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Extracellular l-asparaginase productive potential of the *Priestia megaterium* strain GB911 from Khanh Hoa sea of Viet Nam

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Abstract. Currently, L-asparaginase isolated from terrestrial microorganisms has been employed for anticancer industrial production. This enzyme has the function of inhibiting the growth of acute lymphoblastic leukemia, Hodgkin's lymphomas and other cancers. In this study, for the first time, L-asparaginase (ASNase) productive capacity of forty marine bacteria originating from Khanh Hoa sea of Viet Nam was screened and reported. Among those, twenty eight isolates were clarified for their extracellular ASNase activity based on their ability to form a pink zone around colonies on asparagine agar plates. As a result, four strains GB910, GB911, GB976 and GB982 showed high secreted extracellular ASNase activity, ranging from 8.64 to 13.22 IU/mg. Among these four most potential strains, GB911 exhibited better enzyme production capacity and was subjected to taxonomy identification. According to the 16S rRNA gene sequence on the GenBank database, strain GB911 was identified as *Priestia megaterium* and registered in the GenBank database with accession number MW407014. The purified enzyme obtained from strain GB911 exhibited strong cytotoxic activity against five monolayer and suspension cancerous cell lines with IC_{50} values ranging from 39 mU/mL to 98 mU/mL. The strain could serve as a promising source for industrial production of ASNase for clinical chemotherapeutic treatment.

Keywords: Bacillus megaterium, L-asparaginase, cytotoxicity, extracellular enzyme, marine bacteria.

Classification numbers: 1.5.3, 2.7.1, 2.7.2

1. INTRODUCTION

Presently, the most popular enzymes used in industry are extracellular enzymes extracted from microbial strains of the genera *Aspergillus*, *Bacillus*, including α-amylase, β-glucanase,

cellulase, dextranase, protease, etc. [1]. L-asparaginase (ASNase) is an amidase group enzyme, that has been mainly used for clinical chemotherapy. ASNase is the first enzyme to be profoundly studied for anti-leukemic activity in human beings [2]. Currently, ASNase is approved for clinical therapy to treat various cancers such as acute lymphoblastic leukemia, malignancies of the lymphatic system and Hodgkin's lymphomas [3]. ASNase are widely present in mammals, birds, plants, yeasts, and many bacteria. At present, microorganisms are considered the primary source for ASNase synthesis. The production of this enzyme is mainly carried out by submerged fermentation. In previous studies, the production of this enzyme from some microorganisms was reported, including *Pseudomonas fluorescens*, *Serratia marcescens*, *Escherichia coli*, *Erwinia carotovora*, *Proteus vulgaris*, *Saccharomyces cerevisiae*, *Streptomyces karnatakensis*, *Streptomyces venezuelae*, and some fungi such as *Aspergillus*, *Penicillium* and *Fusarium* [4]. The best ASNase-producing strains belong to the Enterobacteriaceae family. In the pharmaceutical industry, *Escherichia coli* and *Erwinia carotova* are common sources of producing ASNase to treat leukemia and lymphoma, in which L-asparaginase II (EC2) is known as an exogenous enzyme with anticancer activity [5]. However, industrial production of ASNase faces with a number of challenges such as finding new microorganisms expressing extracellular ASNase with fewer side effects and higher production efficiency. Recently, marine microorganisms have been reported as promising pharmaceutical resources for new compounds with a variety of medical applications [6]. However, there has not been any report on Vietnamese bacteria which produce extracellular ASNase. Therefore, in this study, for the first time, the ASNase production capacity of 42 microorganisms isolated from Khanh Hoa sea in Viet Nam will be assessed.

2. MATERIALS AND METHODS

2.1. Chemicals

QIAamp® DNA mini Kit for bacteria genomic purification was purchased from Qiagen (Germany). PCR master mix was purchased from Bioneer (Korea) and all other chemicals (for media) were obtained from Himedia (India) and Sigma-Aldrich (Chemical Co., USA).

