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Screening of Halophilic Archaea from Indonesia that Produce L-Asparaginase Enzyme

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Abstract**:** *L-asparaginase enzyme that uses for treatment of acute lymphoblastic leukemia (ALL) is still produced from Escherichia coli dan Erwina carotovora. The use of this enzyme for medicine in a sustainable way is thought as the most common cause the allergies to the user, so it needs an L-asparaginase enzyme with different characteristics than before. Halophilic archaea is a microorganism that can live in extreme environmental condition and probably have the capability to produce L-asparaginase enzyme with different characteristics than another microorganism. 71 halophilic archaea isolated from Indonesia was studied. 9 isolates of Halophilic archaea show a positive enzyme L-asparaginase activity. Identification based on 16S rRNA for selected isolates shown that Halobaculum sp. Has the highest activity (0.3033 U/mL) and then followed by Halostagnicola kamekurae (0.2134 U/mL), Halogranum rubrum (0.0927 U/mL), Haloferax, sp (0.0916 U/mL), Halococcus thailandensis (0.0808 U/mL), Halogranum, sp (0.0646 U/mL), Halococcus, sp (0.0326 U/mL), Halalkalicoccus paucihalaphilus (0.0200 U/mL) and Halococcus hamelinensis (0.0056 U/mL).*

Keywords: *Archaea, L-Asparaginase, Halophilic archaea, acute lymphoblastic Leukemia*

1. Introduction

Acute Lymphoblastic Leukemia (ALL) is included as one of cancer that usually attacks children, but sometimes it also found in adults. ALL is one of cancer that attacks lymphocytes that usually found in spinal. L-asparaginase enzyme had a function as a catalyst on hydrolysis of L-asparagine amino acid process become L-aspartate and ammonia (Wakil & Adelegan, 2015). L-asparagine is non-essential amino acid that used by lymphocytes in proliferation process, this biosynthesis process makes oxaloacetate become aspartate by transaminase enzyme with the transfer of amino group from glutamate into oxaloacetate and the result of this reaction are α-ketoglutarate and aspartate (Kumar & Sobha, 2012).

L-asparaginase enzyme clinically used as anti-cancer and very effective as medicine for lymphoma and leukemia. This enzyme also tested to animal and human for treatment of lymphoblastic leukemia, reticle sarcoma, Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma and also in melanosarcoam chemotherapy (Pradhan, Dash, & Sahoo, 2013).

Halophilic archaea are one of microorganism that could survive in high salt concentration area. Halophilic archaea also could grow in the condition of salt concentration near saturation point, this microorganism also could be found in rocks that contain salt or old rocks with aged 195-250 years old (Fendrihan et al., 2007). This environment makes a unique challenge for this organism, just like the difference of salt concentration between inside and outside of **the** cell, this difference produces high of osmotic pressure. Meanwhile, this condition makes others organism will be lost all of their water inside of their cell and then they will die. This halophilic archaea need nutrition and have a different metabolism pathway different with another microorganism (Falb et al., 2008).

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2. Materials and Methods

2.1 Chemicals

L-asparagine, phenol red, Mercury(II) iodide, Kalium hydroxide, Kalium iodide from Merck. Trichloroacetic acid was obtained from Sigma. Molecular biology kit for PCR was supplied by Promega.

2.2 Reviving Halophilic Archaea

Halophilic archaea ampule were open and revive at the specific medium that contains NaCl (150-200 g/L), Yeast extract (0.1-2 g/L), Na_3 citrate (1 g/L), KCl (2 g/L), Casamino acids (0.1-2 g/L), Na glutamate (1 g/L), K_2HPO_4 (0.3 g/L), $CaCl_2$. $2H_2O$ (0.15 g/L), NH_4Cl (1 g/L), MgSO4.$7H_2O$ (20-50 g/L), Trace element (2 mL/L) with the final pH 7.0 – 7.4. The reviving cultures were incubated for 1week at 30˚C.

2.3 Screening for L-asparaginase Enzyme

A rapid plate assay method for screening L-asparaginase enzyme was used (Gulati, Saxena, & Gupta, 1997). Halophilic archaea modified medium for qualitative assay containing: L-asparagine (5 g/L), Na_3 citrate (1 g/L), KCl (2 g/L), K_2HPO_4 (0.3 g/L), $CaCl_2$. $2H_2O$ (0.15 g/L), MgSO4.$7H_2O$ (20-50 g/L), Trace element (2 mL/L) and Phenol red (10 mL/L) with the final pH 7.0 – 7.4. Isolates were incubated for 96 hours at 30˚C. Some of halophilic archaea makes color changes that appeared around of the colonies. The color changes indicate that the L-asparaginase enzyme was produced.

