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Research Article

SCREENING THE POTENTIAL OF BIOACTIVE PROTEIN AS ANTICANCER AGENTS FROM EPIPHYTIC BACTERIA ASSOCIATED WITH BROWN ALGAE, *SARGASSUM* SP.

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ABSTRACT

The exploration of bioactive protein from epiphytic bacteria associated with marine algae has not much been revealed. One of the symbiotic bacteria producing bioactive protein was successfully isolated from brown algae *Sargassum* sp. Molecular identification of bacteria was done by using PCR 16S rRNA sequencing. Based on the results, the active strains of bacteria isolate SG-A1 belonging to *Enterobacter hormaechei* with 96% sequence similarities. The crude extract protein of symbiotic bacterial isolates then was determined for the toxicity assay by using the *Artemia salina* Leach. The optimum time to produce bioactive proteins was evaluated. *Enterobacter hormaechei* showed the highest bioactivity at the time fermentation of 24 h with LC_{50} is 1,73 µg/mL. These findings suggest that the identified strains are very potential as protein sources for anticancer agent. Further research will be carried out using the optimum time for producing bioactive protein and more purification.

Keywords: Algae, Anticancer, Bioactive protein, Epiphytic bacteria, *Sargassum* sp.

INTRODUCTION

Cancer is the largest cause of death in humans and has been responsible for 9,6 million deaths in 2018. Globally, about 1 of 6 deaths is due to cancer [1,2]. In recent years, there has been significant progress in the diagnosis, treatment and prevention of several types of cancer, but the prevalence of deaths from this disease is still very high and continues to increase, but the prevalence of deaths from this disease is still very high and continues to increase. This is due to the resistance of cancer cells to anticancer agents that have been there [3]. This makes research on the discovery and development of new anticancer drugs is a top priority [4].

Marine resources have highly potential in the development of bioactive compounds. Based on existing research data, it is known that them provide medicinal raw materials with a variety of chemical and pharmacological novelty and diversity [5]. Researchers have reported the uses of marine natural resources, especially algae as agents for antibacterial, antifungal, antiviral, antitumor, anticancer, antioxidant, etc [6].

Algae are known to be one of the marine eukaryotes that are rich in symbiotic bacteria [7]. It is known that bacterial symbionts contain thousands of chemical compounds that have the potential as medicines with characteristics and unique physiological properties and functions that are very diverse [8,9].

The epiphytic bacteria and the bioactive protein from brown algae, *Sargassum* sp, and its bioactivity as anticancer drug raw materials have not much explored. In this research isolation and identification of symbiotic bacteria, and identification bioactive proteins from isolate bacteria have been carried out.

MATERIALS AND METHODS

Materials

The materials were used the brown alga *Sargassum, sp*, Nutrient Broth (NB), Nutrient Agar (NA), Buffer A (Tris (hydroxymethyl) aminomethane, NaCl, $CaCl_2$, β-mercaptoethanol, Triton X-100), Buffer B and Buffer C (Tris(hydroxymethyl) aminomethane, NaCl, $CaCl_2$), distilled water, Bovine Serum Albumin (BSA), Ammonium sulphate, reagent Lowry A and Lowry B, HCl, sea water, eggs of the shrimp *Artemia salina* Leach.

Instruments

Instruments were use analytical balance, fisher magnetic stirrer, micropipette (10-1000 µl), shaker incubator, magnifying glass, Eppendorf tubes, vials, 40-60 watt incandescent/neon lamp, refrigerator, petri dish, inoculation loop, Spectronic 20D+ spectrophotometer, Veriti™ 96-Well Thermal Cycler (AB Applied Biosystems), BIO-RAD Universal Hood ii Gel Doc System, Heraeus™ Biofuge™ Stratos™ Centrifuge Series, spray bottles and glass tools commonly used in laboratories.

Isolation of Epiphytic symbiotic bacteria

A total of 45 mL sterile seawater used to rinse 5 grams of algae samples. The rinse water added into NB medium and then incubated in the shaker at 37 ºC for 24 hours. Samples refreshed on NB media were taken as much as 1 mL and put into a test tube

containing 9 mL of seawater. A multilevel dilution was performed from 10^{-1}-10^{-5} dilution. After that, each dilution was grown in NA media at 37 ºC for 2 x 24 hours. Colonies that have the same shape and color are considered as the same isolate. Each colony is then transferred into a marine agar medium and incubated for 24 hours to obtain a single isolate [8].

Identification of bacterial species

Biochemical test

Biochemical tests of the isolated bacteria were conducted according to Bergey's Manual of Systematic Bacteriology method, included Triple Sugar Iron Agar test (TSIA), Sulfide Indole Motility test (SIM), Methyl Red Voges Proskauer test (MR-VP), Simon Citrate Test Agar (SCA), and carbohydrate fermentation test.

Molekuler identification

DNA extraction is done by selecting inoculated colonies in 50-100 µl ddH_2O. The extract solution was heated at 95 °C for 30 min, then put into refrigerator an overnight.