2.2. Sample collection

Thirty-one random marine samples, including sediments (11 samples), sponges (8 samples), mollusks (5 samples), seaweeds (4 samples), corals (2 samples), and sea cucumber (1 sample) were collected from different locations at a depth of 5 - 22 m in Van Phong bay, Khanh Hoa sea, Viet Nam. The samples were put into 15 mL or 50 mL sterile Falcon tubes, preserved in an ice-box and processed within 24 h.

2.3. Isolation of marine bacterial strains

An amount of 0.5 g of sample was ground and suspended in 4.5 mL of sterile distilled water, homogenized by vortexing for 1 min. Next, 0.5 mL of the homogenized solution was used for serial dilution in sterile distilled water to 10^{-3}. In the final dilution step, 50 µL aliquots were spread over five different solid media, including A1 (soluble starch 10 g/L; yeast extract 4 g/L; peptone 2 g/L; instant ocean 30 g/L; agar 15 g/L); NZSG (soluble starch 20 g/L; yeast extract 5 g/L; glucose 10 g/L; NZ amine A 5 g/L; instant ocean 30 g/L; agar 15 g/L); M1 (soluble starch 5 g/L; yeast extract 2 g/L; peptone 1 g/L; instant ocean 30 g/L; agar 15 g/L); ISP1 (yeast extract 2

g/L; casitone 5 g/L; instant ocean 30 g/L, agar 15 g/L); ISP2 (soluble starch 5 g/L; yeast extract 2 g/L; glucose 10 g/L, malt extract 10 g/L; instant ocean 30 g/L; agar 15 g/L). Plates were incubated at 30 °C for 5 - 10 days. Single colonies of bacteria were transferred onto new Petri dishes of A1 medium for further purification steps.

2.4. Screening of bacterial isolates for ASNase production

The bacterial isolates were screened for ASNase production using the rapid plate assay method [7, 8], which was performed using M9 medium ($Na_2HPO_4.2H_2O$ 6 g/L; KH_2PO_4 3 g/L; L-asparagine 5 g/L; 2 mL of 1 M $MgSO_4.7H_2O$; 1 mL of 0.1 M $CaCl_2.2H_2O$; 10 mL of 20% dextrose, instant ocean 30 g/L; agar 15 g/L) supplemented with 0.3 mL of 2.5 % phenol red, pH 7.0 medium. Control plates were of medium M9 containing 0.3 mL of 2.5 % phenol red, without L-asparagine (instead, containing $NaNO_3$ as nitrogen source). After incubating at 30 °C for 24 h, the pink zone diameter around the colonies (Enzyme index) was measured (mm).

2.5. Identification of promising strain via 16S rRNA gene sequence analysis

Genomic DNA of GB911 was extracted by using QIAamp® DNA mini Kit for bacteria genomic (Qiagen, Germany). The 16S rRNA gene amplifications were performed in a 25.0 µL mixture containing 10.5 µL of sdH_2O, 12.5 µL of Master Mix 2X, 1.0 µL of 0.05 mM for both primers 16SF (5'- AGAGTTTGATCATGG CTCA-3'), 16sR (5'- AAGGAGGTGATCCAGCC - 3'), and 1.0 µL of genomic DNA. The thermocycling process was performed on an MJ Thermal cycler (Bio - Rad), with a preheating step at 94 ^oC for 3 min, followed by 30 cycles of denaturation at 94 ^oC for 1 min, annealing at 62 ^oC for 30 s, extension at 72 ^oC for 45 s before a final extension at 72 ^oC for 10 min. The PCR product size was about 1500 bp. PCR products were purified using a DNA purification kit (Invitrogen) and sequenced on a DNA Analyzer (ABI PRISM 3100, Applied Bioscience). Gene sequences were handled by BioEdit v.2.7.5. and compared with bacterial 16S rRNA gene sequences available in the GenBank database using the NBCI Blast program. The alignment was verified and adjusted manually prior to the reconstruction of a phylogenetic neighbor-joining tree using the MEGA version 7.2 program.