2.4 Molecular Identification

Physiological and morphological characterization is impossible to describe the species of the halophilic archaea that already positive produce the L-asparaginase enzyme. The DNA from targeted isolates was extracted by using heat shock method (95˚C) for 10 min. Targeted gene (16S rRNA) were amplified using Ar109F (5'-ACKGCTCAGTAACACGT-3') and Ar915R (5'-GTGCTCCCCCGCCAATTCC -3'). The PCR condition are pre denaturation at 98˚C for 10 min, denaturation 98˚C for 30 s, annealing 55˚C for 1 min, extention 72˚C for 90 s with 30 cycle and then final extention 72˚C for 10 min. PCR product were sequenced at Macrogen (Korea) by using ABI 3370XL. The sequence similarity were proceed by using Chromaspro and the align result were BLAST to National Centre for Biotechnology Information (NCBI).

2.5 Enzyme Assay

Nesslerization method was used for determined enzyme activity (Gulati et al., 1997). Targeted isolates were inoculated in the modified medium (without phenol red) for halophilic archaea for 24-96 hours at 30˚C. 1 mL of crude enzyme was centrifuge at 5.000 xg for 20 min. 0.5 mL supernatant were added with 1 mL sodium acetate 0.1 M (pH 8.5) and L-asparagine 0.5 mL (0.05 M). The solution were mixed then incubated for 10 min at 37 ˚C. The enzyme reaction was stopped by added 0.5 mL trichloroacetic acid (1.5 M). Solution of enzyme were centrifuge for 20 min with speed 10.000 xg. Ammount of ammonia in 1 mL of supernatant was determined by added 0.5 Nessler reagent (1 L distilled water containing 112 g KOH was added with 45.5 g HgI_2 and 35 g KI). The absorbance of solution was determine at 400 nm by using Varioskan UV/VIS spectrophotometer from Thermo Scientific. Ammonium sulfate ($(NH_4)_2SO_4$) with different concentration was used as standards on this assay. Amount of 1 µmole of ammonia in 1 min reaction at the condition of assay were catalyzes by enzyme is defined as 1 unit (U) of enzyme activity.

3. Results

Halophilic archaea which stable and active in the environment with a high concentration of salt, have good opportunities in biotechnologies applications. In this research, we have studied the halophilic archaea for the screening of L-asparaginase enzyme activity. 71 isolates halophilic archaea were isolated from solar saltern in Bali and Madura. The first step to identify the isolates that have an activity of L-asparaginase enzyme is by using

the qualitative method. Those isolates belonging to *Haladaptatus*, *Halalkalicoccus*, *Haloarchaeobius*, *Haloarcula*, *Halobacteriaceae*, *Halobaculum*, *Halobium*, *Halococcus*, *Haloferax*, *Halogeometricum*, *Halogranum*, *Halomarina*, *Halorubrum*, *Halostagnicola*, *Haloterrigena*, *Halovivax*, and *Natrialba*.

This screening process gave a result that 9 isolates of halophilic archaea have an L-asparaginase enzyme activity. The qualitative analysis result shows that 9 isolates have an L-asparaginase enzyme activity (**Fig. 1**). Those 9 isolates make color changes from yellow to slightly red around of the colony grow area.

