces avermitilis*-produced L-glutaminase exhibited its maximum activity at 30°C as Mostafa et al. (2021) indicated.

Effect of incubation time variation on enzyme activity

The characterization was conducted under the previously identified optimum conditions, specifically at pH 8 and a temperature of 37°C, with varied incubation times of 20, 30, 40, and 50 minutes. As depicted in Figure 3.C, L-glutaminase activity increases with extended incubation time, reaching its peak activity at the 30th minute with a value of 0.9 U/mL. However, beyond this point, enzyme activity decreases with prolonged incubation. This decline can be attributed to the enzyme's inability to maintain its conformation, resulting in a mismatch between its active

site and the substrate. Consequently, the enzyme loses its activity under certain time conditions. These results align with Ahmed et al. (2016) research, which studied the marine soft sponge *Aplysina fistularis* symbiont endophytic *Aspergillus* sp. and found that the optimum incubation time for L-glutaminase was 30 minutes.

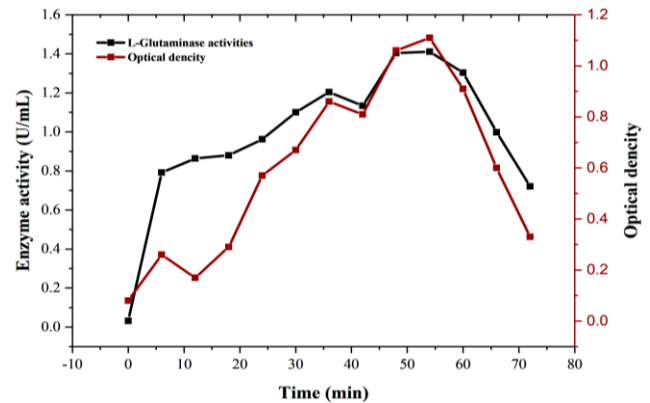


Figure 2. The effect of fermentation time on optical density and protein levels on the growth of symbiont bacteria

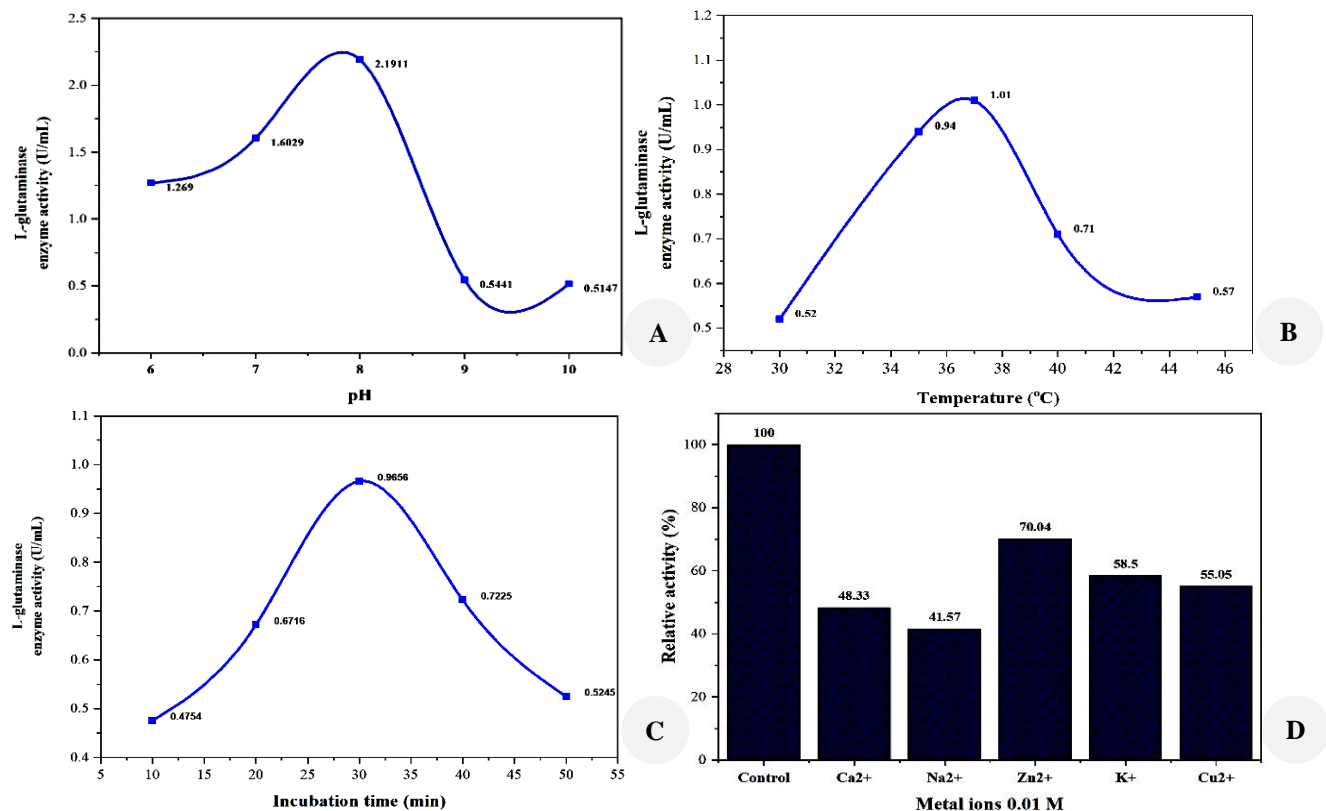


Figure 3. Characterization of L-glutaminase enzyme from symbiotic *Cobetia marina*. A. Effect of pH; B. Effect of temperature; C. Effect of incubation time; D. Effect of addition of metal ions on enzyme activity