2.6. Purification and bioactivity of isolated ASNase

100 mL of supernatant from the culture medium of the four selected bacterial strains was collected and then precipitated with 80 % ammonium sulfate solution [9]. 1 mL of precipitated protein was then put into a dialysis bag with a cut-off size of 15 KDa and soaked in 50 mM Tris-HCl buffer overnight at 4 ^oC to remove remained chemicals and undesired proteins. After that, the protein solution was loaded on Sephadex G-100 chromatography and CM-Sephadex C50 for purification. The protein concentration in the obtained fractions were determined using a Pierce BCA Protein Assay Kit (Thermo Scientific). 5 µg of the precipitated protein and 3 µg of the purified enzyme (24 µL/well) were used for quality assessment using SDS-PAGE. In addition, ASNase activity was measured using Nessler's reagent (Sigma Aldrich, USA) [10].

2.7. Cytotoxicity assay

Different human adherent cancer cell lines including lung cancer cells (A549), colon cancer cells (HT-29), cervical cancer cell line (Hela) and two human leukemic suspension cancer cell lines (Jurkat and K562) were used for determining the cytotoxic activity of ASNase. The anti-proliferative MTT (3-(4,5-dimethylthiazol-2 - yl)- 2, 5 - diphenyltetrazolium) assay was

employed and conducted following the previously described protocols [9]. Briefly, cells were pre-cultured in 96-well microtiter plates (190 µL/well) at a suitable seeding concentration which depends on each cell line. After 24 h, 10 µL of ASNase at different concentrations diluted in sterile saline was added to the cell-prepared wells. Experiments were carried out in triplicate for the accuracy of data. The cells were then cultured for an additional 48 h. At the end of the treatment period, MTT (final concentration 1 mg/mL) was added to each well, which was then incubated at 37 ^{o}C in 5 % CO_2 for 4 h. The colored crystals of produced formazan were dissolved in DMSO (dimethyl sulfoxide, Sigma, USA). The absorbance was measured at 630 nm using an ELx800 Microplate Reader (Bio-Tek Instruments). The inhibitory rate of cell growth (IR) was calculated according to the following equation:

$$IR = 100\ \% - [(ODt - ODblank)]/[(ODc - ODblank)]\times 100$$

where ODt is the average OD value of the treated sample; ODc as the average OD value of the normal saline control wells; ODblank is the average OD value of the blank wells with culture medium only. The TableCurve 2Dv4 software was used for data analysis and for IC_{50} calculation. The IC_{50} values should be in small deviation throughout the experiments.

3. RESULTS AND DISCUSSION

3.1. Isolation and screening of marine bacteria producing ASNase

From the marine samples randomly collected, serial dilution and plating on different media were carried out for bacterial isolation, after 5 - 10 days of incubation, fifty distinct colonies were isolated, purified and further cloned. All isolated bacterial strains were screened for their ability to produce extracellular ASNase using a qualitative rapid plate assay. The principle of the assay is based on the ability to form a pink zone around colonies on asparagine agar plates containing a phenol red indicator. According to Gulati's method [8], the appearance of pink colored zones in an overnight incubator clearly indicates that the organism is capable of producing the required enzyme. As a result, twenty eight isolates showed intense pink zones around the colonies (**supplementary**). Most notable were the four strains with high ASNase production ability, namely GB910 (11 mm), GB911 (12 mm), GB976 (12 mm), and GB982 (11 mm) (Figure 1). These four strains were further studied for their capacity to produce extracellular ASNase.