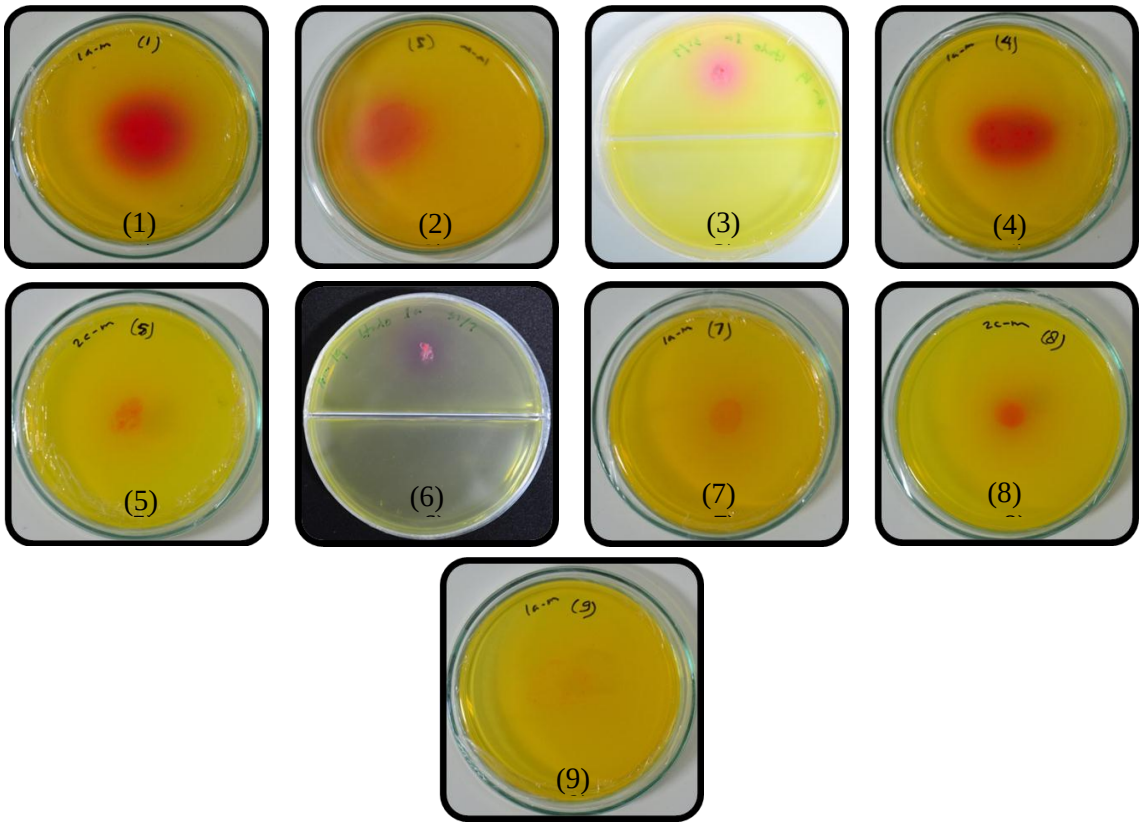

Fig. 1: (1) 1a_35, (2) 1a_43_1, (3) 2c_61_2, (4) 1a_43_2, (5) 2_36_4, (6) 1a_37, (7) 1a_31_1, (8) 2c_11_1, (9) 1a_23_1

The identification process for 9 isolates of halophilic archaea by molecular approach shows that those isolates are belonging to *Halostagnicola*, *Halococcus*, *Halobaculum*, *Halalkalicoccus*, *Haloferax*, and *Halogranum* (**TABLE**).

TABLE I: Molecular Identification Result

No	Isolate	Identification Result	Similarity (%)	acc. No
1	1a-35	*Halostagnicola kamekurae*	99	AB663440.1
2	1a-43-1	*Halococcus hamelinensis*	99	KJ875309.1
3	2c-61-2	*Halococcus* sp.	100	KJ875346.1
4	1a-43-2	*Halobaculum* sp.	97	KY114620.1
5	2c-36-4	*Halococcus thailandensis*	99	JX481758.1
6	1a-37	*Halalkalicoccus paucihalophilus*	99	JF449426.1
7	1a-31-1	*Haloferax* sp.	99	DQ373057.1
8	2c-11-1	*Halogranum rubrum*	99	NR_113453.1
9	1a-23-1	*Halogranum* sp.	100	KJ917655.1

