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Production, Characterization, and Toxicity Test of L-asparaginase from *Vibrio alginolyticus* Bacterial Symbiont of Green Algae *Caulerpa lentillifera*

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ABSTRACT. L-asparaginase is an enzyme that can be used as an anticancer therapeutic agent. This study aimed to optimize fermentation time for production, optimization, and to test the toxicity of L-asparaginase from *Vibrio alginolyticus* (*V. alginolyticus*) bacterial symbiont of green algae *Caulerpa lentillifera* (*C. lentillifera*). The activity of L-asparaginase enzyme assay and the toxicity test were done by using the Nessler method and the Brine Shrimp Lethality Test (BSLT) method respectively. The results show L-asparaginase with 66 hours of fermentation time indicates the highest enzyme activity. L-asparaginase mentioned has an optimum enzyme activity of 17.99 U/mL for 30 minutes of incubation time at 37 °C, and pH 7.5. The BSLT results show LC_{50} value of 17.83 µg/mL indicating the enzyme is bio-toxic with high-level toxicity, which can continue for cytotoxicity tests on cancer cells.

Keywords: Enzyme, L-asparaginase, L-asparagine, toxicity

INTRODUCTION

L-asparaginase (E.C.3.5.1.1) is an enzyme of the hydrolase group that catalyzed the hydrolysis reaction of L-asparagine to ammonia and L-aspartic acid (Lopes et al., 2017; Nadeem et al., 2022; van Trimpont et al., 2022). L-asparagine is an important nutrition of cancer cells (Jiang et al., 2021). Hydrolyzed L-asparagine caused protein synthesis of the cancer cell to be disrupted and eventually, cancer cell death (Shrivastava et al., 2016).

L-asparaginase enzyme can be found in animals, microorganisms, and plants (Cachumba et al., 2016; Doriya & Kumar, 2016). Isolation of L-asparaginase from animals and plants encountered several obstacles, including the need for animal and plant samples in great quantity for enzyme production. Therefore, the production of the L-asparaginase enzyme from microorganisms is preferred because of its ease and effectiveness (Raveendran et al., 2018). Some L-asparaginase-producing microorganisms were *Bacillus licheniformi* (Alrumman et al., 2019), *Serratia marcescens* (Ghosh et al., 2013), *Pyrococcus abyssi* (Nadeem et al., 2022), and many others.

Bacterial symbionts of algae can also produce L-asparaginase (Karim et al., 2020). It can happen because bacterial symbiont has almost the similar capability as their hosts to produce bioactive compounds. Interaction between the secondary metabolite by marine biota (algae) and the symbiotic bacteria causes the induced bacteria to produce specific bioactive metabolites (Dharmayanti et al., 2021; Menaa et al., 2020). *Caulerpa lentillifera* is one of the green algae commonly called "sea grapes" (Sopon et al., 2020), with a potential chemo-preventive source. *Caulerpa* species contains metabolites, flavonoids, and phenolics, so it possesses antioxidant and anti-proliferative activities (Tanawoot et al., 2021). Khairunnur et al. (2022) found a bacterial symbiont from the green algae *C. lentillifera* that yields a bioactive protein with a high toxicity value.

In various studies on L-asparaginase production, optimization of fermentation time was used to obtain high enzyme activity. Characterization and the toxicity test of L-asparaginase were also performed to identify the characteristics and anticancer potential of the enzyme that has been produced (Yusriadi et al., 2019). Based on the explanations above, this research aims to explore L-asparaginase from *V. alginolyticus,* which is a symbiosis of the green algae *C. lentillifera*.

EXPERIMENTAL SECTION

Materials

The materials used were *V. alginolyticus* bacterial symbiont from green algae *C. lentillifera*, which is the

bacterial collection of Biochemistry Laboratory, Natural Science Faculty, Hasanuddin University, Makassar, Indonesia, distilled water, sterile seawater, nutrient agar (Merck), nutrient broth (Merck), KH_2PO_4 (Merck), L-asparagine (Merck), phenol indicator (Merck), egg *Artemia salina* Leach, buffer Tris-HCl, Lowry reagent, Bovine Serum Albumin (BSA) (Merck), 70 % alcohol (One Med), Nessler reagent, ammonium chloride (Merck), and trichloroacetate acid (TCA) (Merck).