Effect of addition of metal ions on enzyme activity

Adding metals to enzyme activity aims to assess whether these metals can either enhance or inhibit L-glutaminase enzyme activity. The calculated values for L-glutaminase activity from red algae symbiont bacteria in the presence of metals are presented in Figure 3.D. As indicated in Figure 3.D, the highest relative activity is observed in sodium metal, amounting to 41.57%. However, it is noteworthy that this metal inhibits the activity of the L-glutaminase enzyme. Following sodium, calcium, copper, potassium, and zinc exhibit successive inhibition with relative activities of 48.31, 55.05, 58.80, and 70.04%, respectively. Therefore, it can be concluded that these metals have a suppressive effect on L-glutaminase enzyme activity. This aligns with previous research by Saleem and Ahmed (2021), which demonstrated that Co^{+2} , Hg^{+2} , Zn^{+2} , and Fe^{+2} metals ion act as inhibitors and can reduce L-glutaminase enzyme activity.

The study on the effect of metal ions on L-glutaminase produced by *Bacillus* sp. B12 reported that the enzyme was activated by Mn^{2+} , Mg^{2+} , Ca^{2+} , and Na^{+} , while no effect was observed with the addition of K^{+} , Co^{2+} , and Ni^{2+} . However, the enzyme was inhibited by Hg^{2+} , Cu^{2+} , Fe^{2+} , and Zn^{2+} . These findings suggest that the enzyme is a metalloenzyme, which requires activation by specific metals for optimal activity. The enzyme's activation or inhibition appears to depend on the presence of certain metals, highlighting the specificity of the enzyme's metal cofactors and their roles in its catalytic activity (Vimal and Kumar 2019).

Symbiont bacteria L-glutaminase enzyme antibacterial test

The antibacterial test for L-glutaminase was conducted through the agar diffusion method, employing Gram-negative bacteria *E. coli* and Gram-positive *S. aureus*. The positive control (ampicillin) is crucial in serving as a reference for the test substance. By comparing the diameter of the inhibition area formed, this control helps assess the efficacy of the tested substance, as stated by Hossain (2024).

Table 2 shows that F1 demonstrated the most potent antibacterial activity against the tested bacteria *E. coli* and *S. aureus*. Among the various fractions tested, F1 formed an inhibitory or clear zone around the 6 mm paper disc. These observations were made following incubation at 37°C for 2×24 hours. The clear zones suggest that the substance in F1 has inhibitory effects on bacterial growth, particularly notable for Gram-positive bacteria.

Potential of anticancer of L-glutaminase enzyme from symbiotic *C. marina* against MCF-7 cell line

The BSLT analysis results reveal varying toxicity levels for each enzyme fraction. F1 exhibited the highest toxicity, followed by F3 and F2 (data not shown). The toxicity level can indicate potential anticancer activity, whereas a smaller LC_{50} value suggests higher toxicity and potential anticancer properties, as described by Hanafi et al. (2020). The fraction with the highest toxicity activity, F1, was further evaluated for cytotoxicity through the MTT test. The anticancer

bioactivity of the purified L-glutaminase enzyme on MCF-7 breast cancer cells is detailed in Table 3 and Figure 4.

According to Figure 4, the inhibition of MCF-7 breast cancer cell viability by the fraction of L-glutaminase enzymes at concentrations ranging from 10 to 160 $\mu\text{g}/\text{mL}$ demonstrates a successive reduction in cell viability, with values of 84.62, 66.67, 43.59, 33.33, and 25.64%. This indicates that as the concentration of the L-glutaminase enzyme increases, the percentage of live cells in MCF-7 breast cancer cells decreases. The elevated enzyme concentration inhibits the growth of MCF-7 cells, leading to the death of cancer cells. In cytotoxic testing, cell development inhibition is calculated as the IC_{50} (50% Inhibitory Concentration).

Table 2. L-glutaminase enzyme activity in each enzyme fraction

Enzyme fractions	Diameter of inhibition zone (mm)			
	<i>E. coli</i>		<i>S. aureus</i>	
	24 h	48 h	24 h	48 h
Crude extract (F0)	8.30	11.40	8.40	13.50
0-40% (F1)	7.50	8.30	8.20	15.30
40-60% (F2)	7.20	8.70	8.20	14.00
60-80% (F3)	6.80	7.60	7.80	14.30
Control (+)	19.30	19.50	20.10	21.70
Control (-)	0.00	0.00	0.00	0.00

Table 3. IC_{50} value of the enzyme fraction on MCF-7 breast cancer cells

Concentration ($\mu\text{g}/\text{mL}$)	% Inhibition	IC_{50} ($\mu\text{g}/\text{mL}$)
10	15.38	64.26
20	33.33	
40	56.41	
80	66.67	
160	74.36	

