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EFFECT OF SUBSTRATE CONCENTRATION AND $FeCl_2$ ADDITION ON L-ASPARAGINASE ACTIVITIES OF BACTERIA SYMBIONTS BROWN ALGAE *Sargassum* sp. AND ITS POTENTIAL AS A NEW ANTI-DENGUE AGENTS

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ABSTRACT

The disintegration of the amide link, causing L-Asparaginase catalyzes the hydrolysis process of L-Asparagine into ammonia and aspartic acid. The purposes of this study were to determine the optimal conditions for enzyme production and enzymatic activity, including their potential as a new anti-dengue of L-asparaginase from *Klebsiella* sp. The Nessler method used a UV-Visible spectrophotometer to determine the L-asparaginase activity by measuring ammonia levels produced by L-asparaginase catalysis. The ideal fermentation period was at the 48th hour, and the optimal substrate concentration was 10 g/L with an activity value of 49.572 U/mL, and the optimal $FeCl_2$ concentration was 0.5 g/L with an activity value of 60.1183 U/mL, and protein content of 0.1878 mg/mL. The optimum conditions for L-asparaginase, which has an activity value of 61.1182 U/mL, were observed at pH 8 and 37 °C. The optimum cofactor concentration of $FeCl_2$ was 0.5 mM, which resulted in an activity value of 106.5046 U/mL. Both the inhibition percentage and CC_{50} value were 80% and 167.15 µg/mL respectively, showing that the L-asparaginase has a high anti-dengue potential.

Keywords: L-Asparaginase, *Klebsiella* sp., Anti-dengue, $FeCl_2$ Cofactor.

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INTRODUCTION

The L-Asparaginase enzyme is an enzyme that has been commonly applied in the clinical field, due to its ability to fight cancer. Cancer cells are cells that have lost the arranging power. For cell growth, cancer cells need L-asparagine amino acids. Cancer cells do not have asparagine synthetase enzyme that serves to synthesize asparagine from aspartic acid, hence the cancer cells then take asparagine into the blood. The given asparaginase will inhibit the synthesis of cancer cell protein by outlining asparagine into aspartic acid and ammonia. This condition will lack asparagine cancer cells, which lead to the death of these cells.[1]

The production of bacterial symbionts macroalga enzyme has high activity. One of the potential algae species to be developed and utilized is *Sargassum* sp. which grows abundantly and widely spread in Indonesian waters.[2] The identification of bacterial symbionts brown algae, *Sargassum* sp. then acquired a bacterial species of *Klebsiella* sp. An initial report showed *Klebsiella* sp. bacteria have potential as a producer of the L-Asparaginase enzyme,[3] although *Klebsiella* sp. bacteria has been reported as phytase enzyme resources[4,5]. Identification of macroalgae symbionts bacterial conducted by Karim *et al.* 2020, obtained two species of bacteria, *Enterobacter agglomerans* SB 5(1) and *Enterobacter aerogenes* SP 3(2), that have potential as L-Asparaginase enzyme producer.[6] On the other hand, that *Enterobacter agglomerans* SB 5(1) contain proteins with high potential as anti-dengue activities.[7]

Over the last few years, researchers have revealed that marine algae symbiont bacteria comprise about 10,000 bioactive compounds, which have the potential to become a medicinal product with various characteristics and typical physiological features and utility. It is reported that marine algae known as *Sargassum vulgare* provide antiviral screening, as well as cytotoxicity testing results for human hepatoma cells (Huh 7.5). Their test results include an MNTD (Neutral Maximum Non-Dose-Based on MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) and Neutral Red Assays value of 62.5 µg/mL.[8]
The enzyme requires a cofactor, which is a nonprotein compound with inactive proteins (the apoenzyme) are combined to form a catalytically active complex. Cofactors can be inorganic molecules, such as Fe^{2+}, Mn^{2+}, Zn^{2+}, Na^{+}, Mg^{2+}, Cu^{2+}, Ca^{2+}, or also a complex organic molecule called a coenzyme such as thiamine pyrophosphate, flavin adenine dinucleotide (FAD), and coenzyme A. Some enzymes require coenzymes or one or more metal ions for their activity.[9]
The aim of this research was to determine the optimum concentration of L-substrate asparagine's, $FeCl_2$, the optimal temperature and pH to produce the enzyme L-asparaginase of bacteria *Klebsiella* sp. This study would address the question of how to increase the activity of the L-asparaginase enzyme, an enzyme produced by brown algae symbiont bacterium *Klebsiella* sp. using the $FeCl_2$ cofactor.
Currently, the research investigating groups of protein compounds or enzymes derived from algae-symbiont bacteria as natural resources for anti-dengue has not been done, although phospholipase A2 (PLA2) enzyme has been reported as a natural anti-dengue agent.[10] The L-asparaginase enzyme of *Klebsiella* sp., an algae-symbiont bacteria, was isolated using the fractionation method of ammonium sulphate followed by dialysis. Brine shrimp lethality test (BSLT) and MTT assay were conducted subsequently to analyse its toxicity and cytotoxicity, respectively. The results showed that extracellular L-asparaginase fraction from brown algae *Sargassum* sp. symbiont *Klebsiella* sp. contained L-asparaginase in the crude extracts with 9.92 µg/mL LC_{50} values (high toxicity). The Vero cell activity against the dengue virus showed an inhibition percentage and the CC_{50} value of 80% and 162.15 µg/mL, both of which were demonstrated to have anti-dengue virus potential.