As reported elsewhere, ASNase selectively targets the deficiency of metabolic pathways in cancer cells, leading to serious nutrition limitations and causing cell death [7]. Besides, marine microorganisms that have been shown to be potential sources for ASNase with improved thermal stability, lower glutaminase activity and higher substrate affinity have attracted much attention. In terms of this approach, for the first time, fifty bacteria from randomly collected samples (sediments, seaweeds, sponges, Molluscas, etc.) at different points and deep water zones in Van Phong bay, Khanh Hoa sea of Viet Nam, presented twenty eight strains expressing extracellular ASNase. In a similar research by Alrumman *et al*. [8], there were also twenty eight microbial strains that produced ASNase out of 40 isolates from the Red Sea of Saudi Arabia. Besides, from a study by Ameen *et al*., it was found also from this Red Sea that only *Bacillus subtilis* strain isolated from sponges exhibited ASNase production ability among twenty bacterial isolates [11]. There was also another report on the isolation and identification of ASNase producing bacteria from Persian Gulf, Iran [12]. This study recognized 57 strains that secreted ASNase out of 181 isolates. These numbers demonstrate the varying potential for

ASNase production by marine bacteria from different oceans over the world and raise the need for more similar studies.

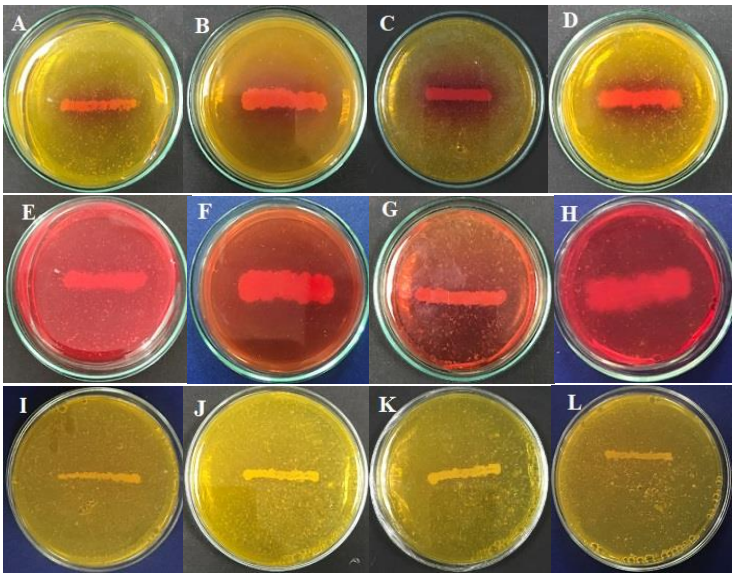

Figure 1. Enzyme production pink zone of the four screened strains (GB910, GB911, GB976, GB982) after 24 h (A, B, C, D); 48 h (E, F G, H) and control plates after 48 h (I, J, K, L) in an incubator at 30 °C.

3.2. ASNase enzyme activity of the four selected strains

The results, which were obtained from screening assay, showed ASNase activities in culture medium from four selected bacterial strains of which GB911 exhibited the highest enzymatic activity reaching 13.22 IU/mg, followed by strains GB982, GB910 and GB976 (Table 1).

Table 1. L-asparaginase activities of the most potential bacterial strains from isolates.

Strain	**Extracellular Enzyme Activity (IU/mg)**	**Enzyme production pink zone *D-d* (mm)**
GB 910	9.93 ± 0.54	11
GB 911	13.22 ± 1.08	12
GB 976	8.64 ± 0.48	11
GB 982	11.12 ± 0.92	12

As reported by Moushree *et al*. (2019), ASNase activity from *Bacillus megaterium* MG1 strain isolated from water bodies of Moraghat forest, Jalpaiguri, India was 6.82 IU/mg (equal to 215 IU/mg after purification) [13]. In another study by Alrumman *et al*. (2019), it was found that the highest enzyme production was the *Bacillus licheniformis* isolate obtained from the Al-Haridah coast, reaching 8.1 IU/mL [8]. The information allows predicting that bacteria from Khanh Hoa sea of Viet Nam could be a good potential source for industrial ASNase production.