Instruments

The instruments used in this study were UV-Visible spectrophotometer T60 (PG Instruments), Incubator (Memmert), Autoclave (Pressure Sterilizer 1925X Wisconsin Aluminium), Spectronic $20D^+$ (Thermo Scientific), Centrifuge (Hermle Z 366 K), Analytical Balance (OHAUS).

Bacterial Rejuvenation

Rejuvenation of *V. alginolyticus* bacterial symbiont of green algae *C. lentillifera* was done by growing the bacteria on an L-asparagine agar medium (Alrumman et al., 2019), that has been modified. The medium components were L-asparagine 10 g/L, K_2HPO_4 3.3 g/L, Nutrient agar 16 g/L, and red phenol indicator 0.6 L. The growing colonies of bacteria were incubated in an incubator for 24 hours.

Determination of Optimum Fermentation Time for L-Asparaginase Production

The pure isolate of *V. alginolyticus* bacterial symbiont from *C. lentillifera* was inoculated into 100 mL inoculum medium (L-asparagine substrate 1.5 g, K_2HPO_4 0.33 g, and Nutrient Broth 0.8 g) in 250 mL Erlenmeyer flask. The inoculum medium of 100 mL was incubated in a shaker water bath at 37 °C, 180 rpm, and for 24 hours. After that, the active inoculum medium of 10 mL was inoculated into 90 mL of production medium. The mixture was then shaken in a shaker water bath at 37 °C, 180 rpm, for up to 78 hours. Sampling was held every 6 hours to measure bacterial cell growth, shown by its optical density (OD) by using a UV-VIS spectrometer at λ 650 nm. Then, L-asparaginase crude enzyme and bacterial cell deposits were separated by using centrifuge Hermle Z 366 K at 4 °C, 5000 rpm, and for 30 minutes. The L-asparaginase crude enzyme was assayed for its enzyme activity using the Nessler Method (Meghavarnam & Janakiraman, 2015).

Production of L-Asparaginase from *V. Alginolyticus*

The pure isolate of *V. alginolyticus* bacterial symbiont from *C. lentillifera* was inoculated into 100 mL inoculum medium (L-asparagine substrate 1.5 g, K_2HPO_4 0.33 g, and Nutrient Broth 0.8 g) in 250 mL Erlenmeyer flask. The inoculum medium of 100 mL was incubated in a shaker water bath at 37 °C, 180 rpm, and for 24 hours. After that, the active inoculum medium of 10 mL was inoculated into 90 mL of production medium. The mixture was then shaken in a shaker water bath at 37 °C, 180 rpm, and for 66 hours (optimum fermentation time that was obtained). The L-asparaginase crude enzyme and bacterial cell deposits obtained from the fermentation were then separated by using centrifuge Hermle Z 366 K at 4 °C, 5000 rpm, and for 30 minutes. The L-asparaginase crude enzyme was then characterized and tested for toxicity test.

Assay of L-Asparaginase Activity

Creating a standard ammonium chloride curve

The standard curve equation of ammonium chloride solution is required to determine L-asparaginase activity (Karim et al., 2020). 0.5 mL of the standard solution of ammonium chloride was pipetted with each concentration of 0.1; 0.2; 0.4; 0.6; 0.8; 1 mg/mL, and then 0.5 mL L-asparagine 0.04 M, 0.5 mL Tris-HCl buffer 0.05 M pH 8, and trichloroacetate acid 1.5 M of 0.5 mL was added, and then centrifuged for 5 minutes at 10.000 rpm. 0.1 mL of the filtrate was diluted with 3.75 mL distilled water and 0.2 mL Nessler reagent. The absorption was measured at λ 385 nm.