EXPERIMENTAL

Materials

The materials employed were brown algae *Sargasssum* sp., distilled water, M9 medium [$MgSO_4.7H_2O$ (pa), KH_2PO_4 (pa), NaCl, $CaCl_2.2H_2O$, L-asparagine (pa), gel, glucose, the indicator phenol red], Egg of *Artemia salina*, Leach, BSA (Bovine Serum Albumin), 1% (w/v) L-glutamine, DENV-2 virus, Vero cells, Nutrient Broth media (NB), DMEM media, Nutrient Agar (NA), normal saline, alcohol 70%, $FeCl_2$, Lowry A (phophotungstat phosphomolybdat acid solution with dH_2O 1: 1), Lowry B (sodium potassium tartrate 2%; Na_2CO_3 2%; $CuSO_4.5H_2O$ 1%; 0.1 N NaOH), buffer A (0.1 M Tris(hydroximethyl) amino methane pH 8.3; β-mercaptoethanol 1%; Triton X-100 0.5%; 0.01 M $CaCl_2$; 2 M NaCl), buffer B (0.1 M Tris (hydroximethyl)amino methane pH 8.3; 0.01 M $CaCl_2$; 0,2 M NaCl;), buffer C (0.01 M Tris (hydroximethyl) amino methane pH 8.3; 0.01 M $CaCl_2$; 0.2 M NaCl), ammonium chloride, ammonium sulphate, 1 M HCl, 0.1 M borate buffer pH 7.0, Tris-HCl buffer pH 8.6, Nessler reagent, trichloroacetic acid, aluminium foil, denatured alcohol, filter paper, tissue roll, and cotton.

Instruments

Instruments used were test tubes, Petri dishes, a loopful, Bunsen, test tube rack, incubators, ovens, the UV light, water bath shaking, centrifugation, rod stirrer, pH meter, Erlenmeyer, hot plate, magnetic stirrer, refrigerator, tweezers, spectrophotometry UV-VIS $20D^{+}$, micropipette, microscopes, microscope slides, cover glass, analytical scales, Eppendorf tubes, gloves, masks, as well as laboratory glassware is regularly in use.

Rejuvenation of Bacteria

The bacterium stock collection of the Biochemistry Laboratory at Hasanuddin University in Makassar, Indonesia was used to rejuvenate *Klebsiella* sp. bacteria that colonize brown algae *Sargassum* sp. The medium components such as L-asparagine, KH_2PO_4, NaCl, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, glucose, agar, and phenol red indicator were mixed, and the resulting mixture was incubated for a total of 24 hours at 37 °C. Selective medium M-9, which had been modified, was used to produce L-asparaginase.[11,12]

Optimal Incubation Production and Determination of L-Asparagine Substrate Concentration Enzymes of L-Asparaginase

The procedure on determining the optimal concentration of the L-Asparagine substrate began with the creation of inoculum, which was then followed by a fermentation process (production of enzymes). The inoculum was cultured for 24 hours at 37 °C and 200 rpm before being inoculated into 100 mL of production medium containing various L-asparagine substrate concentrations of 6, 8, 10, 12, and 14 g/L. Every 12 hours to 72 hours, samples were taken. The OD (Optical Density) of the microorganisms was measured using UV-VIS spectrophotometry at a wavelength of 646 nm, and the maximum of L-Asparaginase activity was determined for each modification of the L-Asparagine substrate concentration.

