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Identification of Protease Producing Halophilic Bacteria from Bledug Kuwu-Mud Volcano

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Abstract

The objective of this research was to isolate and identify the halophilic bacteria from Bledug Kuwu-mud volacano having an ability to produce proteolytic enzyme. From this work, 6 bacterial isolates were obtained from mud and water samples using artificial sea water media after incubation at room temperature. Three out of the 6 isolates (BKL-3, BKL-5, and BKA-1) were selected for further analysis. BKL-3, BKL-5 and BKA-1 exhibited an ability to grow at salt concentration greater than 10%. BKL-3 could grow on media supplemented with 15% of salt, meanwhile BKL-5 and BKA-1 could grow at 20% of salt, respectively. Furthermore, those isolates also exhibited proteolytic activity when they were grown on casein media. The phylogenetic analysis based on the 16SrRNA gene sequences showed that the BKL-3 belong to the group of *Bacillaceae,* whilst BKL-5 and BKA-1 isolates were relatively distance from the group of *Halomonadaceae*. Therefore, BKL-5 and BKA-1 could be considered as the allegedly new species that were separated from *Halomonadaceae.*

Keywords : halophilic, halotolerant, protease, 16SrRNA gene, Bledug Kuwu-mud volcano,phylogenetic tree

Introduction

Hypersaline environments are defined as those in which salt concentration exceed that of seawater. They include artificial and naturally occuring solar saltern in arid areas such as hypersaline lakes, dead sea, hypersaline soils and deep sea zone (Rodriguez-Velera *et al*., 1979; Ma *et al*, 2010; Grant, 2004). Such environments are considered to be extreem for microbial life, because of water availability limitation (Oren, 2008). Yet, the diverse taxa of halophilic (i.e requiring salt for growth) or halotolerant bacteria have been recovered from a wide variety of hypersaline environments (Grant, 2004; Ventosa *et al*., 1998).

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One common characteristic of halophiles is their predicted proteomes have dramatically low isoelectric points (pI). For example, *Halobacterium* sp. NRC-1 proteome contains protein which have an avarage pI of only ~ 4.9. By contrast, the pI of nearly non halophilic bacteria proteomes are close to neutral (Das Sarma, 2006). Furthermore, the proteins of halophilic bacteria are dominated by the negative charge residues on their surfaces. The acidic surface of the halophilic proteins are readily dissolved in high salinity solution and reduce the water activity. These observations help to explain how the metabolic functions can be maintain in hypersaline media (Das Sarma, 2006).

The isolation and identification of halophilic or halotolerant from hypersaline environment is of pratically important because of biotechnological potential with regard to the production of biomolecules such as osmolytes (compatible solutes), hydrolytic enzymes and exopolysaccharides

(Gomez *et al.*, 2004; Oren, 2002; Oren, 2010).

Bledug Kuwu which is located in Kuwu village, Centarl Java, Indonesia is a natural phenomenon that is formed as a result of geological activity over longer period of time. The gas explosion accompanied by mud flow is the unique phenomenon of Bledug Kuwu. Mud mixed with formation water possesses a high content of salt. Therefore, it is interesting to explore the halophilic bacteria inhabit in the Bledug Kuwu-mud volcano. Previous work carried out by Pangastuti *et al.* (2002), reported that they were successfully to isolate the moderately halophilic bacteria which were closely related to marine bacteria.

As this group of bacteria may have biotechnological significant, therefore the objective of this work was to isolate and identify the halophilic bacteria from Bledug Kuwu-mud volcano having an ability to produce proteolytic enzyme. Bacterial isolates were screened using enrichment method in liquid artificial seawater media and sebsequently plated onto agar plate of the same media. The selected isolates were further examined for the salt tolerance test, proteolytic activity and identified by 16SrRNA gene sequences analysis.

Materials and Methods

Samples handling and preparation

Samples for halophilic bacterial isolation were collected from Bledug Kuwu mud-volcano included mud and water samples. The samples were then transported to the laboratory for the analyses or kept in cold storage not more than a week.

Cultivation media

Bacterial isolation was performed using artificial sea water media with the composition of salt were adjusted according to the requirement concentration, except for the $CaCl_2$ and $NaHCO_3$ concentration that were kept similar to their concentration in natural sea water (Subov, 1931). The medium with the 20% (w/v) of salt should have the following composition (%w/v): NaCl 15.6; $MgCl_2.6H_2O$ 1.3; $MgSO_4.7H_2O$ 2.0; $CaCl_2.6H_2O$ 0.1; KCl 0.4; $NaHCO_3$ 0.02; NaBr 0.05 and yeast extract (Difco) 0.1. For making the agar media, the 2% (w/v) of agar (Bacto) was added to the above composition (Rodriguez-Velera *et al.*, 1980). The final pH of each media was adjusted to 7.2.