Sample analysis

The enzyme activity of L-asparaginase was determined by measuring the ammonia from L-asparaginase catalysis using the Nessler reagent. The solution containing of 0.5 mL crude L-asparaginase enzyme, 0.5 mL of L-asparagine 0.04 M substrates, 0.5 mL pH 8 of Tris-HCl buffer 0.05 M was incubated for 30 minutes at 37 °C. The reaction was stopped using 0.5 mL trichloroacetate acid 1.5 M. After centrifuged for 5 minutes at 10.000 rpm, 0.1 mL of the filtrate was diluted with 3.75 mL distilled water, and 0.2 mL Nessler reagent was added (Meghavarnam & Janakiraman, 2015). The absorption was measured at λ 385 nm. One unit of L-asparaginase enzyme activity is defined as the amount of L-asparaginase to hydrolysis asparagine to μmol ammonia per minute at the assay conditions (Qeshmi et al., 2018). The L-asparaginase activity can be obtained using Equation 1 (Karim et al., 2020):

$$\text{EA (U/mL)} = \frac{\text{y-b}}{\text{a}} \times \frac{\text{V.total}}{\text{V. analysis}} \times \frac{1}{\text{V.enzyme} \times \text{t. incubation}} \quad (1)$$

Where:
EA= Enzyme Activity
y = absorbance
a = slope
b = intercept
V.tot =buffer+substrate+enzyme+TCAvolume (2 mL)
V. analysis= volume analyzed (0.1 mL)
V. enzyme= volume of enzyme analyzed (0.5 mL)
t. incubation= time of incubation (30 minutes)
The specific activity of L-asparaginase can be obtained using equation 2 (Yusriadi et al. 2020):

$$\text{EA specific (U/mg)} = \frac{\text{EA (U/mL)}}{\text{Protein level (mg/mL)}} \quad (2)$$

Determination of Protein Content

The protein levels were assayed by using the Lowry method and Bovine Serum Albumin (BSA) as a standard solution (Ahmad et al., 2013). In order to

determine the protein content of the enzyme, about 2 mL L-asparaginase crude enzyme and 2.75 mL of Lowry B reagent were pipetted and mixed then hushed for 10-15 minutes at room temperature. The mixture was added with 0.25 mL Lowry A reagent, then shaken rapidly. After being left for 30 minutes, the absorption was measured at λ 650 nm. The protein content can be obtained by using equation 3 (Khairunnur et al., 2022) :

$$\text{Protein content (mg/mL)} = \frac{\text{Absorbance} + \text{Slope}}{\text{Intercept of standard curve}} \quad (3)$$

Characterization of L-Asparaginase from *V. alginolyticus*

pH effect on L-asparaginase enzyme activity

The solution to determine pH effect on L-asparaginase enzyme activity contains 0.5 mL of L-asparaginase crude enzyme, 0.5 mL of L-asparagine 0.04 M substrates, 0.5 mL of pH 6.0-7.0 of potassium phosphate buffer and pH 7.5-9.0 Tris-HCl buffer. The solution was then incubated at 37 °C for 30 minutes. The reaction was stopped using 0.5 mL trichloroacetate acid 1.5 M. After centrifuged for 5 minutes at 10.000 rpm, 0.1 mL of the filtrate was diluted with 3.75 mL distilled water and added with 0.2 mL Nessler reagent. The absorption was measured at λ 385 nm.

Incubation time effect on L-asparaginase enzyme activity

The solution to determine pH effect on L-asparaginase enzyme activity contains 0.5 mL of L-asparaginase crude enzyme, 0.5 mL of L-asparagine 0.04 M substrates, 0.5 mL pH 7.5 Tris-HCl buffer (buffer at optimum pH that was obtained). The solution was then incubated at 37 °C for 0; 30; 60; 90; and 120 minutes. The reaction was stopped using 0.5 mL trichloroacetate acid 1.5 M. After centrifuged for 5 minutes at 10.000 rpm, 0.1 mL of the filtrate was diluted with 3.75 mL distilled water, and added with 0.2 mL Nessler reagent. The absorption was measured at the λ 385 nm.