Determination of Concentration of $FeCl_2$ and Optimal Production Incubation Time of L-Asparaginase Enzyme

The procedure of determining the ideal concentration of $FeCl_2$ began with the generation of inoculum, followed by a fermentation process (enzyme synthesis) utilizing an optimal concentration of L-Asparagine substrate. After 24 hours of incubation at 37 °C and 200 rpm, 10% of the inoculum was transferred to 100 mL of production medium with varying $FeCl_2$ concentrations (0.25, 0.5, 0.75, 1.00, and 1.25 g/L). The growth of microorganisms, or OD (Optical Density), was monitored using UV-VIS spectrophotometry at a wavelength of 646 nm, and a maximum of L-Asparaginase activity was determined for each modification of the L-Asparagine substrate concentration.

Extracellular L-asparaginase Production on Optimal Conditions

L-Asparaginase was produced following the optimization of its L-asparagine substrate, fermentation period, and Fe^{2+} metal ion concentrations. The enzyme activity was at its peak in the 1000 mL batch, and hence the enzyme synthesis process was carried out in a huge volume. The composition of the production medium was 6.2 grams of $Na_2HPO_4.2H_2O$, 3 grams of KH_2PO_4, 0.5 grams of NaCl, 0.3 grams of L-asparagine, and 2 grams of $MgSO_4.7H_2O$. Into this mixture, 0.1 grams of $CaCl_2$ was added, followed by the addition of 10 mL of 20 percent glucose solution (pH 7.0). Furthermore, the enzyme was centrifuged at 3,000 rpm for 10 minutes at 4 °C, to separate the filtrate from the cells. Prior to purification, the crude extracts were obtained by ammonium sulphate precipitation. Lowry method was used to analyse protein content. UV-VIS spectrophotometry was used to measure the activity of L-asparaginase by Nessler methods.

L-asparaginase Enzyme Activity Measurement

The Nessler method was used to analyse the L-asparaginase activity at 37°C, with a UV-visible spectrophotometer. This procedure employed Nessler reagent and L-asparaginase to discover the ammonia created from L-asparaginase catalysis. In this case, the reaction mixture contained 0.1 mL of the enzyme, 0.2 mL of buffer with a pH of 8, and 1.7 mL of L-asparagine at a concentration of 0.01 M. The reaction mixture was incubated at 50 °C for 10 minutes. When 0.5 mL of trichloroacetic acid 1.5 M was added, the process was halted. The resulting solution was diluted with 8.5 mL of sterile water, and then a 1 mL portion of Nessler reagent was added. Hydrolysis of the L-Asparaginase enzyme results in the production of ammonia that reacts with the Nessler reagent. The compound was then tested using a standard solution of ammonium chloride. The enzyme, L-asparaginase, catalyses the creation of one micromole of ammonia per minute under the stated circumstances.

Protein Content Measurement

Using bovine serum albumin as a standard, the Lowry method was used to identify protein levels.

Response of L-Asparaginase Enzyme Activity on pH

The activity of L-asparaginase was assessed under varied pH. The enzyme was incubated in the buffer that contained 0.05 M of pH 6-10. The total amount of ammonia released was calculated in each of the three conditions. During incubation, the solution was centrifuged, and the resultant filtrate was assayed.[13]

Response of L-Asparaginase Enzyme Activity on Temperature

Optimal pH, at different temperature regimes, was used to determine the activity of L-asparaginase. The enzyme was stored at the following temperatures: 30, 35, 37, and 45 degrees Celsius. After centrifuging, incubation was performed for 10 minutes, and the filtrate enzyme activity was then measured.[13]

Effect of Addition of $FeCl_2$ on the L-Asparaginase Enzyme Activity

Buffer solution at the optimal pH was added to 0.1 mL of the enzyme (sample). Furthermore, the various concentrations of $FeCl_2$ metal ions (0.075 mM, 0.100 mM, 0.250 mM, 0.500 mM, and 0.750 mM) were added. Finally, 1.7 mL of L-asparagine was added to a final concentration of 0.01 M. In addition, the solution was incubated for 10 minutes at the ideal temperature and then centrifuged. Afterward, the enzyme activity in the solution was determined by the spectrophotometry method. [6,14]

Anti-Dengue Test

As in prior research, toxicity experiments were performed utilizing the BSLT assay.[12] To determine the cytotoxic effects of the *Klebsiella sp*. L-asparaginase enzyme on Vero cells, DENV-2-infected Vero cells were used. Following prescribed the test protocol, the cytotoxic tests were performed.[7] Viability percentage (%) and CC_{50} value (µg/mL) were calculated using the aforementioned probit technique to determine the inhibition percentage.[15]

