Short Communication:

Terasi, an Indonesian fermented shrimp paste, a new reported source of L-Asparaginase-Producing Bacteria

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Abstract

Terasi, a fermented fish/or shrimp paste, is the main food ingredient in Indonesian cuisine. It can never be explored as a source of L-asparaginase. The objective of the research was to investigate the bacterial producing L-asparaginase derived from terasi. The isolated bacteria were investigated for their capacity to produce L-asparaginase. The highest producer of L-asparaginase has further analyzed its species by using a Scanning electron micrograph and 16s rDNA molecular method. Eleven of bacterial were successfully cultured and screened for its ability to produce L-asparaginase.

Two isolates indicated high activity, among others. 16s rDNA analysis revealed that the highest producer was identified as Bacillus cereus strain T4. Terasi is a potential source for L-asparaginase-producing bacteria. In this study, we reported the Indonesian fermented shrimp, terasi as a source for L-asparaginase-producing bacteria.

Keywords: *Terasi*, L-asparaginase, Indonesia, *Bacillus cereus*, fermented product.

Introduction

Fermented products are well known as one of the sources generally recognized as safe (GRAS) microorganisms. These products have been used for the mining of useful metabolites, either primary or secondary metabolites. Lactic acid bacteria (LABs) are perhaps the most famous bacteria related to the food fermentation process. The bacteria included in this fermentation possess beneficial properties toward food production or conversion of chemical compounds. They also improve food safety and quality[11]. Therefore, by exploring and isolating the bacteria from a fermented product, the discovery of GRAS microorganisms with desired properties is possible.

Fermentation product is usually rich in functional compounds. Several fermented foods have been reported as a source of antioxidants, enzymes and also anti converting enzyme inhibitor[4,9,19]. *Terasi*, an Indonesian food fermented product made of fish/or shrimp, is the main food ingredient in almost every Indonesian cuisine. It is a paste form and has been used as a condiment and umami enhancer due to its high glutamic acid and its specific flavour. A study indicated that this product could be used as a source for isolating enzyme-producing bacteria[2,14]. Hence, in this study, we screened L-ASNase-producing bacteria from these two Indonesian fermented foods. The objective of the research was to explore the bacterial which is producing L-asparaginase derived from *terasi*, an Indonesian fermented shrimp paste.

Material and Methods

Terasi preparation: *Terasi* was bought from the local market near Brawijaya university. *Terasi* was weighted for 1 gr. The samples were mixed with Na physiologic (NaCl 1%). Hundred µl samples were spread onto LB agar medium containing 1, 5, 10 % NaCl. The plates were incubated at 37 °C for 24-48 h. Bacterial colonies were transferred to a new medium to obtain a single pure colony.

Enzyme preparation: Bacterial strains obtained from Indonesia fermented foods and cyanobacteria were cultured in a different medium. The bacteria were cultured in LB medium (peptone 1%, yeast extract 0.5%, NaCl 1%). The bacteria were pre-cultured in 5 ml before transferring into a 200 ml LB medium. Harvesting was conducted after 24-h incubation at 37 °C. The medium was centrifuged for 15 min at 12.000 rpm. Pelleted cells were washed twice with ten mM KPB (pH 7). The cells were disrupted using sonication for 10 min (10 min on and 40 min off). The solution was centrifuged to separate a crude enzyme extract with cell debris. Before enzyme assay, the crude extract was dialyzed against KPB at pH 7.

L-Asparaginase Assay: The proteins were assayed using the Lowry et al[7] method using egg albumin as a standard. ASNase activity was measured by Nessler method. A mixture of 150 µl of a crude enzyme, 50 µl of 1 M KPB (pH 7), 200 µl of deionized water, and 100 µl of 150 mM L-asparagine was incubated for 30 min at 30 ˚C. A solution of 125 µl of 20% trichloroacetic acid (TCA) was added to the mixture for stopping the reaction. The mixture was then centrifuged at 2,000 rpm for 15 min. To 450 µl of pipetted supernatant, 125 µl of the Nessler solution was added. The solution was maintained for 15 min to allow the appropriate reaction between the Nessler solution and ammonia. The results were read at OD480 nm. One unit of enzymatic activity was defined as 1 µmol of ammonia per minute under the described condition.

Bacterial Identification: The identification of bacteria was only performed for the best L-asparaginase production. The analysis was performed using SEM and 16S rDNA analysis. Bacterial cells were examined via scanning electron micrograph with HITACHI-S-530 at a voltage of 5 kV. For molecular analysis, total genomics was extracted using a standard phenol-chloroform method. Universal primers, 27F (5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R 5'-TACGGYTACCTTGTTACGACTT-3') were used for PCR amplification of the 16S rDNA gene.

PCR reaction mixture (50 µl) contained 34 µl miliQ, 1 µl of DNA, 2.5 U of blend Taq (Toyobo Co. Japan), 5 µl of blend Taq buffer, 5 µl of dNTP mixture (0.2 mM each), and 50 pmol of each primer. PCR amplification had the following program: 94°C for 2 min, followed by 32 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 2 min and a final extension period of 72°C for 4 min. PCR products were purified using a PCR purification kit (Viogene Co.).

Amplified 16S rDNA fragment was sequenced for both strands determined using an ABI Prism Big Dye Terminator cycle sequencing. The 16S rDNA gene sequence was analyzed using BLAST (NCBI GenBank database) to find closely related bacterial 16S rDNA sequences. Clustal X analysis was performed for investigating multiple alignments among the sequences. The phylogenetic tree was constructed using the Neighbor-Joining method[15]. MEGA 5.2.2 was used for the drawing of a phylogenetic tree.