Isolation of bacteria and test of salt tolerance

Isolation of halophilic bacterial was carried out using enrichment method in artificial sea water media (Subov, 1931). One gram or 1 ml of sample was inoculated into artificial sea water media and then incubated in reciprocal incubator at room temperature for one week. Fifty or one hundreds microliter of aliquots was then surface plated on respective agar media. The plates were then incubated at room temperature for 3 to 7 days and the individual bacterial colonies selected on basis of differences of morphology and Gram reaction.

Salt tolerance for growth was tested by streaking fresh bacterial cultures in duplicate on artificial sea water agar media containing appropriate salt concentration and plates were incubated at room temperature.

Proteolytic activity assay

Proteolytic activity assay was carried out using minimal agar media supplemented with 1% casein. The colonies which indicated the formation of clear zones were judge as positive colonies (Kannan *et al.*, 2001).

Amplification of 16SrRNA gene

16SrRNA genes were amplified from bacterial genomic DNA using bacterial universal primers (forward primer: 5′-AGAGTTTGATCCTGGCTCAG-3′, reverseprimer: 5′-ACGGCAACCT TGTTACGACT-3′, both were manufactured by 1st Base Inc.). The forward primer correspond to the position of 8-27 and the reverse primer to position 1,493-1,512 of the 16SrRNA gene sequence of *E. coli* (Accession

no. U00096) (Edwards *et al*., 1989). PCR was performed using thermal cycler (BioCycler-TCS) with 25 µl of reaction mixture containing 12.5 µl of KAPA2G Fast PCR kit (Kappa Biosystem Inc.). PCR cycling condition were denaturation at 95°C for 2 min, 30 cycles of 95°C for 15s, 55°C for 15, and 72°C for 20s and the final extension at 72°C for 2 min.

Amplified products were separated by 1% agarose gel electrophoresis (Takara Biotechnology-Dalian). The DNA fragments of the correct size (1,500 bases) were excised from the gel and purified using Econo Spin IIa (Gene Design). The purified fragments were then subjected for sequencing by ABI Prism DNA sequencer.

Phyologenetic analysis

16SrRNA gene sequences of bacterial reference strains, type strains and closest phylogentic relatives were selected from GeneBank by subjecting the nucleotide sequences of bacterial isolates to similarity searches using BLASTn (http://www.ncbi.nlm.nih.gov/blast) and SeqMatch (Release 10 update 27, Ribosomal Database Project) (Cole *et al*., 2009). The multiple alignment sequences were done using ClustalW as implemented in MEGA5 (Tamura *et al*., 2011).

Phylogenetic tree was reconstructed using MEGA 5 (Tamura *et al*., 2011). The model of nucleotide substitution was selected on the basis of the Bayesian Information Criterion implemented on MEGA 5 (Tamura *et al*., 2011). Evolutionary histories were inferred using neighbor-joining method (Saitou and Nei, 1987) and bootstrap consensus trees inferred from 1,000 permutations of the data sets (Felsenstein, 1985). Evolutionary distances were computed using the Tamura-Nei method (Tamura and Nei, 1993) as the number of base substitutions per site. The rate variation among sites was modeled with gamma distribution (shape parameter = 1.68). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

Results and Discussion

Six isolates were successfully obtained from mud and water samples of Bledug Kuwu mud volcano. Although the range of different colony morphology is similar, at least, two different colony morphology appearances of isolates were successfully discriminated from each enriched media containing 10%, 15% and 20% of salt. In addition to the differences of colony morphology, those isolates were also tested of their reaction against Gram-staining. Five of six isolates (BKL-1, BKL-2, BKL-4, BKL-5 and BKA-1) were Gram-negative and only one isolate (BKL-3) gave Gram-positive reaction (Table 1).

Among six isolates, five of these were isolated from mud sample, while one isolate was obtained from water sample. Data from salt tolerance test of 6 isolates indicated that all were capable to grow on agar media containing between 10 to 20% (w/v) of salts (Table. 1).