RESULTS AND DISCUSSION

Substrate Concentration of L-Asparagine and Optimal Incubation Time

During the fermentation process of L-asparagine substrate content, the highest optical density of *Klebsiella* sp. cultures (OD_{646} nm = 1.47) was reported at a fermentation time of 24 hours, as shown in Fig.-1A. The L-Asparaginase activity's peak was 60.11827 U/mL, produced in L-Asparagine substrate concentration of 10 g/L after 48 hours of fermentation time, as shown in Fig.-1B.

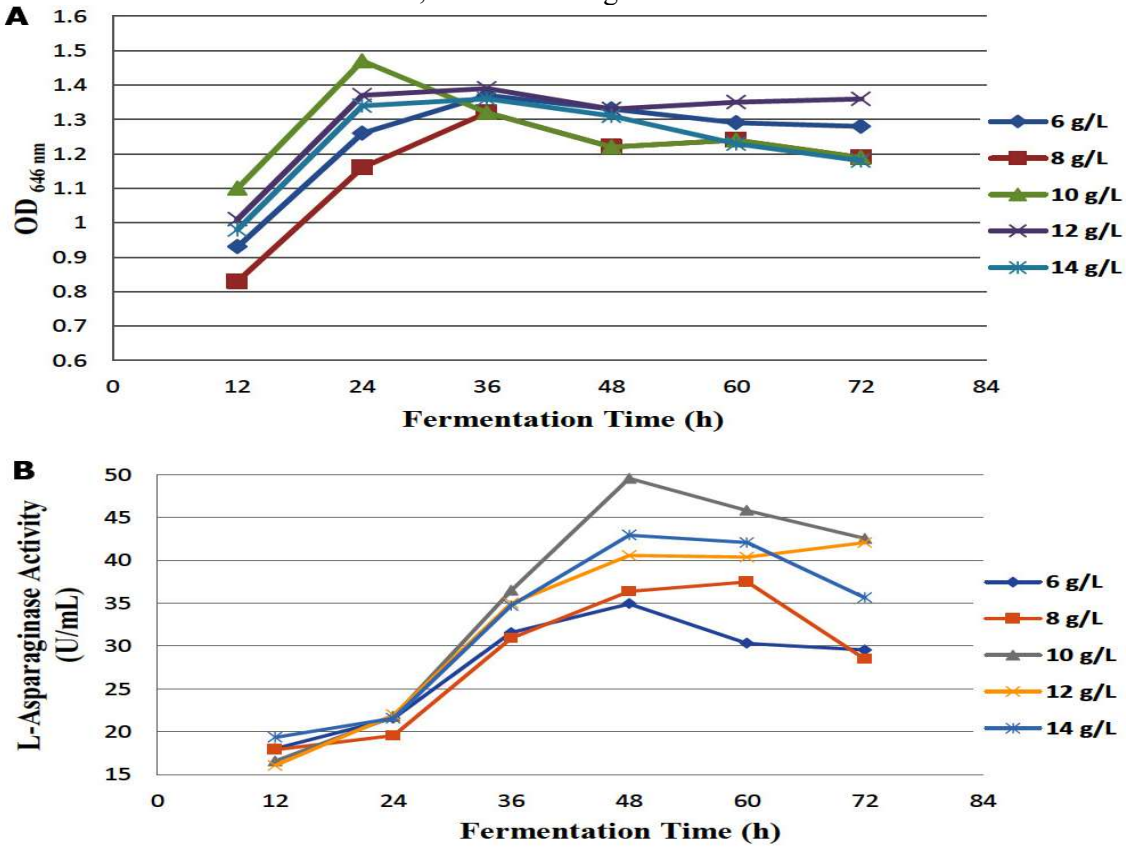

Fig.-1: Effect of L-asparagine Substrate Concentration and Fermentation Time on (A) Optical Density of *Klebsiella* sp Cultures and (B) L-Asparaginase Activity produced during Fermentation Medium Cultivation

$FeCl_2$ Concentration and Optimal Incubation Time of L-Asparaginase Enzyme Production

The highest Optical Density of the cultures OD_{646nm} was 0.79. In the L-asparaginase fermentation medium, the highest enzyme activity was obtained at $FeCl_2$ concentration of 0.5 g/L which was incubated for 60 hours, as shown in Fig.-2.