3.3. Identification of the GB911 strain

The most potential strain GB911 was subjected to identification by 16S rRNA gene

sequencing using the bacterial identification method of Alrumman *et al*. (2019) and Moushree *et al*. (2019) [8, 13]. The 16S rRNA gene was amplified by PCR using specific primers 16sR and 16sF, giving a product of 1500 bp (Figure **1** in the supplement). The results of comparative analyses by 16S rRNA gene sequence of this isolate on the GenBank database showed that strain GB911 exhibited high similarity (99.93 %) with strain *Priestia megaterium* strain ATCC 14581 (NR_117473). Various sequences taken from the GenBank database were used to build the phylogenetic tree to determine the phylogenetic position of the strain (Figure 2). The nucleotide sequence of the amplified 16S rRNA gene of GB911 was registered in the GenBank database with accession number MW407014. *Priestia* is a genus of Gram-Positive rod-shaped bacteria belonging to the family *Bacillaceae* of the order *Bacillales*. The type species of this genus is *P. megaterium*. Members of *Priestia* are formerly species belonging to *Bacillus*.

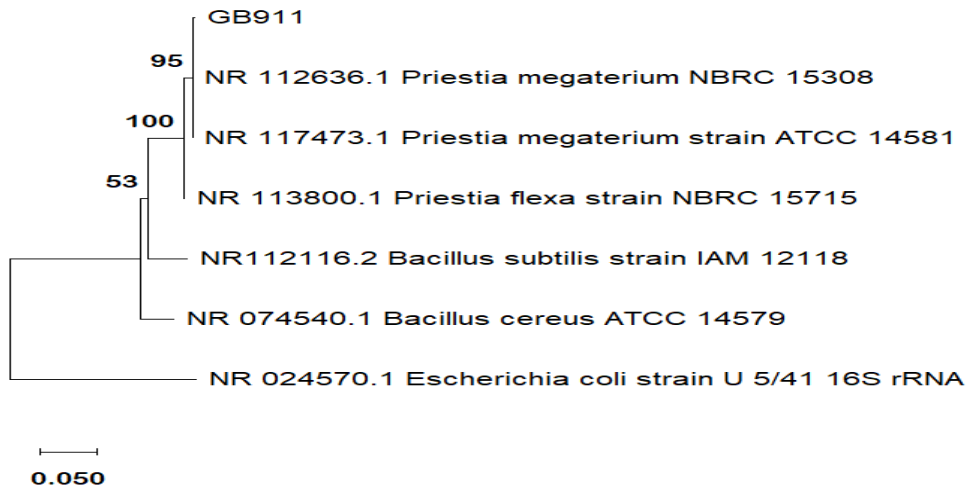

Figure 2. Neighbor-joining tree based on 16S rRNA gene sequences showing relationships between strain GB911 and representative members of the genera *Priestia* and *Bacillus*. The bootstrap values below 50 % were eliminated.

3.4. Purification of ASNase

Strain GB911 was cultured at a scale of seven liters/batch using M9 medium. After the collection and purification process, the purified ASNase obtained has a specific activity of 249.20 IU/mg, an increase of 18.87 times compared to the ASNase activity before purification (13.22 IU/mg). As reported by Michalska and Jaskolski, bacterial-type ASNases are classified into two subtypes, defined by their intra- or extracellular location [14]. Extracellular enzymes are easily extracted and processed downstream [15]. Thus, extracellular enzymes have gained more attention as well as applications. From our study, ASNase producing capacity of strain GB911 is at a high level and similar to that of other reported Bacillus strains [16 - 18]. The genus *Bacillus* is the group most commonly reported to carry asparaginase genes and gene products [19]. These strains produce extracellular ASNase. For example, *B. megaterium* from the water bodies of Moraghat forest in India has a specific activity of 215 IU/mg.

The purified protein appeared as a single band on the SDS-PAGE gel stained with coomassie brilliant blue. The SDS-PAGE result showed that the molecular weight of purified ASNase was about 37 kDa (Figure 3), similar to that from *B. aryabhattai* (38.8 kDa), but quite lower than that from *Corynebacterium glutamicum* (80 kDa) [20, 21]. In other previous studies, *B. megaterium* from sponges of the Red Sea produced 45 kDa ASNase, while this strain from

the water bodies of Moraghat forest in India was roughly 47 kDa of molecular mass [8, 22].