Table. 1. Characteristics of halophilic bacteria isolated from Bledug Kuwu-mud volcano

Characteristics	Isolates					
	BKL-1	BKL-2	BKL-3	BKL-4	BKL-5	BKA-1
Colony morphology	Circular, umbonate, entire	Circular, raised, entire	Irregular, crateriform, undulate	Circular, umbonate, entire	Irregular, convex, undulate	Circular, raised,entire
Colony color	Brown	Transparent	White	Yellowish-white	Yellowish-white	White
Cell morphology	rod	rod	rod	rod	rod	rod
Gram reaction	Negative	Negative	Positive	Negative	Negative	Negative
Salt concentration	10%	10%	15%	15%	20%	20%
Proteolytic activity	+	-	+	+	+	+
Sources	mud	mud	mud	mud	mud	water

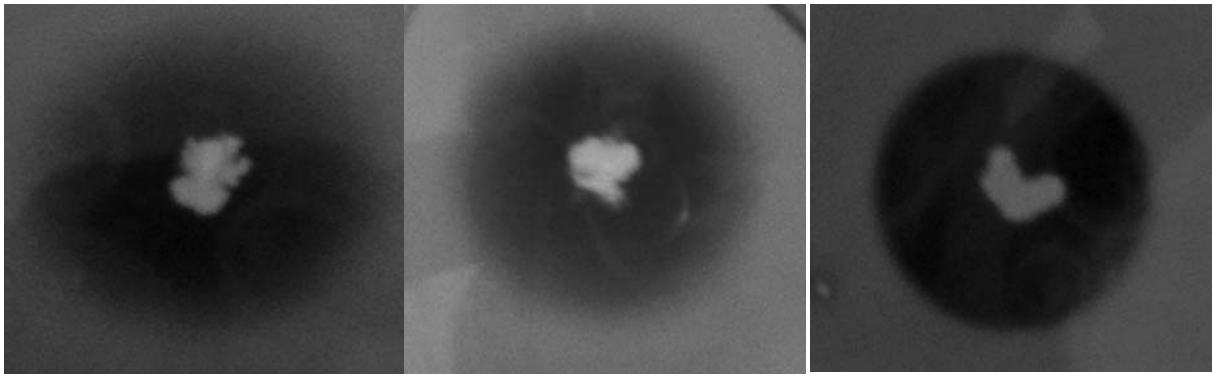

Figure 1. Proteolytic activity test of selected isolates. The protoelytic assay was performed on minimal media supplemented with 1% casein.

The proteolytic activity analysis of isolated halophilic bacteria showed that among the 6 isolates only 1 which gave the negative reactions (Table 1). From the 5 isolates which gave the positive result on proteolytic activity, 3 isolates (BKL-3, BKL-5 and BKA-1) were selected for further analysis (Figure 1).

The proteolytic index analysis which was defined as the ratio of clear zone and colony diameter showed that BKL-5 gave the highest proteolytic index compared to BKL-3 and BKA-1. The proteolytic index values of BKL-3 and BKA-1 were not significantly different (Table 2). However, the result suggests that the BKL-3, BKL-5, and BKA-1 are good candidate for protease production. In order to understand more the protease from these bacteria, mainly BKL3, BKL-5 and BKA-1, further characterization on the enzyme is necessary. The work on proteases characterization is currently underway.

Table 2. The proteolytic activity of isolated halophilic protease producing bacteria

Isolates	Proteolytic index*)
BKL-3	3.33 ± 0.42a
BKL-5	4.25 ± 0.43b
BKA-1	3.02 ± 0.31a

*) The proteolytic index was calculated by: $\frac{\theta \text{ of clear zone}}{\theta \text{ of colony}}$

The letters following the protolytic index values which represent 95% significant levels are shown. Errors which represent 68% confidence limits are also shown.

For phylogenetic analysis, the 16SrRNA genes from selected 3 isolates, BKL-3, BKL-5 and BKA-1 were amplified using PCR and the resulting fragments (1,500 bp) were then subjected for sequencing (Figure 2).