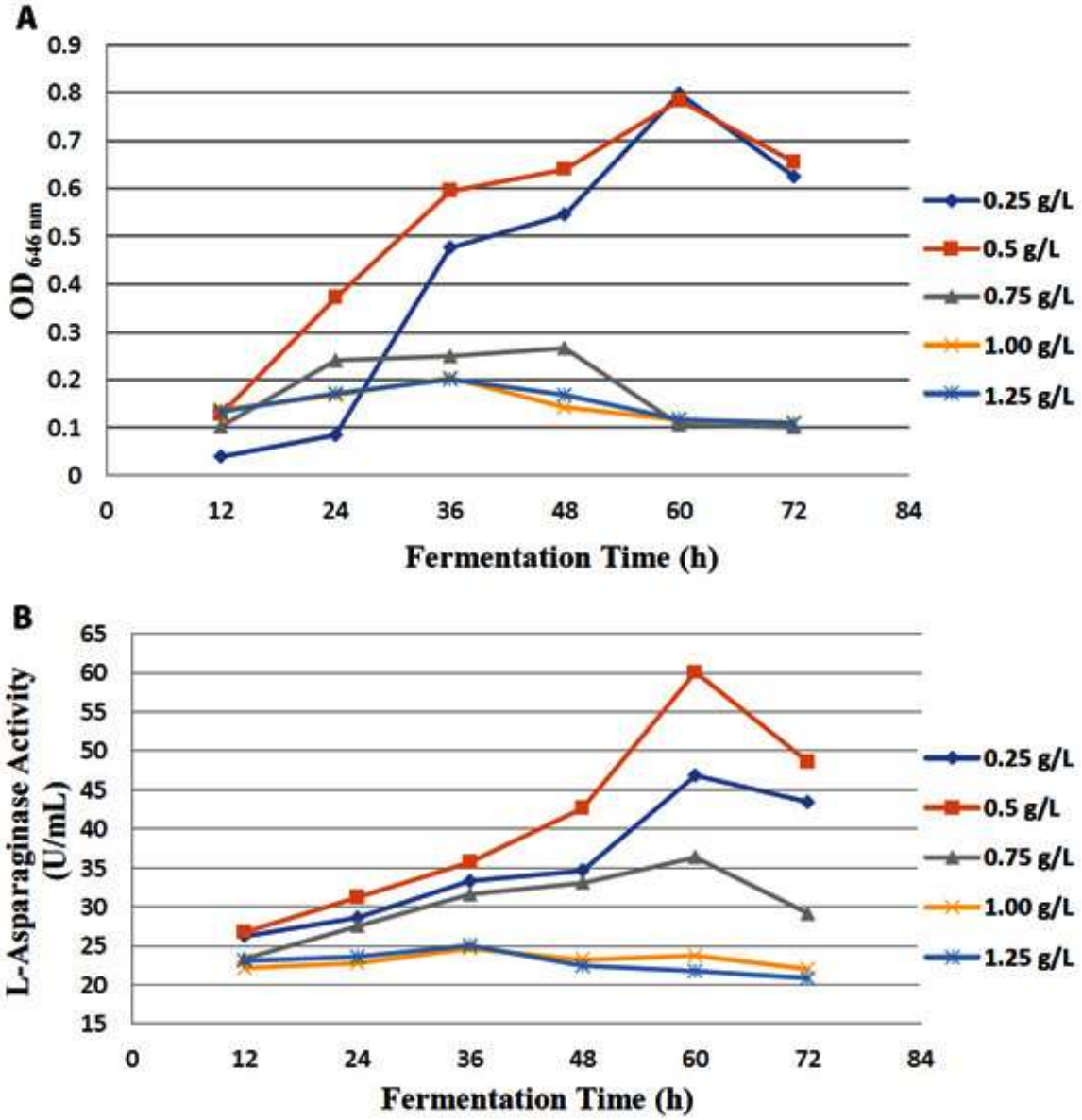

Fig.-2: Optical Density (A) and L-Asparaginase Enzyme Activity Pattern (B) during the Cultivation of *Klebsiella* sp bacteria grown in the Fermentation Medium both depend on the Concentration of $FeCl_2$ and Fermentation Time.