Temperature effect on L-asparaginase enzyme activity

The solution to determine pH effect on L-asparaginase enzyme activity contains 0.5 mL of L-asparaginase crude enzyme, 0.5 mL of L-asparagine 0.04 M substrates, 0.5 mL pH 7.5 Tris-HCl buffer. The solution was then incubated at 27; 32; 37; 42; and 47 °C for 30 minutes (optimum incubation time that was obtained). The reaction was stopped using 0.5 mL trichloroacetate acid 1.5 M. After centrifuged for 5 minutes at 10.000 rpm, 0.1 mL of the filtrate was diluted with 3.75 mL distilled water, and added with 0.2 mL Nessler reagent. The absorption was measured at the λ 385 nm.

Test The Potential for Anticancer of L-Asparaginase with The BSLT Method

10 larvae of *A. salina* Leach were inserted into each tube containing seawater and L-asparaginase crude enzyme with various concentrations of 1, 10, and 100 µg/mL. Seawater without added enzyme was used as a control. This test was left under the lamps for 24 hours. After that, dead larvae were observed to determine LC_{50} values (Khairunnur et al., 2022; Yusriadi et al., 2019).

RESULTS AND DISCUSSION

Determination of Optimum Fermentation Time for L-Asparaginase Production

Optimization of fermentation time of L-asparaginase production was carried out through the determination of bacterial cell growth by assaying the optical density (OD) and enzyme activity. The stages of cell culture growth include adaptation, exponential, stationary, and death stage (Khairunnur et al., 2022; Murtius et al., 2022). The optical density (OD) value was determined every 6 hours at λ 650 nm. The enzyme activity value was determined using the Nessler method and calculated using the formula in Equation 1. The relationship between fermentation time, OD, and enzyme activity of L-asparaginase production was shown in (**Figure 1).** The results showed that bacterial cell growth has maximum growth at 66 hours which an OD value is 1,66 and L-asparaginase produced with the highest enzyme activity of 11 U/mL, which means the optimum fermentation time for L-asparaginase enzyme production from *V. alginolyticus* was at 66 hours.

Production of L-Asparaginase from *V. alginolyticus*

L-asparaginase production was carried out after obtaining data on optimum fermentation time. Based on these data (**Figure 1**), the L-asparaginase which has the highest enzyme activity was produced after 66 hours. After the production, the medium was fermented for 66 hours and then centrifuged at 4 °C, 5000 rpm, and for 30 minutes to separate filtrate and bacterial cell deposits. The filtrate obtained was a crude enzyme.

L-asparaginase activity was determined by using the Nessler method with ammonium chloride as a standard solution (Karim et al., 2020). One unit of L-asparaginase activity is defined as the amount of L-asparaginase that can break down L-asparagine to µmol ammonia per minute at the assay conditions (Qeshmi et al., 2018). Ammonia is produced from the hydrolysis of L-asparagine substrates by the catalytic L-asparaginase enzyme. Ammonia reacts with the Nessler reagent to form a yellow-colored compound (Utomo et al., 2023). The absorbance was determined by using UV-Visible spectrophotometer T60 at the maximum wavelength that was obtained (λ 385 nm) and using Equation 1 to obtain enzyme activity value.

Protein contents were determined by using the Lowry method (Ahmad et al., 2013). The protein reacts with copper in alkaline conditions, then reduces the Folin reagent to form a blue color compound, indicating amino acids, which are tyrosine and tryptophan (Vallabh et al., 2019). The absorbance was measured by using Spectronic $20D^+$ at the maximum