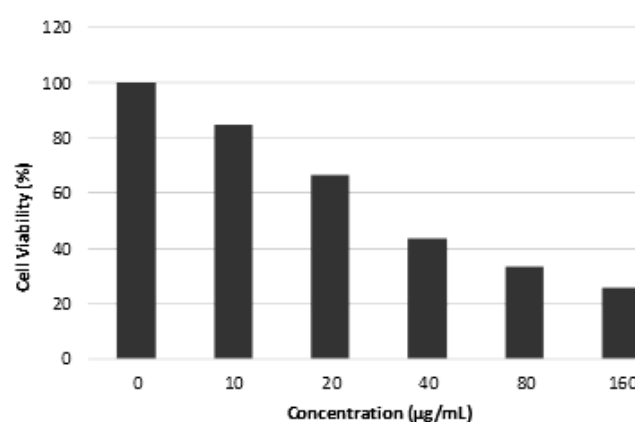


Figure 4. Level of inhibition of MCF-7 breast cancer cell viability from the L-glutaminase enzyme fraction synthesized by the symbiotic *Cobetia marina*

The IC₅₀ determination aims to identify the lowest concentration of the test compound that acts as an inhibitor, causing a 50% reduction in cell growth. This is consistent with Hanafi et al. (2020) statement, where cytotoxic activity is evaluated based on the IC₅₀ value. The IC₅₀ value is derived from the percent inhibition values and is processed using the probit log graph method, as detailed in Table 3. As date per Table 3, the percentage of inhibition at concentrations ranging from 10 to 160 µg/mL is as follows (i) 15.38; (ii) 33.33; (iii) 56.41; (iv) 66.67; and (v) 74.36%, with an IC₅₀ value of 64.26 µg/mL, indicating moderate cytotoxic activity. This suggests that the L-glutaminase enzyme fraction can be developed as an anticancer drug. These findings are similar to research conducted by Reda (2015), who obtained an IC₅₀ value of 64.70 µg/mL against HCT-116 cancer cells, indicating the potential development of the test substance as an anticancer drug. Although the IC₅₀ value in this study is still below the standard, the L-glutaminase enzyme from the red alga symbiont *E. spinosum* demonstrated its ability to inhibit MCF-7 cells, albeit to a small extent.

Anti-dengue potential activity test

The toxicity test was performed on the L-glutaminase enzyme from the extracellular symbiotic *Cobetia marina* using *Artemia salina* Linnaeus 1758, Leach (data not shown). The high toxicity of the L-glutaminase fractions was further evaluated for anti-dengue properties through cytotoxicity tests on Vero cells infected with the DENV-2 virus. The crude extract demonstrated the highest anti-dengue activity, with an inhibition percentage of 78% and a CC₅₀ value of 167.15 µg/mL. The F1 fraction (0-40%) showed an inhibition percentage of 60% and a CC₅₀ value of 200.01 µg/mL (Table 4). The anti-dengue activity differed between the fractions, with F1 demonstrating the most significant potential due to its higher protein content and possibly more active components against the dengue virus. F3 and F2 also exhibited anti-dengue effects, but their activities were comparatively lower than F1.

Based on the criteria established by previous research by Sood et al. (2015) and Rosmalena et al. (2019) a natural compound is considered to have potential as an anti-dengue agent if it exhibits cell viability of over 50%, percent

inhibition of over 90%, and a CC₅₀ value of less than 250 µg/mL. According to these conventions, the F0 fraction (crude extracts) and the F1 fraction (0-40%) of the L-glutaminase enzyme from the extracellular bacteria *E. spinosum* demonstrate potential as antiviral agents for dengue.

These findings suggest that the L-glutaminase enzyme from symbiotic *C. marina* holds promise as a potential anti-dengue agent, particularly from the F1 fraction, which exhibited the strongest protein concentration and antiviral activity. This study is the first to report on natural products derived from this type of enzyme with potential against the dengue virus. Although previously reported, the L-asparaginase and Phospholipase A2 enzymes also had antiviral potential against dengue (Muller et al. 2012; Reda 2015; Ahmad et al. 2021).

To enhance the antiviral dengue activity, further exploration of other types of proteins from the symbiotic *E. spinosum* is necessary. Additionally, conducting partial hydrolysis of proteins to obtain peptides with smaller molecular sizes could improve their ability to penetrate the dengue virus that causes Dengue Hemorrhagic Fever (DHF). The theoretical mechanism of antibacterial, anticancer, and antiviral action for the L-glutaminase enzyme involves inhibiting protein synthesis in bacterial, cancer, and dengue virus cells without harming normal cells. L-glutaminase catalyzes the hydrolysis of L-glutamine to L-glutamate and ammonia, disrupting protein synthesis and inhibiting cell growth, ultimately leading to cell death (Teixeira et al. 2020).