Protein Content of L-Asparaginase Fractions

Fermentation for 60 hours in each fraction (F0, F1, F2, F3, and F4) resulted in 8.698 mg/mL; 4.188 mg/mL; 1.058 mg/mL; 2.549 mg/mL; and 0.778 mg/mL for crude extracts (F0), 0-20% (F1), 20-40% (F2), 40-60% (F3), and 60-80% (F4), respectively (Table-1).

The Acidity (pH) effect on L-Asparaginase Activity

As can be seen in Fig.-3, the pH increases when the L-Asparaginase activity increases up to its optimum level at pH 8. L-Asparaginase activity at pH 6 was 44.2181 U/mL, at pH 7 and 8, its activity was 59.0607 U/mL, and at pH 9 and 10, it was 45.1618 U/mL.

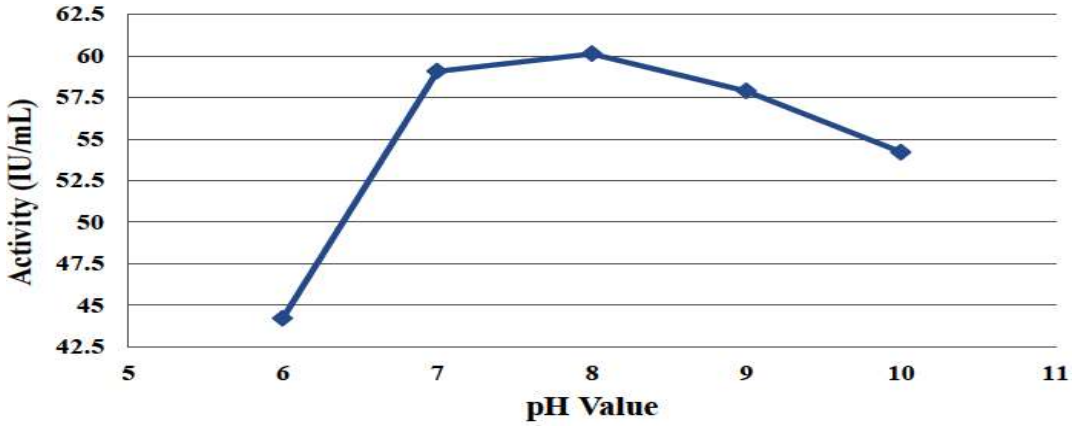

Fig.-3: Effect of pH on Enzyme Activity of L-Asparaginase

Effect of Temperature on Activity of L-Asparaginase Enzyme

Fig.-4 depicts the L-Asparaginase activity increased exponentially from 30°C, reached its maximum at 37°C, and decreased at a constant temperature of 40°C, and 45°C. At 37°C, L-Asparaginase activity was 61.1182 U/mL.

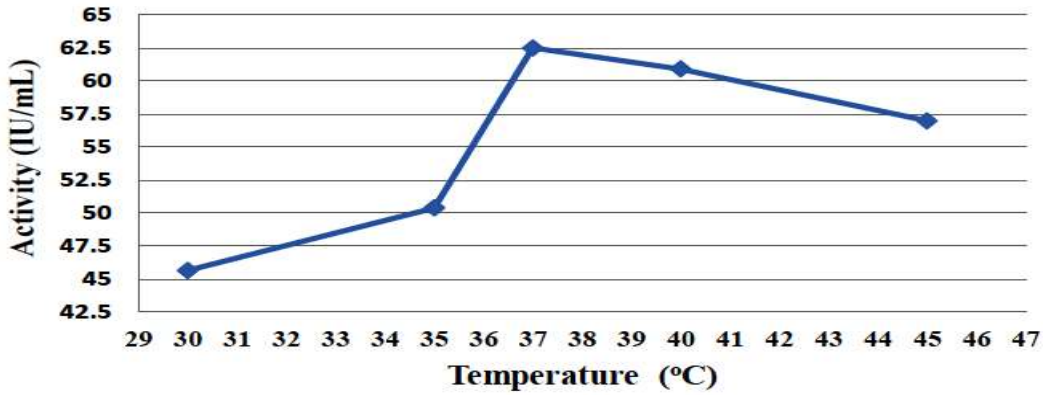

Fig.-4: Effect of Temperature on the Enzyme Activity of L-Asparaginase

Effect of $FeCl_2$ Cofactors Addition on the L-Asparaginase Enzyme Activity

The L-asparaginase enzyme activity with the $FeCl_2$ cofactor increased at concentration of 0.75 mM, however, it was decreased as the concentration decreased to 0.5 mM. The L-asparaginase activity (in U/mL) can be found as follows: 91.2615, 97.8318, 104.4021, 106.5046, and 87.3193 at 0.075, 0.100, 0.250, 0.500, and 0.750 mM $FeCl_2$ concentrations, respectively (Fig.-5).