DNA extracts were amplified by PCR using primers 63F (5'CAGGCCTAACACATGCAAGTC-3') and 1387R (5'GGGCGGTGTGTACAAGGC-3'). The PCR mixture consisted GoTaq®Green Master Mix Promega (25 µl), primer 63F (1-2 µl), primer 1387R (1-2 µl), DNA extract (2-5 µl), and sufficient volume up to 50 µl with Nuclease-Free Water. For PCR amplification, the initial stage is denaturation at 95 °C for 2 minutes, then 95 °C for 1 minute, annealing at 55 °C for 30 seconds followed by final extension temperature 72 °C for 5 minutes for 35 cycles. The PCR products analysed with agarose 1.5% gel electrophoresis, and then imaging by using Gel Doc System.

DNA sequencing was conducted at First Base Pte. Malaysia using the Bioedit software program. For sequence alignment analysis, it is done by comparing the sequences obtained (query) with those already in the Gene Bank with the NCBI internet searches database (http://blast. ncbi.nlm.nih.gov) using BLAST (Basic Local Alignment Search Tool).

Optimization of protein bacteria production

The isolate Epiphytic bacteria was inoculated as many as 10% into the fermentation medium with the same composition as the medium inoculum. The isolate was then shaken at 180 rpm, 37 °C for 1-3 days. Sampling was taken every 6 hours for analysis of the Optical Density (OD), the protein content, and screening of the anticancer activity test by using *Artemia Salina* Leach.

Determination of protein content

The levels of protein in buffer A are determined based on the Lowry method, the standard solution used is Bovine Serum Albumin (BSA) and distilled water as a blank solution. Absorbance is measured by using spectrophotometer UV-Vis at the maximum wavelength [10].

The anticancer activity test

The anticancer activity test with BSLT method. Eggs of *Artemia Salina* Leach were inoculated with seawater and incubated at 30 ºC with strong aeration with continuous incandescent light. After 24 hours of hatching eggs, 10 individuals were pipetted with micropipettes and placed in the small tube containing test extracts and controls (in different tubes). The number of dead individuals is calculated after 24 hours of exposure. The LC_{50} value was determined with probit analysis [11].

RESULTS AND DISCUSSION

Isolation of Epiphytic symbiotic bacteria

The samples of brown alga *Sargassum* sp. used in this study were obtained from Lae-lae Island, Makassar, South Sulawesi. Bacterial isolates were grown through multilevel dilution techniques, 10^{-1}-10^{-5} with pour plate method. Selection of dilution is based on the estimated number of microbes that are suspended in the liquid. One of the epiphytic bacteria was selected and then labeled SG-A1. Table 1 illustrated biochemical characteristics for isolate. Based on those test isolate SG-A1 was identified as *Enterobacter sp*. The result was confirmed by molecular analysis.

Molecular identification of bacteria using PCR 16S rRNA sequencing (Figure 1). The results of electrophoresis contained DNA fragments measuring around 1200 bp that were compared to DNA markers as standard DNA fragments (Figure 1a). Nucleotide sequence showed in figure 1b. The BLAST homology of isolate SG-A1 showed that this bacteria is affiliated to genus Enterobacter. The isolate SG-A1 with phylogenetic tree showed most closely related to *Enterobacter hormaechei* with 96% sequence similarities (Figure 2)

Determination of protein production time

The dynamics of bacterial growth can be understood from the growth curve, which is made by measuring the growth rate at a certain time interval and then measuring the measurement results in a graph that shows the relationship between biomass on y-axis versus the time period of measurement on the x-axis. Growth curves in a limited number of mediums will experience the following phases: lag phase (adaptation), exponential phase, stationary phase, and death phase.

Determination or calculation of cell numbers can be done by the direct method or spectrophotometric method by measuring the value of OD (Optical Density = density of bacteria seen as turbidity medium).

In this study, measurements of protein levels from bacterial cells were carried out. This is done to determine the level of intracellular protein from isolate SG-A1. Measurement of protein content was carried out based on the Lowry method with BSA as a standard solution.

The relationship between Optical Density (OD) and intracellular protein content is shown in Figure 3. Data shows that the adaptation phase occurs at 0-18 hours, then the growth phase occurs at 24-36 hours, and optimum at 36 hours. While the phase of death occurs at 42 hours which marked by a dramatic decline in growth. There was an increasing in protein levels along with the length of incubation time with the highest protein content of 1.2 mg/mL.

Toxicity test

The confirmation of LC_{50} values purposes to determine the toxicity of the crude extract of the protein from isolate SG-A1 on various times. The BSLT method is one method for screening compounds that have bioactivity as anticancer because it is cheaper, need shorter time, easier to be developed and there are no ethical rules in the use of test materials. The activity of each crude extract can be determined in LC_{50} values using the probit-

log concentration graph. Data resulting from the calculation of LC_{50} values for *Artemia salina* L. shrimp from crude extract protein can be seen in Table 2.