In conclusion, the red algae symbiont bacteria isolate identified as *C. marina*, particularly *E. spinosum*, has demonstrated the production of L-glutaminase enzyme. The highest enzyme activity was observed in fraction F3, measuring 27.9 U/mL, with a specific activity of 13.3 U/mg protein. The optimum conditions for L-glutaminase enzyme activity were identified as pH 8, a temperature of 37°C, maximum activity after 30 minutes of incubation time, and the ability to enhance enzyme activity with the addition of metal ions Mg²⁺, Co²⁺, and Mn²⁺, while decreasing activity with the addition of metal ions Ca²⁺, Na²⁺, Zn²⁺, K⁺, and Cu²⁺.

Table 4. Summary of protein concentration and anti-dengue potential after the administration of 10 µg/mL of L-glutaminase fractions from extracellular bacteria symbiotic *Cobetia marina* of red algae *Eucheuma spinosum*

Samples no.	L-glutaminase fractions	Protein concentration (mg/mL)	LC50 (µg/mL)	Cell viability (%)	Inhibition (%)	CC50 (µg/mL) Vero cells
F0	Crude extracts	22.0	10.01	53	78	167.15
F1	0-40%	9.9	14.43	60	86	200.01
F2	40-60%	1.4	15317	ND	ND	ND
F3	60-80%	2.1	38.870	72	90	250.12

In antibacterial tests using the agar diffusion method, the L-glutaminase enzyme fraction exhibited bactericidal properties, with a 7.50 mm inhibition diameter in F1 against *E. coli* and a 15.30 mm inhibition diameter in F1 against *S. aureus*. In cytotoxic tests using the MTT method, the most potent fraction was F1, showing a percent inhibition value of 25.65% at a concentration of 160 µg/mL and an IC₅₀ value of 64.26 µg/mL. Additionally, the crude extracts of L-glutaminase exhibited an inhibition percentage of 78% and a CC₅₀ value of 167.15 µg/mL against DENV-2 when tested on vero cells. These results collectively indicate the potential of L-glutaminase enzyme fractions from symbiotic *C. marina* for antibacterial and anticancer applications, including antiviral activity against dengue. The limitations of this study include the need for further purification of the obtained L-glutaminase enzyme to achieve a pure enzyme that meets the requirements as a therapeutic agent. Additionally, the bioactivity data for antibacterial, anticancer, and antiviral dengue effects are limited to in vitro testing results. Therefore, it is recommended that future research focuses on further enzyme purification and confirms the bioactivity through in vivo testing on experimental animals.

ACKNOWLEDGEMENTS

The authors would like to thank the Biochemistry Laboratory staff of Faculty of Mathematics and Natural Sciences, Universitas Hasanuddin, Makassar, Indonesia and Laboratory of Microbiology, School of Pharmacy YAMASI, Makassar, Indonesia for their assistance in completing this study; also, special thanks to Mrs. Kurniati Umrah for proofreading these manuscripts. This work was supported by DRTPM grant vocation, PKDN schema with contract No. 245/LL9/PK.00.PGPV/2024 and I.2.1.082.113/IV/2024.