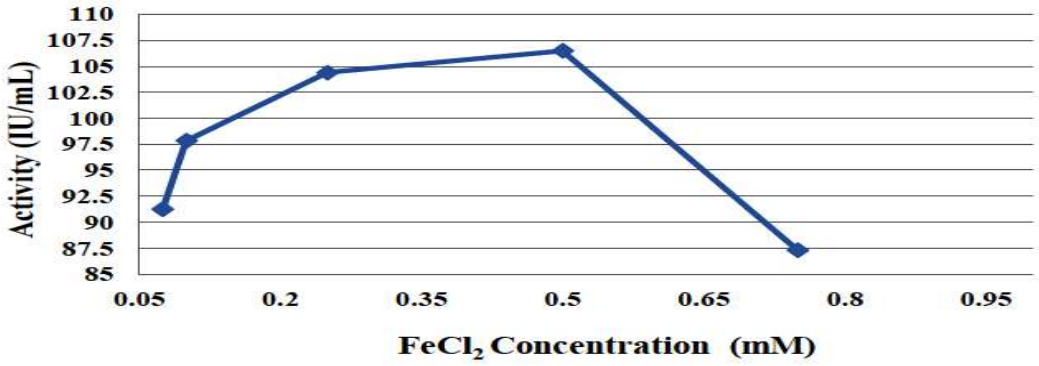

Fig.-5: Effect of Cofactors $FeCl_2$ on Activity of L- Asparaginase

Anti-dengue Potential Activity Test

The toxicity test was conducted on the L-asparaginase enzyme derived from *Klebsiella* sp. The results shown in Table-1 indicate that the extract and L-asparaginase components have different values. F0 (crude

extracts) and F2 (20-40% fraction) are extremely hazardous, although F1 (0-20% fraction) is slightly harmful. While the F3 (40-60% fractions) and F4 (60-80% fractions) have no hazardous values, F1 (0 - 20% fraction) and F2 (40 - 60% fraction) have hazardous value. For example, the L-asparaginase fraction with the saturation of 0 - 20% (F1) had a toxic response to *Artemia salina* Leach, which is a type of shrimp larva. The LC_{50} has a maximum of 24.14 µg/mL. However, intriguingly, the toxicity of L-asparaginase fraction in crude extracts and the L-asparaginase saturation value of 20-40% (F2) to *Artemia salina* Leach both produced a toxic response for shrimp larvae. The LC_{50} value for the toxicity of L-asparaginase in crude extracts (F0) was 9.92 µg/mL, while the L-asparaginase saturation value of 20-40% fraction (F2) was 11.54 µg/mL. The next part is further proof that L-asparaginase high toxicity fractions are anti-dengue compounds because Vero cells were infected with the DENV-2 virus. The crude extracts from L-asparaginase inhibited the dengue virus in Vero cells by 80% and 167.15 µg/mL. In 20-40% fraction (F2), it had a percentage of inhibition and CC_{50} value of 75% and 250.12 µg/mL (Table-1).

Table-1: Summary of protein concentration and anti-dengue potential after the administration of 10 µg/mL L-Asparaginase fractions from extracellular from *Klebsiella* sp. bacterial symbiont of algae *Sargassum* sp.

Samples No.	L-Asparaginase Fractions	Protein Concentration (mg/mL)	LC_{50} (µg/mL)	Cell Viability (%)	Inhibition (%)	CC_{50} (µg/mL) Vero cells
F0	Crude Extracts	8.698	9.92	60	80	167.15
F1	0-20%	4.188	24.14	ND	ND	ND
F2	20-40%	1.058	11.54	52	75	250.12
F3	40-60%	2.549	7898.22	ND	ND	ND
F4	60-80%	0.778	167.155	ND	ND	ND

Note: ND (Not Detected)

When the L-Asparagine substrate is a component of the growth media at a given concentration and incubation time, bacterial enzyme production is at its highest. The research team aimed to determine the optimum L-asparagine concentration for the production of L-asparaginase production media, a procedure that involved inoculating a batch of bacteria *Klebsiella* sp. that had been growing for a full day in an L-asparaginase liquid inoculum medium with 6, 8, 10, 12, and 14 g/L of L-asparagine. The inoculum media was incubated in a shaker that ran at 200 rpm for a total of 24 hours at 37°C. An additional portion of incubated inoculum media was mixed with L-asparaginase production media, resulting in a ratio of 1:10. Determining the ideal incubation time can be carried out by measuring the activity of L-Asparaginase's every 12 hours over the course of 72 hours. The results showed that the highest activity was detected in L-Asparagine concentration of 10 g/L in a 48-hour incubation period.