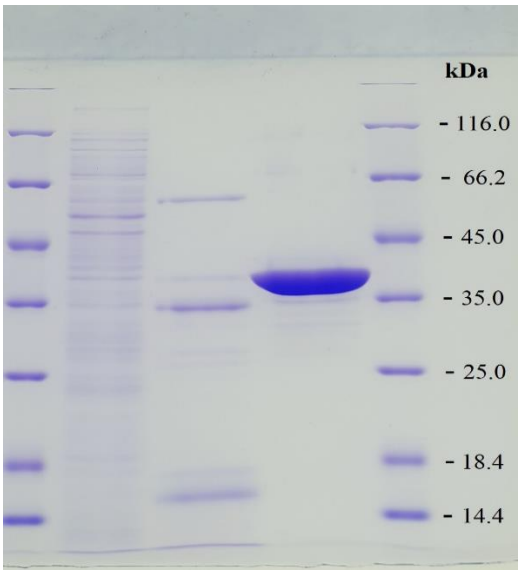

Figure 3. Molecular weight analysis of purified ASNase from strain GB911 using SDS-PAGE; The left lane is precipitated protein of GB911; The middle lane is purified ASNase; The right lane is Fermentas ladder.

3.5. Cytotoxic activity

ASNase is used in anticancer therapy, based on its specific ability to catalyze the conversion of L-asparagine to L-aspartate and ammonia. Cancer cells are unable to synthesize endogenous L-asparagine, so a lack of amino acid leads to cell death [23]. The effects of ASNase purified from the culture medium of *Priestia megaterium* strain GB911on several cancer cell lines were evaluated using the MTT assay. As shown in Figure 4, the cytotoxicity of purified ASNase was different from cell lines and was dependent on ASNase concentration. The purified ASNase was evaluated for cytotoxicity on the 3 monolayer adherent and the 2 suspension leukemic cancer cell lines. A concentration of 2 IU/mL showed the highest effect on the cytotoxicity of all the tested cancer cell lines. The cell growth was strongly suppressed at concentrations above 80 mU/mL with less than 50 % cell proliferation. However, the cytotoxic activity of ASNase decreased quickly at a concentration of 16 mU/mL. There was less than 30 % of cells that died at this concentration. Using Tablecurve software, IC_{50} values of ASNase across cell lines were calculated. First, ASNase showed the highest activity on Jurkat cells with an IC_{50} of 39 ± 6 mU/mL. The cytotoxicity of ASNase on Hela cells was lower than Jurkat with an IC_{50} of 49 ± 6 mU/mL. The results showed significant efficiency when compared to the results of Bhat and Marar [24], in which the IC_{50} values were 172 mU/mL and 96 mU/mL for HeLa and Jurkat cell lines, respectively. The results showed high selectivity of ASNase indicating high specific toxicity of this enzyme towards leukemia cell lines (Jurkat), similar to previous studies [25].

However, the enzyme showed lower activity on the K562 myelogenous leukemic cell line with an IC_{50} of 61 ± 4 mU/mL, meaning there was a difference in activity for typical leukemias. As reported, the purified ASNase from *Bacillus licheniformis* showed a cytotoxic effect against

Jurkat clone E6-1, K-562 and MCF-7 [26]. ASNase also exhibited sensitivity against fibrosarcoma and liposarcoma [27]. *In vitro* cytotoxicity of ASNase originated from the pathogenic strain *Helicobacter pylori* showed that AGS and MKN-28 gastric epithelial cells were most affected [28]. As such, the anti-proliferative activity of ASNase from strain GB911 exhibited strong growth inhibition of colorectal carcinoma (HT-29 cells) with an IC_{50} of 64 ± 8 mU/mL but the lowest cytotoxicity on lung carcinoma (A549 cells) with an IC_{50} of 98 ± 15 mU/mL. Those data showed that ASNase from the *Priestia megaterium* GB911 strain isolated from Khanh Hoa sea of Viet Nam maintained its strong anticancer potential in *in vitro* experiments.