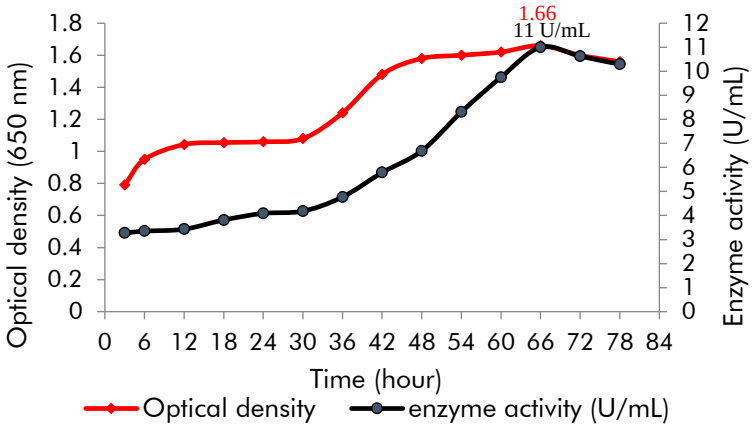

Figure 1. Effect of fermentation time on OD and enzyme activity of L-asparaginase enzyme produced from *V. alginolyticus* bacterial symbiont from *C. lentillifera*

Table 1. Results of crude enzyme of L-asparaginase from *V. alginolyticus*

Sample	Enzyme activity (U/mL)	Protein content (mg/mL)	Specific activity (U/mg)
Crude enzyme	17.99	4.26	4.22

wavelength (650 nm). The protein contents can obtain by substituting the absorbance into equation 3, therefore specific activity can be determined by comparing enzyme activity and protein content.

The result in **Table 1**. showed the crude enzyme of L-asparaginase extract from *V. alginolyticus* has an enzyme activity value of 17.99 U/mL. This signifies that *V. alginolyticus* as a bacterial symbiont of *C. lentillifera* can be a new source of L-asparaginase enzyme. The comparison between this research with previous research is that the enzyme activity showed in this research is lower than *Enterobacter agglomerans* SB 221 (Karim et al., 2020), and *Escherichia coli* MF-107 (Shahnazari et al., 2022). It shows higher activity than *P. carotovorum* and *S. marcescens* (Darwesh et al., 2018), and *Sterptobacillus* sp. KK2S4 (Makky et al., 2014).

Characterization of L-Asparaginase from *V. alginolyticus*

pH effect on L-asparaginase enzyme activity

pH had affected the active site of L-asparaginase, further affecting L-asparaginase activity (Hassan et al., 2018) because the concentration of hydrogen ions affects the dimensional structure of the enzyme. Enzyme has the most conducive three-dimensional structure in binding substrates at optimum pH (Nirmala et al., 2021). L-asparaginase has optimum enzyme activity at a pH range between 6-10 (Muneer et al., 2020). **Figure 2.** showed enzyme activity of L-asparaginase from *V. alginolyticus* bacterial symbiont from green algae *C. lentillifera* increased with increasing pH until it reached the highest enzyme activity in pH 7.5 with value of 17.99 U/mL. Then, in pH 8 to pH 9, the value of enzyme activity decreased to 14.27 U/mL. Qeshmi et al. (2015), also obtained the maximum L-asparaginase enzyme activity by *Bacillus* sp. at pH 7.5. In addition, L-asparaginase from *Escherichia coli* MF-107 was reported to have stable enzyme activity at pH 7.5-8 (Shahnazari et al., 2022).

Incubation time effect on L-asparaginase enzyme activity

The enzyme activity was also influenced by incubation time. The incubation time determines the optimum time of the active site of an enzyme reacting with the substrate. In this study active site of the L-asparaginase enzyme react with L-asparagine as a substrate. **Figure 3**. showed that enzyme activity increased until 30 minutes and then decreased until 120 minutes. In this research L-asparaginase from *V. alginolyticus* symbiont bacteria of green algae *C. lentillifera*, shows optimum enzyme activity of 17.99 U/mL after 30 minutes of incubation, similar to L-asparaginase from *Pseudomonas aeruginosa* WCHPA075019 (Amany et al., 2021) and *P. aeruginosa* (Sheltagh & Ali, 2022), both shows maximum activity after 30 minutes of incubation.