REFERENCES

- Ahmad A, Asmi N, Karim H, Massi MN, Wahid I, Sugrani A. 2021. Characterization of anti-dengue and cytotoxic activity of protein hydrolysates from the exophytic bacteria of brown algae *Sargassum* sp.. *J Appl Pharm Sci* 11: 039-045. DOI: 10.7324/JAPS.2021.110205.
- Ahmad A, Asmi N, Karim H, Massi MN, Wahid I. 2022. Effect of substrate concentration and FeCl₂ addition on L-asparagine activities of bacteria symbionts brown algae *Sargassum* sp. and its potential as a new anti-dengue agent. *Rasayan J Chem* 15 (1): 659-667. DOI: 10.31788/RJC.2022.1516742.
- Ahmad K, Mohd H, Nor FZ, Muhammad FA, Aziz H, Mohd RCS, Thirukanthan J, Malinna. 2024. Genetically engineered microalgae for enhanced bioactive compounds. *Discover Appl Sci* 6 (9): 482. DOI: 10.1007/s42452-024-06116-5.
- Ahmed M, Taha TM, Abo-Dahab NF, Hassan FS. 2016. Process optimization of L-glutaminase production; a tumour inhibitor from marine endophytic isolate *Aspergillus* sp. ALAA-2000. *J Microb Biochem Technol* 8: 1-8. DOI: 10.4172/1948-5948.1000313.
- Akbar, Fitri H, Tsania R, Fakhriana FB, Choirunnisa H, Nurul S, Irfan. 2022. Potential extract of red algae (*Euclidean spinosum*) on wound healing: Literature review. *Makassar Dent J* 11 (2): 148-153. DOI: 10.35856/mdj.v11i2.577.
- Anand U, Dey A, Chandel AS, Sanyal R, Mishra A, Pandey DK, De Falco V, Upadhyay A, Kandimalla R, Chaudhary A, Dhanjal JK, Dewanjee S, Vallamkondu J, Pérez de la Lastra JM. 2023. Cancer chemotherapy and beyond: Current status, drug candidates, associated risks and progress in targeted therapeutics. *Gene Dis* 10 (4): 1367-1401. DOI: 10.1016/j.gendis.2022.02.007.
- Andinata B, Bachtiar A, Oktamianti P, Partahi JR, Dini MSA. 2023. A comparison of cancer incidences between Dharmais Cancer Hospital and Globocan 2020: A descriptive study of top 10 cancer incidences. *Indones J Cancer* 17 (2): 119. DOI: 10.33371/ijoc.v17i2.982.
- Anusree MK, Manasa LK, Sreehari M, Raj S, Sreenikethanam A, Bajhaiya AK. 2023. Marine microalgae: An emerging source of pharmaceuticals and bioactive compounds. In: Meena SN, Nandre V, Kodam K, Meena RS (eds). *New Horizons Natural Compound Research*. Academic Press, Cambridge. DOI: 10.16/B978-0-443-15232-0.00025-4.
- Astriani A, Nurjanah N. 2023. Bioactive components of seaweed *Euclidean cottonii* potential as antibacterial. *Jurnal Biologi Tropis* 23 (2): 341-347. DOI: 10.29303/jbt.v23i2.4882.
- Atanasov AG, Zotchev SB, Dirsch VM, Orhan IE, Banach M, Rollinger JM, Barreca D, Weckwerth W, Bauer R, Bayer EA, Majeed M. 2021. Natural products in drug discovery: Advances and opportunities. *Nat Rev Drug Disc* 20 (3): 200-216. DOI: 10.1038/s41573-020-00114-z.
- Biris-Dorhoi ELS, Michiu D, Pop CR, Rotar AM, Tofana M, Pop OL, Socaci SA, Farcas AC. 2020. Macroalgae-a sustainable source of chemical compounds with biological activities. *Nutrients* 12 (10): 3085. DOI: 10.3390/nu12103085.
- Clark C, Lim A, Gupte P, Pigott DM, Brady OJ. 2024. A global dataset of publicly available dengue case count data. *Sci Data* 11 (1): 296. DOI: 10.1038/s41597-024-03120-7.
- CLSI [Clinical and Laboratory Standards Institute]. 2019. National Committee for Clinical Laboratory Standards. 2000. Performance Standard for Antimicrobial Susceptibility Testing. Document M100-S10. https://clsi.org/media/1930/m100ed28_sample.pdf
- Cyriac R, Lee K. 2024. Glutaminase inhibition as potential cancer therapeutics: Current status and future applications. *J Enzyme Inhibit Med Chem* 39 (1): 2290911. DOI: 10.1080/14756366.2023.2290911.
- Damongilala L, Dotulong V, Apriyanti E, Kurnia D. 2023. Antioxidant and antibacterial activities of the tropical red alga *Euclidean spinosum*: In silico study. *Nat Prod Commun* 18 (7): 1934578X231187467. DOI: 10.1177/1934578X231187467.
- Daniotti S, Re I. 2021. Marine biotechnology: Challenges and development market trends for the enhancement of biotic resources in industrial pharmaceutical and food applications. A statistical analysis of scientific literature and business models. *Mar Drug* 19 (2): 61. DOI: 10.3390/md19020061.
- Dhankhar R, Gupta V, Kumar S, Kapoor RK, Gulati P. 2020. Microbial enzymes for deprivation of amino acid metabolism in malignant cells: Biological strategy for cancer treatment. *Appl Microbiol Biotechnol* 104 (7): 2857-2869. DOI: 10.1007/s00253-020-10432-2.
- Dong SH, Duan ZK, Bai M, Huang XX, Song SJ. 2024. Advanced technologies targeting isolation and characterization of natural products. *Trend Anal Chem* 175: 117711. DOI: 10.1016/j.trac.2024.117711.
- Edwards DN, Ngwa VM, Raybuck AL, Wang S, Hwang Y, Kim LC, Chao SH, Pain Y, Wang Q, Zhang S, Manning HC, Rathmell JC, Cook RS, Boothby MR, Chen J. 2021. Selective glutamine metabolism inhibition in tumor cells improves antitumor T lymphocyte activity in triple-negative breast cancer. *J Clin Invest* 131 (4): e140100. DOI: 10.1172/JCI140100.
- El-Beltagi HS, Mohamed AA, Mohamed HI, Ramadan KMA, Barqawi AA, Mansour AT. 2022. Phytochemical and potential properties of seaweeds and their recent applications: A review. *Mar Drug* 20 (6): 342. DOI: 10.3390/md20060342.
- Ferrol-S, Ferse CA, Glase M. 2014. Patron-client relationships, livelihoods and natural resource management in tropical coastal communities. *Ocean Coast Manag* 100: 63-73. DOI: 10.1016/j.ocecoaman.2014.07.016.
- Fouda A, Al-Otaibi WA, Saber T, AlMotwaa SM, Alshallash KS, Elhady M, Badr NF, Abdel RM. 2022. Antimicrobial, antiviral, and in-vitro cytotoxicity and mosquitocidal activities of *Portulaca oleracea*-based green synthesis of selenium nanoparticles. *J Funct Biomat* 13 (3): 157. DOI: 10.3390/jfb13030157.
- Ghosh S, Sarkar T, Pati S, Kari ZA, Edinur HA, Chakraborty R. 2022. Novel bioactive compounds from marine sources as a tool for functional food development. *Front Mar Sci* 9 (2): 832957. DOI: 10.3389/fmars.2022.832957.
- Gomaa, Eman Z. 2022. Production, characterization, and antitumor efficiency of L-glutaminase from halophilic bacteria. *Bull Nat Res Centre* 46 (1): 10. DOI: 10.1186/s42269-021-00693-w.

