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P-ISSN: 2302-8270
E-ISSN: 2808-1048

Screening and Identification of Fibrinolytic Bacteria From Tauco

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Article History:
Received: August 1, 2022; Revised: September 22, 2022; Accepted: October 29, 2022; Online: December 1, 2022

ABSTRACT

Tauco was one kind of traditional fermented food from Indonesia. Tauco was processed from soybean paste with a salty taste and commonly had yellow and black colors. Generally, tauco was used as a spice or seasoning in various daily dishes. The study aimed to find and identify the bacteria that produced fibrinolytic enzymes in tauco. The sample used in this research was tauco from six different manufacturers. Two tests were conducted in this study: a proteolytic test on Skim Milk Agar and a fibrin plate test. Both tests were used to identify the bacteria producing fibrinolytic enzymes. The bacteria were identified macroscopically, through gram staining, and by 16S rRNA sequencing. In the proteolytic test, the sample was diluted with 0,9% NaCl to 10^-7 and then inoculated on Skim Milk Agar medium, incubated at 37ºC for 24 hours. The samples had proteolytic activity, which was shown by the formation of clear zones around the bacteria. Seventeen isolates of proteolytic bacteria were incubated at 37ºC for 24 hours and then used for the fibrin plates. All proteolytic bacteria produced fibrinolytic activity, with the most extensive fibrinolytic index in the TC4a isolate of 3,46. Furthermore, TC4a bacteria were genotypically identified by sequencing the 16S rRNA gene. According to the results of tauco identification, the bacteria that strongly produced fibrinolytic enzymes in the tauco sample TC4a were found to be similar to *Bacillus cereus* TC4a.

Keywords: Fibrinolytic enzyme, Fibrinolytic activity, Tauco, Screening, identification, 16s rRNA, *Bacillus cereus*

INTRODUCTION

A fibrinolytic enzyme is a group of protease enzymes that can destroy blood clots (fibrin). Several studies have been conducted to find sources of fibrinolytic enzymes that can be obtained cheaply and quickly. Fibrinolytic enzymes are proteins obtained from plants, animals, or microorganisms. Some fibrinolytic enzymes found in microorganisms are bacteria, actinomycetes, and fungi (Peng et al., 2005). There are many advantages when consuming foods with fibrinolytic enzymes, so there is an opportunity to explore new sources of fibrinolytic enzymes from Indonesian fermented foods, for example, in Tempe, soy sauce, and tauco. Based on research conducted by Anh et al. (2015), it is known that fibrinolytic enzymes are produced from *Bacillus* sp. CK 11-4 strain obtained from screening results of Korean traditional fermented food named chungkookjang. In Indonesia, one of the chungkookjang.

Fermentation products are tauco. Tauco is also processed from soybeans in the form of pasta, some are yellow, and some are black; the taste is somewhat salty, often consumed by most Javanese, made by a fermentation process and commonly used as a seasoning or flavoring food in various daily dishes. The Tauco fermentation process has two ways: mold fermentation and salt. In salt fermentation, the microbes that play a role are *Lactobacillus delbrueckii*, *Hansenula* sp, and *Zygosacchar myces*. At the same time, the Microbial mold fermentation plays a role in *Aspergillus oryzae*, producing enzymes such as amylase, protease, and lipase. With this mold, there will be a breakdown of the components of the material (Handoyo et al., 2006). Qualitatively, fibrinolytic activity can be proven using the fibrin plate method. Positive results can be seen from forming clear zones due to the degradation of fibrin clots(Ashipala & He, 2008). The characterization of fibrinolytic

The enzyme that produces bacteria can be carried out by microscopic characterization of bacterial morphology by doing gram staining to determine the type of bacteria, including gram-positive or gram-negative, and the form of bacteria and endospores staining. Then, bacterial morphology will be characterized macroscopically (Poernomo & Sudjarwo, 2020). This study was conducted to identify bacteria that can produce fibrinolytic enzymes in tauco. With this study, the researcher is expected that the bacteria producing fibrinolytic enzymes in tauco can improve alternative foods to prevent thrombosis and cardiovascular disease (CVD).

MATERIAL AND METHODS

The sample used in this study is tauco which is sold in the market around Surabaya (T1, T2, T3, T4, T5, and T6). T1, T2, and T3 samples are tauco obtained from traditional markets, while T4, T5, and T6 are tauco obtained from non-traditional markets in East Surabaya. In this study used 0,9% NaCl Solution, high casein Skim Milk So (SMA), fibrinplate. This study uses other solutions and chemicals: spirtus, 70% alcohol, distilled

Cite this as Setyawati, E., Poernomo, A. T., and Sudjarwo, S. (2022) 'Screening And Identification of Fibrinolytic Bacteria From Tauco', *Berkala Ilmiah Kimia Farmasi*, 9(2), pp.44-48. DOI: 10.20473/bikfar.v9i2.42707

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E-ISSN: 2808-1048

water, phosphate buffer, fatty cotton, methylene blue, crystal violet, iodine, safranin, and malachite green solution.