The phylogenetic tree was reconstructed on the basis of 16SrRNA gene sequences of

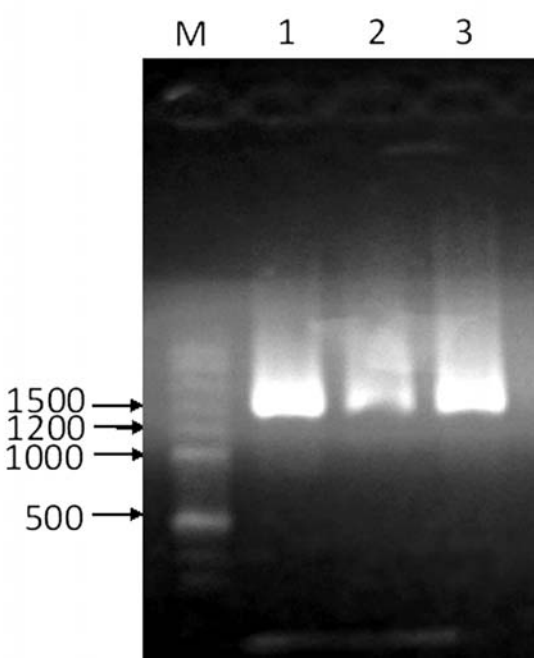

Figure 2. Amplified 16SrRNA gene from halophilic bacteria. Lane M : *100 bp DNA ladder;* lane 1: BKL-3; lane 2: BKL-5; lane 3: BKA-1;

BKL-3, BKL-5 and BKA-1. The 16SrRNA gene sequences of those three isolates were subjected for the BLASTn analysis (http://www.ncbi.nlm.nih.gov/blast) for searching the closest phylogenetic relatives (Table 3). On the basis of BLAST analysis, BKL-3 was closely related to the group of *Bacillaceae* by which the closest relative was *non type strain*, *Bacillus* sp (99% identity). However, BKL-5 and BKA-1 showed the lower identity value (≤ 98.7%) to the *non type strain* species *Halomonas* sp. strain S2 and B140_1. Strains with sequence identity of 98.7% or less can be generally considered as a member of different species (Schleifer, 2009). Therefore, those two isolates could be considered as allegedly new species.

The phylogenetic analysis against *type strain* of the group of *Bacillaceae* and *Holomonadaceae* showed that BKL-3 certainly belong to group of *Bacillaceae* with the bootstrapping value of 100%. Meanwhile, BKL-5 and BKA-1 were out grouping from the group of *Halomonadaceae* and also it could be inferred from the phylogenetic tree that those two isolates were closely related to each other (*bootstrapping* value of 100%) (Figure 3).

Table 3. The closest phylogenetic relatives of bacterial isolates from Bledug Kuwu-mud volcano

Isolates	Closest relatives*)	Sequence identity
BKL-3	*Bacillus* sp.	99%
BKL-5	*Halomonas* sp. S2	98%
BKA-1	*Halomonas* sp. B140_1	96%

In this work, the numbers of obtaining isolates were too low, because the work was done by culture dependent approaching. The culture dependent will not be able to produce large diversity since many bacteria inhabiting saline environment are intractable to cultivation. Perhaps, it is not surprising that culture independent approaches have identified far greater bacterial diversity (Tang *et al*., 2011).

From this work the three halophilic protease producing bacteria were successfully isolated and identified. From our results suggested that BKL-3 was considered as the member of *Bacillaceae,* whilst BKL-5

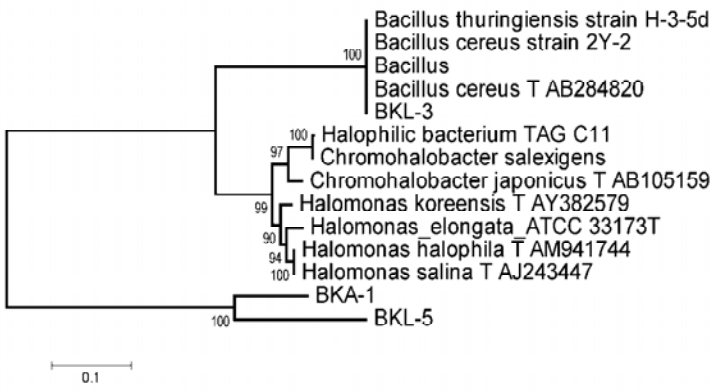

Figure 3. Evolutionary relationships of bacterial isolates from Bledug Kuwu mud volcano. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.19 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura-Nei method and are in the units of the number of base substitutions per site. The analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 671 positions in the final dataset. Evolutionary analyses were conducted in MEGA5. The 16SrRNA gene sequence of BKL-5 has been deposited at Gene Bank data base with Gene Bank Accession Number JQ791106.

and BKA-1 neither belongs to the group of *Halomonadaceae* nor *Bacillaceaea*. Therefore, BKL-5 and BKA-1 were considered as allegedly new species which were separated from the group of *Halomonadaceae*. The results also suggested that BKL-5 and BKA-1 were closely related species.

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