- Hanafi H, Irawan C, Sirait SM, Sulistiawaty L, Setyawati SR. 2020. Toxicity test with BSLT (Brine Shrimp Lethality Test) method on methanol, ethyl acetate extract, hexane on seeds, and rind of mataoa extract (*Pometia pinnata*). *Orient J Chem* 36 (6): 1143-1147. DOI: 10.13005/ojc/36061.
- Hemmerling F, Piel J. 2022. Strategies to access biosynthetic novelty in bacterial genomes for drug discovery. *Nat Rev Drug Discov* 21 (5): 359-378. DOI: 10.1038/s41573-022-00414-6.
- Hossain TJ. 2024. Methods for screening and evaluation of antimicrobial activity: A review of protocols, advantages, and limitations. *Eur J Microbiol Immunol* 14 (2): 97-115. DOI: 10.1556/1886.2024.00035.
- Ilic I, Ilic M. 2024. Global patterns of trends in incidence and mortality of dengue, 1990-2019: An analysis based on the global burden of disease study. *Medicina* 60: 425. DOI: 10.3390/medicina60030425.
- Jambulingam K, Nachimuthu S, Saranya M. 2018. Maximizing L-glutaminase production from marine *Bacillus subtilis* JK-79 under solid state fermentation. *Afr J Biotechnol* 17 (9): 288-305. DOI: 10.5897/ajb2017.16015.
- Jin J, Byun JB, Choi YK, Park KG. 2023. Targeting glutamine metabolism as a therapeutic strategy for cancer. *Exp Mol Med* 55 (4): 706-715. DOI: 10.1038/s12276-023-00971-9.
- Kaur R, Gupta S, Bhardwaj A. 2023. Cancer treatment therapies: Traditional to modern approaches to combat cancers. *Mol Biol Rep* 50 (11): 9663-9676. DOI: 10.1007/s11033-023-08809-3.
- Leng G, Duan B, Liu J, Li S, Zhao W, Hou G, Qu J. 2024. The Advancements and prospective developments in anti-tumor targeted therapy. *Neoplasia* 56: 101024. DOI: 10.1016/j.neo.2024.10102.
- Lim CL, Koh RY, Haw TY, Boudville LA. 2015. Antioxidant activity of the sea bird nest (*Eucheuma cottonii*) and its radical scavenging effect on human keratinocytes. *J Med Bioeng* 4 (6): 461-465. DOI: 10.12720/jomb.4.6.461-465.
- Liu B, Zhou H, Tan L, Siu KTH, Guan XY. 2024. Exploring treatment options in cancer: Tumor treatment strategies. *Signal Transduc Target Ther* 9 (1): 175. DOI: 10.1038/s41392-024-01856-7.
- Liu J, Obaidi I, Nagar S, Scalabrino G, Sheridan H. 2021. The antiviral potential of algal-derived macromolecules. *Curr Res Biotechnol* 3 (4): 120-134. DOI: 10.1016/j.crbiot.2021.04.003.
- Maharem TM, Enam MA, Said Y. 2020. Purification and characterization of L-glutaminase enzyme from camel liver: Enzymatic anticancer property. *Intl J Biol Macromol* 150: 1213-1222. DOI: 10.1016/j.ijbiomac.2019.10.131.
- Min HY, Ho-YL. 2022. Molecular targeted therapy for anticancer treatment. *Exp Mol Med* 54 (10): 1670-1694. DOI: 10.1038/s12276-022-00864-3.
- Mostafa Y, Alamri SA, Alfaifi M, Alrumman SA, Elbehairi SE, Taha TH, Hashem M. 2021. L-glutaminase synthesis by marine *Halomonas meridiana* isolated from the red sea and its efficiency against colorectal cancer cell lines. *Molecules* 26 (7): 1963. DOI: 10.3390/molecules26071963.
- Mukriani, Ahmad A, Asmi N, Karim H, Massi MN, Prihantono. 2020. Production and characterization of L-asparaginase enzyme from symbiont bacteria of red algae *Eucheuma spinosum*. *Egypt J Chem* 65 (11): 475-481. DOI: 10.21608/ejchem.2022.81705.4045.
- Muller VDM, Russo RR, Cintra ACO, Sartim MA, Alves PRDM, Figueiredo LTM, Sampaio SV, Aquino VH. 2012. Crotoxin and phospholipases A₂ from *Crotalus durissus terrificus* showed antiviral activity against dengue and yellow fever viruses. *Intl Soc Toxicol* 59 (4): 507-515. DOI: 10.1016/j.toxicol.2011.05.021.
- Nakase T, Giovanetti M, Obolski U, Lourenço J. 2023. Global transmission suitability maps for dengue virus transmitted by *Aedes aegypti* from 1981 to 2019. *Sci Data* 10 (1): 275. DOI: 10.1038/s41597-023-02170-7.
- Nieuwkoop T, Claassens N, Van der Oost J. 2019. Improved protein production and codon optimization analyses in *Escherichia coli* by bicistronic design. *Microb Biotechnol* 12 (1): 173-179. DOI: 10.1111/1751-7915.13332.
- Nindhita Y, Kusdarwati R, Sulmartiwi L. 2022. Antibacterial activity of crude extract red algae *Eucheuma spinosum* against *Staphylococcus epidermidis* bacteria growth. *J Mar Coast Sci* 11 (3): 111-118. DOI: 10.20473/jmcs.v11i3.38286.
- Orabi H, El-Fakharany E, Abdelkhalek E, Nagwa S. 2020. Production, optimization, purification, characterization, and anti-cancer application of extracellular L-glutaminase produced from the marine bacterial isolate. *Prepar Biochem Biotechnol* 50 (4): 408-418. DOI: 10.1080/10826068.2019.1703193.