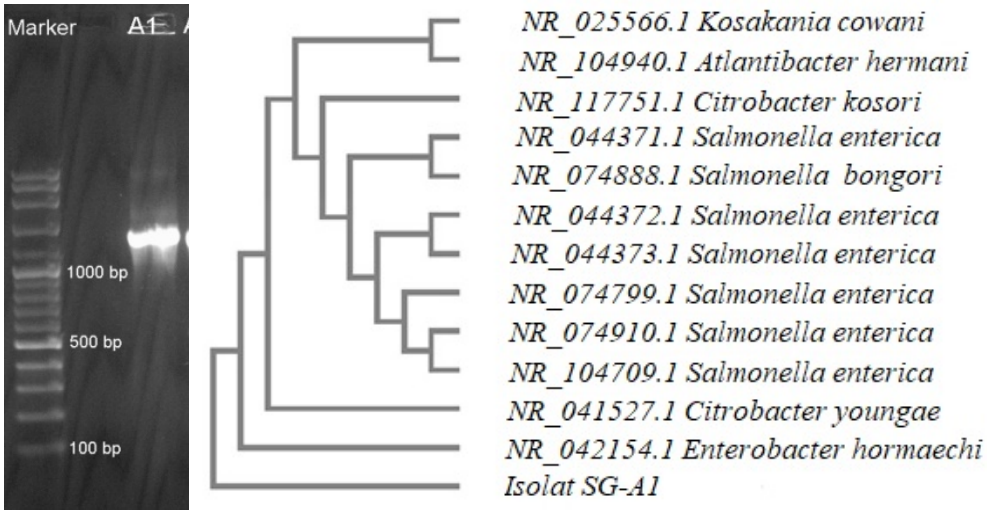
A compound has very toxic activity if its LC_{50} value < 20 µg/mL, toxic activity with LC_{50} value is 20-100 µg/mL, medium toxic activity with LC_{50} value is 100-500 µg/mL, weak toxic activity with LC_{50} value is 500-1000 µg/mL and no toxic activity with LC_{50} value is >1000 µg/mL [12]. From these data, it can be seen that the crude extracts of the protein from Isolate SG-A1 on various time were very toxic and the highest bioactive protein activity was shown with LC_{50} is 1.73 µg/mL at 24 hours fermentation.

Table 1. The biochemical test result for Isolate SG-A1.

Biochemical test		Isolate SG-A1
TSIA	Slant	Alkali
	Butt	Acid
	Gas	+
	H2S	-
SIM	Indole	-
	Motility	-
	H2S	-
MRVP	MR	+
	VP	+
Citrate		+
Urea		-
Glucose		+
Lactose		-
Sucrose		+
Mannitol		+

Table 2. The calculated LC_{50} values of against larvae shrimp (*Artemia salina* Leach.) of protein crude extract of isolate SG-A1

No	Time (hours)	LC_{50} value(µg/mL)	Toxicity
1	6	5.940	Very toxic
2	12	5.782	Very toxic
3	18	4.969	Very toxic
4	24	1.730	Very toxic
5	30	2.042	Very toxic
6	36	2.282	Very toxic



(a) **(b)**

Figure 1. Analysis of 16S rRNA isolat SG-A1. (a) Electrophoresis result of the PCR product; (b) Neighbour-joining phylogenetic tree

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Query  18    TGCTCTCGGGTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGG  77
Sbjct  51    TGCT-TC-GCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGG  108
Query  78    GGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGA  137
Sbjct  109   GGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGA  168
Query  138   CCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACG  197
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Sbjct  349   CTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGG  408
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Sbjct  529   TGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACC  588
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Sbjct  829   ACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGAC  888
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Query  1155  TAGGG  1159
Sbjct  1183  TAGGG  1187
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Figure 2. Nucleotide sequence of isolate SG-A1

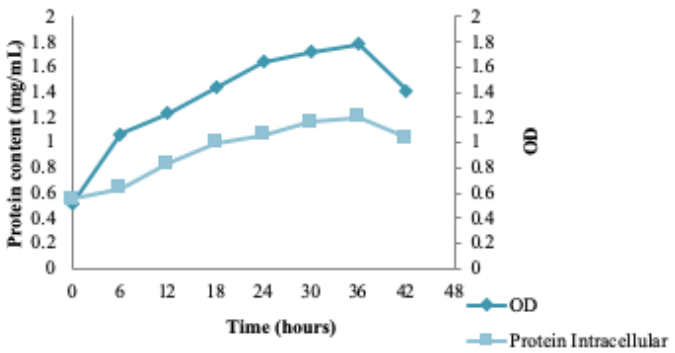

Figure 3. Optimization condition for producing protein by the isolat SG-A1

CONCLUSION

Epiphytic bacteria from marine algae *Sargassum* sp. and the species can be isolated and identified. The optimum time to produce bioactive proteins was evaluated. *Enterobacter hormaechei* showed the highest bioactivity at the time fermentation of 24 h with LC_{50} is 1.73 µg/mL. The results suggest that the bioa ctive protein from isolate SG-A1 are very potential as anticancer agent.

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