Temperature effect on L-asparaginase enzyme activity

Temperature affects the structure and catalytic function of enzymes. At a higher temperature, the enzyme reactions will be faster. However, the reaction of enzyme-catalyzed will be slower or stop at too high a temperature because the enzyme as a protein will be denatured at a high temperature (Mazumder et al., 2018). **Figure 4** showed that enzyme activity increased until at a temperature of 37 °C and then decreased until at a temperature of 47 °C. In this research L-asparaginase from *V. alginolyticus* symbiont bacteria from green algae *C. lentillifera* has an enzyme activity of 17.99 U/mL at an optimum temperature of 37 °C.

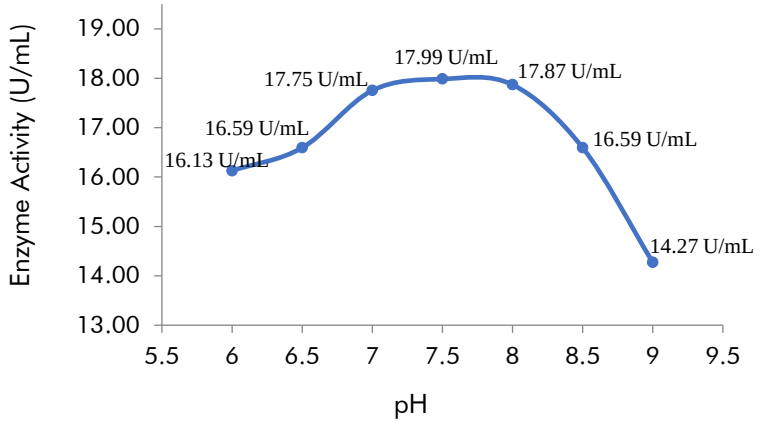

Figure 2. pH effect on enzyme activity of L-asparaginase from *V. alginolyticus* bacterial symbiont of *C. lentillifera*

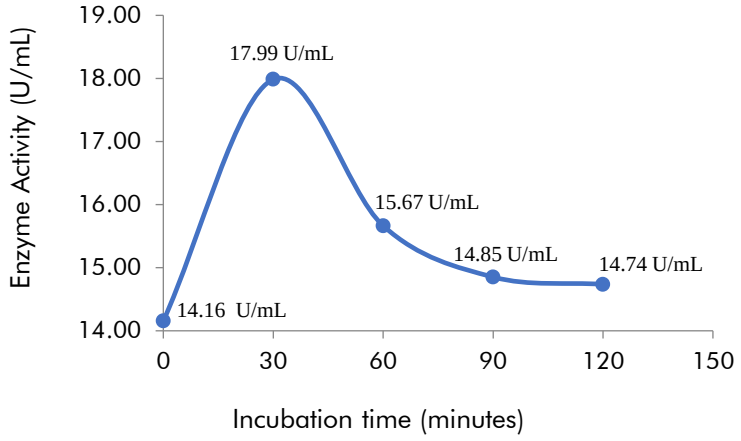

Figure 3. Incubation time effect on enzyme activity of L-asparaginase from *V. alginolyticus* bacterial symbiont of *C. lentillifera*

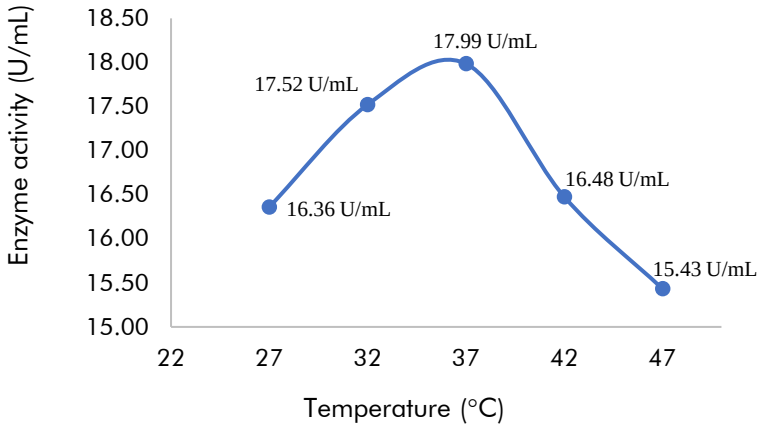

Figure 4. Temperature effect on enzyme activity of L-asparaginase from *V. alginolyticus* bacterial symbiont from *C. lentillifera*