- Prihantono, Rusli R, Christeven R, Faruk M. 2023. Cancer incidence and mortality in a tertiary hospital in Indonesia: An 18-year data review. *Ethiop J Health Sci* 33 (3): 515-522. DOI: 10.4314/ejhs.v33i3.15.
- Punekar NS. 2018. Quantification of catalysis and measures of enzyme purity. *Enzymes: Catalysis, Kinetics and Mechanisms*. Springer Singapore, Singapore. DOI: 10.1007/978-981-13-0785-0_14.
- Purbosari N, Warsiki E, Syamsu K, Santoso J, Effendi I. 2021. Evaluation of the application of seaweed (*Eucheuma cottonii*) extract as fish anesthetic agent. *Aquacult Intl* 29 (4): 1545-60. DOI: 10.1007/s10499-021-00693-7.
- Raghani NR, Chorawala MR, Mahadik M, Patel RB, Prajapati BG, Parekh PS. 2024. Revolutionizing cancer treatment: Comprehensive insights into immunotherapeutic strategies. *Med Oncol* 41 (2): 51. DOI: 10.1007/s12032-023-02280-7.
- Rahman, Lokollo FF, Manuputty GD, Hukubun RD, Krisye, Maryono, Wawo M, Wardiatno Y. 2024. A review on the biodiversity and conservation of mangrove ecosystems in Indonesia. *Biodiver Conserv* 33 (3): 875-903. DOI: 10.1007/s10531-023-02767-9.
- Ramadan R, Ahmad A, Marinda, Natsir H, Karim A, Karim H. 2019. Symbiont bacteria cultures from the red algae *Eucheuma spinosum*, isolation of bioactive proteins and their anticancer potential test. *J Phys: Conf Ser* 1341 (3): p.032020. DOI: 10.1088/1742-6596/1341/3/032020.
- Reda FM. 2015. Kinetic properties of *Streptomyces canarius* L-glutaminase and its anticancer efficiency. *Braz J Microbiol* 46 (4): 957-968. DOI: 10.1590/S1517-838246420130847.
- Rimmer MA, Larson S, Lapong I, Purnomo AH, Pong-masak PR, Swanepoel L, Paul NA. 2021. Seaweed aquaculture in Indonesia contributes to social and economic aspects of livelihoods and community wellbeing. *Sustainability* 13 (19): 10946. DOI: 10.3390/su131910946.
- Rosmalena R, Elya B, Dewi BE, Fithriyah, Desti H, Angelina M, Hanafi M, Lotulung PD, Prasasty VD, Seto D. 2019. The antiviral effect of Indonesian medicinal plant extracts against dengue virus in vitro and in silico. *Pathogens* 8 (2): 85. DOI: 10.3390/pathogens8020085.
- Safitri A, Srihardyastutie A, Roosdiana A, Anna R, Sutrisno S. 2018. Antibacterial activity and phytochemical analysis of edible seaweed *Eucheuma spinosum* against *Staphylococcus aureus*. *J Pure Appl Chem* 7 (3): 308-315. DOI: 10.21776/ub.jpacr.2018.007.03.389.
- Saleem R, Ahmed S. 2021. Characterization of a new L-glutaminase produced by *Achromobacter xylosoxidans* Rshg1, isolated from an expired hydrolyzed L-glutamine sample. *Catalysts* 11 (11): 1262. DOI: 10.3390/catal11111262.
- Sathish T, Kezia D, Bramhachari PV, Prakasham RS. 2018. Multi-objective based superimposed optimization method for enhancement of L-glutaminase production by *Bacillus subtilis* RSP-GLU. *Karbala Intl J Modern Sci* 4 (1): 50-60. DOI: 10.1016/j.kijoms.2017.10.
- Sim S, Hibberd ML. 2016. Genomic approaches for understanding dengue: Insights from the virus, vector, and host. *Genom Biol* 17: 38. DOI: 10.1186/s13059-016-0907-2.
- Simay S, Akbarzadeh KM, Pourseif MM, Barar J, Safary A, Omidi Y. 2022. Recombinant production and characterization of L-glutaminase (GlsA) as a promiscuity therapeutic enzyme. *Appl Microbiol Biotechnol* 106 (17): 5511-5524. DOI: 10.1007/s00253-022-12058-y.
- Sood R, Raut R, Tyagi P, Pareek PK, Barman TK, Singhal S, Shirumalla RK, Kanoje V, Subbarayan R. 2015. *Cissampelos pareira* Linn: Natural source of potent antiviral activity against all four dengue virus serotypes. *PLoS Neglect Trop Dis* 9 (12): e0004255. DOI: 10.1371/journal.pntd.0004255.
- Sugrani A. 2021. Antibacterial and anticancer activity of protein from red algae (*Eucheuma cottonii*). *Saud J Med Pharm Sci* 7 (12): 594-598. DOI: 10.36348/sjmps.2021.v07i12.001.
- Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. 2021. Global cancer statistics 2020: Globocan estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *Cancer J Clin* 71 (3): 209-249. DOI: 10.3322/caac.21660.
- Susanti, Nuryanti S, Rusli. 2024. Potensi ekstrak alga merah jenis *Eucheuma cottoni* sebagai antibakteri. *Makassar Pharm Sci J* 2 (2): 292-298. [Indonesian]
- Tan CH, Chang VS, Teo SS. 2015. In-vitro anticancer activity of *Eucheuma cottonii* extracts against hela cell line, humn lung carcinoma cell line (Sk-Lu-1), human colon carcinoma cell line (Hct-116), and fibroblast. *Malay J Sci* 1 (2): 69-73.
- Teixeira MA, Paiva MC, Amorim PA, Felgueiras, Helene P. 2020. Electrospun nanocomposites containing cellulose and its derivatives modified with specialized biomolecules for enhanced wound healing. *Nanomaterials* 10 (3): 557. DOI: 10.3390/nano10030557.