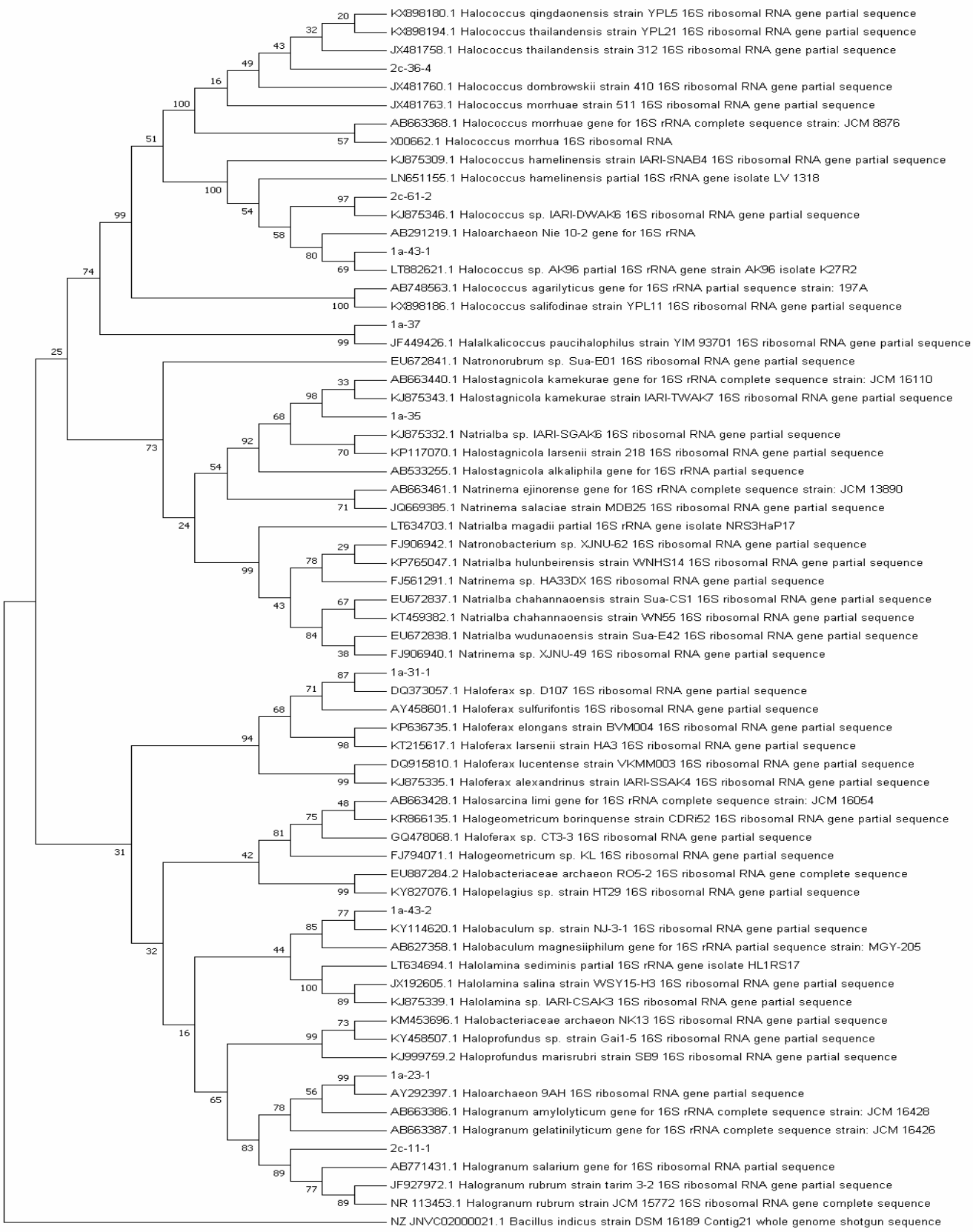

Fig. 2: Halophilic Archaea Produce L-Asparaginase Enzyme Phylogenetic Tree

Studies for the absorbance of ammonium shows that the highest reaction between ammonium with Nessler's reagent has happened to *Halobaculum* sp. with the highest activity and then followed by *Halostagnicola kamekurae*, *Halogranum rubrum*, *Haloferax*, sp, *Halococcus thailandensis*, *Halogranum*, sp, *Halococcus*, sp, *Halalkalicoccus paucihalaphilus* and *Halococcus hamelinensis* (TABLE).

TABLE II: L-Asparaginase Enzyme Activity Result

No	Isolate	96 Jam	ppm	mg	µmol	Unit
1	*Halostagnicola kamekurae*	2.9807	36.2840	0.0363	2.1344	0.2134
2	*Halococcus hamelinensis*	1.0226	0.9459	0.0009	0.0556	0.0056
3	*Halobaculum* sp.	3.8268	51.5542	0.0516	3.0326	0.3033
4	*Halalkalicoccus paucihalophilus*	1.1584	3.3959	0.0034	0.1998	0.0200
5	*Haloferax* sp.	1.8335	15.5804	0.0156	0.9165	0.0916
6	*Halogranum* sp.	1.5786	0.9806	0.0110	0.6459	0.0646
7	*Halogranum rubrum*	1.8430	15.7524	0.0158	0.9266	0.0927
8	*Halococcus thailandensis*	1.7316	13.7418	0.0137	0.8083	0.0808
9	*Halococcus* sp.	1.2771	5.5384	0.0055	0.3258	0.0326

From the result of enzyme activity, shows that the activity of enzyme L-asparaginase are not too high if we do a comparison with the data of enzyme that (Imada, Igarasi, Nakahama, & Isono, 1973) have been done before. The L-asparaginase enzyme activity that halophilic archaea produce is not so high because of the rapidity of growth are not so fast, so the production of the enzyme also not so fast.

4. Acknowledgements

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