Table 2. Result of toxicity test of L-asparaginase from *V. alginolyticus*

Sample	Line Equation	Y Value	LC_{50} (µg/mL)
Crude extract	0.912	50	17.833

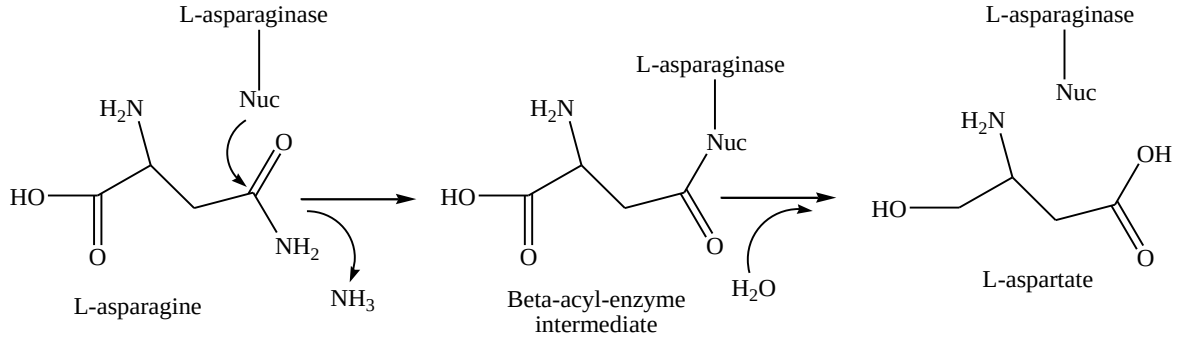

Figure 5. The reaction of L-asparagine hydrolysis by L-asparaginase (Cachumba et al., 2016)

Toxicity Test of L-asparaginase from *V. alginolyticus* with The BSLT Method

The BSLT method is used to test the toxicity of the sample as the initial stage of the anticancer potential test by inspecting the death of *A. salina* Leach larvae with the addition of samples (Sami et al., 2019). *A. salina* Leach larvae were used because it has similar characteristics to mammals that as DNA-dependent RNA polymerase DNA (Nugrahaningsih et al., 2019). The advantages of this method are faster, cheaper, and it can be done in a non-aseptic condition (Yusriadi et al., 2019).

The levels of toxicity of a compound are categorized as very toxic when LC_{50} <30 µg/mL, toxic when LC_{50} 30-1000 µg/mL, and non-toxic when LC_{50} >1000 (Meyer et al., 1982). **Table 2**., it can be seen that the crude enzyme of L-asparaginase from *V. alginolyticus*, a bacterial symbiont of green algae *C. lentillifera* has an LC_{50} value of 17.833 µg/mL, therefore could be categorized as very toxic and could be continued for cytotoxicity test on cancer cells to show its ability as an anticancer agent. According to the theory, L-asparaginase inhibits cancer cells through the hydrolysis of L-asparagine to ammonia and L-aspartate, and in consequence, L-asparaginase disturbs protein synthesis and causes cancer cell death (Yusriadi et al., 2019). The reaction of L-asparagine hydrolysis by L-asparaginase can be seen in **Figure 5**.

CONCLUSIONS

L-asparaginase from *V. alginolyticus*, a bacterial symbiont of green algae *C. lentillifera* indicates optimum activity on the 66th hours of fermentation time, in pH 7.5 at 37 °C after 30 minutes of incubation time and shows LC_{50} <30 µg/mL, which categorized as high toxicity level, therefore can be continued for cytotoxicity test on cancer cells.

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