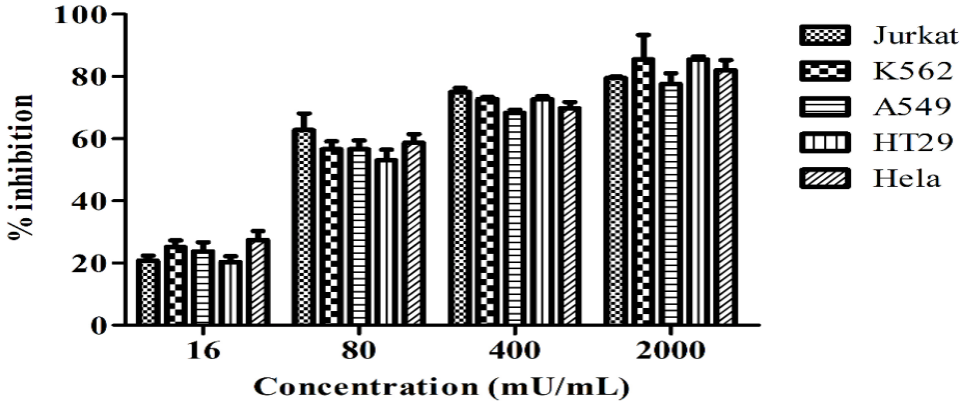

Figure 4. The anti-proliferative activities of L-asparaginase obtained from GB911 bacterial strain against different cancerous cell lines. Cultured cells (6×10^3 cells/well) were treated with different concentrations of L-asparaginase for 72 h. Normal saline served as the negative control. Each value represents the mean ± SD

4. CONCLUSIONS

From fifty strains of marine bacteria íolated in the waters Khanh Hoa, Viet Nam, we have identified twenty eight strains capable of producing extracellular L-asparaginase (ASNase). The most potential strain GB911 could produce ANSase with a specific enzymatic activity of 13.22 IU/mg. According to the 16S rRNA gene sequence on the GenBank database, strain GB911 strain has been identified as *Priestia megaterium* and registered in the GenBank database with accession number MW407014. The purified enzyme obtained from strain GB911 showed strong cytotoxic activity against five cancerous cell lines with IC_{50} values ranging from 39 mU/mL to 98 mU/mL. Based on those characteristics, strain GB911 is considered a very promising source for industrial ASNase production.

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CRediT authorship contribution statement. Do Thi Thao: Conceptualization, Methodology, Supervision and Validation, Review & editing. Do Thi Phuong: Investigation, Data accuration, Methodology, Writing

– original draft. Le Thi Hong Minh: Investigation, Software. Nguyen Thi Nga: Investigation, Software, Review & editing. Nguyen Mai Anh: Investigation. Vu Thi Thu Huyen: Data accuration. Ha Phuong Trieu: Software. Nguyen Thi Cuc: Data accuration.

Declaration of competing interest. The authors declare no conflict of interest.

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SUPPLEMENTARY

Table 1S. Detailed characterization and pink zone diameter after 24 h of marine isolated bacterial strains from Khanh Hoa sea of Viet Nam.