As to assess the percentage of an increase in the synthesis of L-asparaginase, after an initial $FeCl_2$ concentration experiment, L-asparaginase was also tested in the presence of varying concentrations of $FeCl_2$. Although bacteria and products can be accurately monitored during fermentation in this medium, the medium was not capable of rapid growth. L-Asparaginase was produced during the fermentation process every 12 hours for three days. Afterward, sample collection was carried out to determine the ideal production period. *Klebsiella* sp. bacteria cultivated in a medium containing various doses of $FeCl_2$, were also tested for viability at concentrations of 0.25; 0.5; 0.75; 1.00, and 1.25 g/L. After incubated for two hours, the results showed that the L-Asparaginase had the maximum activity in the fermentation medium with 49.5729 U/mL of L-Asparagine solution concentration.

The Nessler method was performed to assess the activity of the crude extracts of L-asparaginase. Ammonia was used as the reference solution to measure the L-asparaginase activity test at the maximal wavelength of 450 nm. To discover how each adjustment of the substrate and the $FeCl_2$ concentration affects the reaction, each experiment was run. The result showed that L-Asparagine concentrations of 10 g/L with the $FeCl_2$ concentration of 0.5 g/L, the L-Asparaginase activity was 60.11827 U/mL.

Utilizing Bovine Serum Albumin (BSA) as a standard, the Lowry method can be used to measure the protein level of the sample. A 640-nm wavelength was used to produce a maximum standard curve, in which a standard concentration was varied to determine the variations. To compute the protein content of L-

asparaginase, the standard curve made a straight-line equation. The protein levels at the optimum substrate concentration of 10 g/L and optimum $FeCl_2$ of 0.5 g/L with variations in incubation time of 12, 24, 36, 48, 60, and 72 hours respectively were; 0.0803 mg/mL, 0.0743 mg/mL, 0.1878 mg/mL, 0.1062 mg/mL, and 0.0801 mg/mL and 0.1482 mg/mL.

The characterization of L-asparaginase was determined by analyzing the enzyme activity of the crude extracts in various ranges of pH, temperature, and the effect of $FeCl_2$ as a metal cofactor. The characterized enzyme was the L-asparaginase enzyme, which has the highest activity at the optimal substrate, the effect of the addition of optimal $FeCl_2$ on enzyme production, and the optimal fermentation time. Based on the previous research convention results in [16,17], a natural compound has criteria and the potential as anti-dengue if the compound has cell viability of >50%, the percent inhibition of >90%, and CC_{50} of <250 g/mL. Based on these criteria, it can be concluded that the F0 fraction (crude extracts) and the F2 fraction (20-40%) of the L-Asparaginase enzyme from the extracellular exophytic bacteria *Klebsiella* sp., has the potential as an antiviral agent for dengue. According to the literature search, this is the first reported study about the natural products from this type of enzyme that have the potential as a new agent against the dengue virus, although there has been previously reported that the PLA2 enzyme also has potential as an antiviral agent for dengue [18,19]. To increase the anti-dengue activity, it is necessary to further explore other types of proteins from the symbiotic bacteria *Sargassum*, sp., or carry out a partial hydrolysis process of proteins to obtain peptides with smaller molecular sizes, thus it can be easier to penetrate the dengue virus that causes dengue hemorrhagic fever (DHF).

CONCLUSION

During a period of 48 hours, maximum extracellular concentrations of L-Asparagine can be produced when *Sargassum* sp. Klebsiella bacterial symbiont was present. The *Sargassum* sp. Klebsiella bacterial symbiont must be present in order to achieve maximum extracellular concentrations of L-Asparagine at the optimal time of 60 hours. The best activity for L-asparaginase was occurred at pH 8 at 37 °C, with the influence of the cofactors molecule at a concentration of 0.5 mM $FeCl_2$, at 106.5046 U/mL. Inhibition percentage and CC_{50} value of 80% and 167.15 µg/mL (DENV-2) respectively for the crude extracts of L-asparaginase, which was tested on Vero cells.

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