Results and Discussion

L-asparaginase-producing bacteria: From *terasi*, eleven bacteria were isolated. Based on the analysis of intracellular L-asparaginase, all isolates had L-ASNase activity. Their total activities ranged from 0.04 to 1.73 U (Table 1). However, only T4 strain exhibited the highest L-ASNase activity. Its specific activity was 34 times higher than that compared to the lowest value of L-ASNase, which was derived from T3 bacteria. Similar research was also conducted by Syal and Vora[17] who isolated L-asparaginase producing microorganisms from several fermented foods. L-ASNase-producing bacteria are widespread in nature[13].

Species identification: The biochemical and molecular techniques were conducted to confirm the identity of the T4 isolate. The phylogenetic tree of the strain, which was based on a comparative analysis of the 16S rRNA gene sequence (1536 bp), is depicted in fig. 1A. On the basis of 16S rRNA gene sequences, a T4 isolate is a new strain of *Bacillus cereus* with 99% similarity compared to other 16SrDNA data from *Bacillus cereus*. Hence then we called *B. cereus* strain T4. It is gram-positive, rod-shaped bacteria. SEM analysis revealed that this *B. cereus* strain T4 has a length of 2.3 µM (Fig.1B).

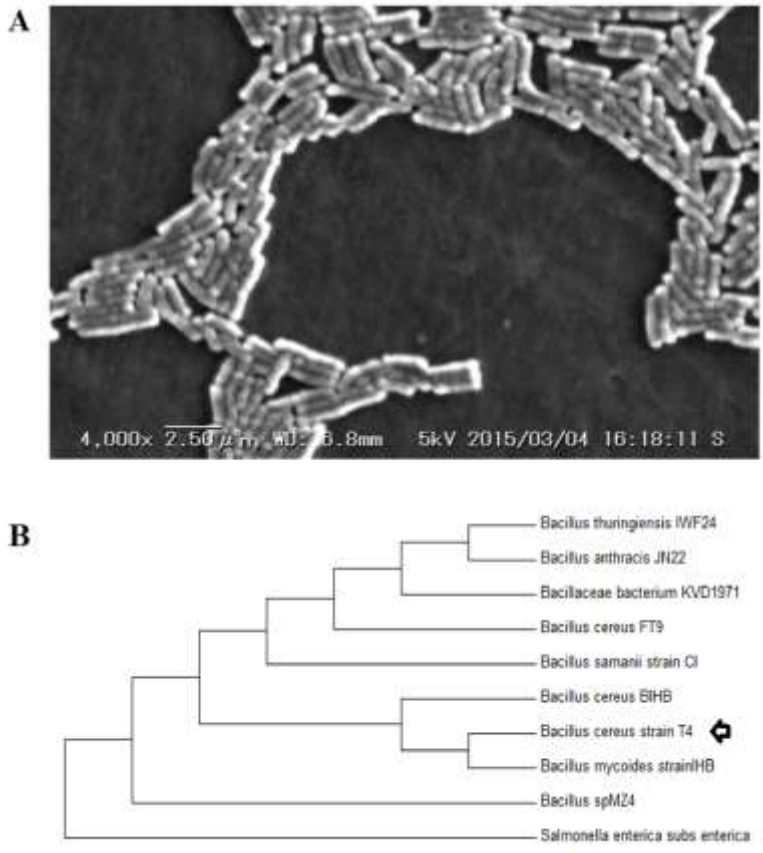

Fig. 1: Identification of isolate. (A) SEM of isolate T4; (B) Neighbor-joining tree showing the position of the *B. cereus* strain T4 based on the partial 16S rRNA sequence comparison. *Salmonella enterica subs enterica* was used as an outgroup

Table 1
L-Asparaginase-producing bacteria isolated from *terasi.*

Code	Total Prot (mg)	Total Activity (U)	Specific Activity x 10^{-3} (U/mg)
T1	112.63	0.33	2.94
T2	84.14	0.07	0.89
T3	97.45	0.04	0.42
T4	126.83	1.73	13.68
T5	107.78	0.08	0.78
T6	46.09	0.09	2.08
T7	58.11	0.10	1.82
T8	68.80	0.70	10.17
T9	48.80	0.20	4.21
T10	70.39	0.19	2.70
T11	42.54	0.13	3.06

In this experiment, only *B. cereus* strain T4 exhibited relatively high activity. *Bacillus* species are well known as one of L-ASNase producers. Other researchers have studied *B. subtilis 168*, *B. circulans*, *B. cereus MAB5*, *B. brevi*s. However, all of them focused on extracellular L-ASNase[5,10,12,18].

B. cereus is a gram-positive, aerobic rod shape, and spore-forming bacterium. It accounted as an opportunistic human pathogen and one of the food poisoning organisms. An emetic or diarrhoea type of food-associated illness was mainly among the reported effect of *B. cereus*-produced toxin. Its enterotoxin and an emetic toxin called circular dodecadepsipeptide (D-O-Leu-D-Ala-L-O-val-L-val3) or cereulide are the most well-known as *B. cereus* virulence factors[1,6]. Moreover, several other diseases in relation to *B. cereus* have also been reported[3]. This fact leads us to halt our chances to precede this bacterium.

Even though *B. cereus* has a wide distribution in nature and can be found in several processed foods, the presence of *B. cereus* in *terasi* was most probably due to contamination. *B. cereus* was frequently isolated from soil and growing plants[16]. Its presence in food is usually related to contamination due to contact with soil or air. Considering the process in *terasi* production where sun drying is commonly involved in air contamination likely happens.

Conclusion

Our investigation revealed that *terasi* is a good source for L-ASNase -producing bacteria. *B. cereus* strain T4 was the best producer of L-ASNase. However, due to its virulence factors, further investigation is needed.

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