No	Name of strains	Mediaof isolation	Name of sample	Type of sample	Geographic coordinates/ Water depth (m)	Pink zone diamete after 24 h (mm)
1	**GB911**	M1	188a	Sediment	12°38’26’’ – 109°21’54’’ 22 m	**12**
2	GB920	A1	186a	Sediment	12°40’10’’ – 109°16’28’’ 16 m	6
3	GB1006	ISP2	186a	Sediment	12°40’10’’ – 109°16’28’’ 16 m	8
4	GB1007	A1	186a	Sediment	12°40’10’’ – 109°16’28’’ 16 m	8
5	GB1010	A1	186a	Sediment	12°40’10’’ – 109°16’28’’ 16 m	7
6	GB975	ISP1	189d	Seaweed	12°38’39’’ – 109°22’38’’ 5 m	6
7	**GB910**	ISP2	190k	Sponges	12°38’43’’ – 109°20’23’’ 15 m	**11**
8	GB940	M1	190m	Molluscas	12°38’43’’ – 109°20’23’’ 15 m	6
9	GB942	M1	190m	Molluscas	12°38’43’’ – 109°20’23’’ 15 m	4
10	GB952	M1	190m	Molluscas	12°38’43’’ – 109°20’23’’ 15 m	6
11	**GB976**	A1	190m	Molluscas	12°38’43’’ – 109°20’23’’ 15 m	**12**
12	GB950	ISP1	191f	Sediment	12°40’30’’ – 109°15’42’’ 5 m	-
13	GB957	M1	192l	Sponges	12°40’30’’ – 109°15’42’’ 12 m	-
14	GB966	ISP1	191l	Sponges	12°40’30’’ – 109°15’42’’ 5 m	4
15	GB972	M1	191a	Sediment	12°40’30’’ – 109°15’42’’ 5 m	-
16	GB973	M1	191a	Sediment	12°40’30’’ – 109°15’42’’ 5 m	4
17	GB919	ISP2	192d	Sediment	12°40’30’’ – 109°15’42’’ 12 m	6
18	GB960	ISP1	192e	Sediment	12°40’30’’ – 109°15’42’’ 12 m	-
19	GB961	ISP1	192d	Sediment	12°40’30’’ – 109°15’42’’ 12 m	6

20	GB965	ISP1	192h	Molluscas	12°40’30’’ – 109°15’42’’ 12 m	-
21	GB968	ISP2	192o	Sponges	12°40’30’’ – 109°15’42’’ 12 m	4
22	GB969	ISP2	192g	Molluscas	12°40’30’’ – 109°15’42’’ 12 m	-
23	GB970	NZSG	192h	Molluscas	12°40’30’’ – 109°15’42’’ 12 m	4
24	GB971	NZSG	192n	Corals	12°40’30’’ – 109°15’42’’ 12 m	-
25	GB974	M1	192c	Sediment	12°40’30’’ – 109°15’42’’ 12 m	6
26	GB977	ISP1	192i	Molluscas	12°40’30’’ – 109°15’42’’ 12 m	8
27	GB980	ISP1	192j	Sponges	12°40’30’’ – 109°15’42’’ 12 m	5
28	GB981	M1	192k	Sponges	12°40’30’’ – 109°15’42’’ 12 m	9
29	**GB982**	M1	192k	Sponges	12°40’30’’ – 109°15’42’’ 12 m	**11**
30	GB1011	ISP2	192i	Molluscas	12°40’30’’ – 109°15’42’’ 12 m	7
31	GB1014	M1	192c	Sediment	12°40’30’’ – 109°15’42’’ 12 m	-
32	GB989	ISP2	193a	Sediment	12°40’44’’ – 109°20’33’’ 5 m	10
33	GB928	A1	195e	Soft corals	12°35’16’’ – 109°24’44’’ 10 m	-
34	GB962	NZSG	195h	Sponges	12°35’16’’ – 109°24’44’’ 10 m	-
35	GB967	SWA	198l	Molluscas	12°37’34’’ – 109°12’49’’ 8.5 m	-
36	GB983	M1	198f	Seaweed	12°37’34’’ – 109°12’49’’ 8.5 m	-
37	GB1013	ISP1	198c	Sediment	12°37’34’’ – 109°12’49’’ 8.5 m	7
38	GB901	ISP2	199b	Sediment	12°37’40’’ – 109°13’46’’ 7 m	6
39	GB907	ISP2	199b	Sediment	12°37’40’’ – 109°13’46’’ 7 m	8
40	GB988	NZSG	200a	Sea cucumber	12°37’57’’ – 109°17’41’’ 10 m	10
45						

Note: - as no pink zone produced