- Teo BSX, Gan RY, Abdul AS, Sirirak T, Mohd A, Mohd FY, Yusuf E. 2021. In vitro evaluation of antioxidant and antibacterial activities of *Eucheuma cottonii* extract and its in vivo evaluation of the wound-healing activity in mice. *J Cosmetic Dermatol* 20 (3): 993-1001. DOI: 10.1111/jocd.13624.
- Vachher M, Sen A, Kapila R, Nigam AN. 2021. Microbial therapeutic enzymes: A promising area of biopharmaceuticals. *Curr Res Biotechnol* 3: 195-208. DOI: 10.1016/j.crbiot.2021.05.006.
- Van DH, Lansbergen R, Axmann H, Soethoudt H, Tacken G, Van DPJ, Rukminasari N. 2022. Seaweed in Indonesia: Farming, utilization and research. Wageningen Centre for Development Innovation, Wageningen. DOI: 10.18174/57800.
- Vijayan N, Swapna TS, Haridas M, Sabu A. 2017. Therapeutic enzymes: L-glutaminase. In: Pandey A, Negi S, Soccol CR (eds). *Current Developments in Biotechnology and Bioengineering*. Elsevier, Amsterdam. DOI: 10.1016/B978-0-444-63662-1.00011-7.
- Vimal A, Kumar A. 2019. Transforming the healthcare system through therapeutic enzymes. In: Kuddus M (eds). *Enzymes in Food Biotechnology*. Academic Press, London. DOI:10.1016/B978-0-12-813280-7.00035-9.
- WHO [World Health Organization]. 2023. Disease Outbreak News; Dengue-Global Situation. <https://www.who.int/emergencies/disease-outbreak-news/item/2023-DON498>.
- Wijayanto D, Bambang AN, Nugroho RA, Kurohman F, Riyadi PH. 2021. The optimization of production and profit of *Eucheuma cottonii* cultivation in Kemojan Island, Indonesia. *AACL Bioflux* 14 (4): 1955-64.
- Wulandari D, Kilawati Y, Fajar M. 2018. Activity of compounds on seaweed *Eucheuma cottonii* extract as antioxidant candidate to prevent effects of free radical in water pollution. *Res J Life Sci* 5 (3): 173-82. DOI: 10.21776/ub.rjls.2018.005.03.
- Yang WH, Qiu Y, Stamatatos O, Janowitz T, Lukey MJ. 2021. Enhancing the efficacy of glutamine metabolism inhibitors in cancer therapy. *Trends Cancer* 7 (8): 790-804. DOI: 10.1016/j.trecan.2021.04.003.
- Zakaria Z, Soekamto N, Dali N, Amalia HA, Syarifuddin SH. 2024. Toxicity test using The Brine Shrimp Lethality Test (BSLT) method on extracts of stem bark, stem wood, and leaves on bayur (*Pterospermum diversifolium* B. Rob.). *Elkawnie* 10 (1): 42. DOI: 10.22373/ekw.v10i1.20380.
- Zhivkopljas E, J Jouffray JB, Dunshirn P, Pranindita A, Blasiak R. 2024. Growing prominence of deep-sea life in marine bioprospecting. *Nat Sustain* 7 (8): 1027-1037. DOI: 10.1038/s41893-024-01392-w.
- Zhong L, Li Y, Xiong L, Wang W, Wu M, Yuan T, Yang W, Tian C, Miao Z, Wang T, Yang S. 2021. Small molecules in targeted cancer therapy: Advances, challenges, and future perspectives. *Signal Transduc Target Ther* 6 (1): 201. DOI: 10.1038/s41392-021-00572-w.