Proteolytic Activity Test

Measure 15 ml of Skim Milk Agar (SMA) media, pour it into a petri dish, and wait until it solidifies. A total of 500 µL of diluted samples were dropped into SMA media and then spread on the surface of solid media using sterile cotton swabs; this method is called a spread plate, and then incubated at 37°C for 24 hours. If bacteria are producing proteolytic enzymes, a clear space will be formed (Poernomo & Sudjarwo, 2020; Modification of Rani, 2013).

Bacteria Maintenance

Bacteria with proteolytic activity were maintained by taking 1 ose of bacteria and then slitting into Nutrient Agar (NA) media, then incubated at 37°C for 24 hours. Colonies that were obtained were stored in a refrigerator at 20ºC.

Fibrinolytic Activity Test

Using the well diffusion method, bacterial isolates obtained from proteolytic assays were tested for fibrinolytic activity. Made using a sterile pit, fill in 50 µL of bacterial suspension of isolates in 0,9% NaCl solution and incubate at 37°C for 48 hours. The formation of clear zones around the wells indicates positive results. Fibrinolytic activity was determined by the Enzyme Activity Index, which compares the diameter of the clear zone with the diameter of the hole / well (modification of Ashipala, O.K and He, Q., 2008).

Phenotype Bacteria Identification

Phenotype identification of bacteria was carried out by microscopic and macroscopic characterization of bacterial morphology. For microscopic, it is done by gram staining test, using glass objects cleaned with 70% alcohol and given distilled water, heated over a flame. Taken 1 ounce of bacterial culture aseptically, flattened on the glass object, and fixed above the flame. Then a crystal violet solution (Gram A) is added, allowed to stand for 1 minute, washed with running water, and dried. Then dripped with iodine solution (Gram B), left for 1 minute, washed with running water, and dried. Then rinse with a bleach/alcohol solution (Gram C) for 30 seconds rinse with running water, and dry. Furthermore, safranin (Gram D) solution or dropping agent is added, allowed to stand for 2 minutes, washed with running water, dried, and observed using a microscope with strong magnification.

The staining indicates that Gram-positive bacteria produce violet color and Gram-negative bacteria produce red. Then also observed the shape of the bacterial cell, whether round (coccus), stem (basil), or wavy (spiral) (Mizanur Rahman et al., 2011; Hastuti et al., 2017; Stephani et al., 2017). Macroscopic characterization of bacterial morphology conducted by bacterial isolates characterized by growing bacteria on Nutrient agar plate media obtained in the form of stab growth and bacterial colony growth on agar plate medium, namely shape, edge, elevation, surface color, colony diameter, and configuration (Poernomo & Sudjarwo, 2020).

Genotype Bakteria Identification

In genotype identification of bacteria, 16S rRNA was identified with several stages, namely making agarose gel electrophoresis, amplification process of the gene encoding 16S rRNA with PCR technique, sequencing of 16S rRNA encoding genes, and constructing three phylogenetic trees. Agarose gel electrophoresis techniques can be used to analyze DNA, RNA, or protein. Agarose gel was made by dissolving 0,4 grams of agarose powder in 40 mL of 0,5x TAE buffer to create a 1% agarose gel. DNA samples were mixed with Dye Loading buffer at a ratio of 3:1, then electrophoresed at 60-70 volts until the blue color migrated approximately ¾ of the gel. The agarose gel was then soaked in 0,5 µg/mL EtBr solution in 0,5x TAE buffer for 5-10 minutes. After this, DNA bands could be observed with UV light and documented with a camera.

For bacterial isolate TC4a, the gene amplification process encoding 16S rRNA using PCR involved denaturation at 96°C for 1 minute, annealing at 53°C and 55°C for 1 minute, and extension at 72°C for 1 minute 30 seconds, with a total of 30 cycles. Amplification began with initiation at 95°C for 50 seconds and ended with final elongation at 72°C for 10 minutes. The PCR product was then electrophoresed to determine the size of the base pairs. Sequencing of the 16S rRNA encoding genes was carried out at the Macrogen laboratory in Korea. The sequencing analysis results of 16S rRNA provided the nucleotide sequence from the bacteria, which was used to determine phylogenetic relationships and serve as molecular markers. Phylogenetic tree construction was performed after obtaining the bacterial nucleotide sequence from 16S rRNA sequencing. The sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) via the site address http://blast.ncbi.nlm.nih.gov/Blast.cgi. Alignment was visualized using the Clustal W program, and the phylogenetic tree was constructed using the MEGA7 program.

RESULT AND DISCUSSION

Proteolytic Activity Test

Using the spread plate method, the proteolytic activity test of 6 tauco samples using Skim Milk Agar (SMA) or skim milk medium was incubated at 37ºC for

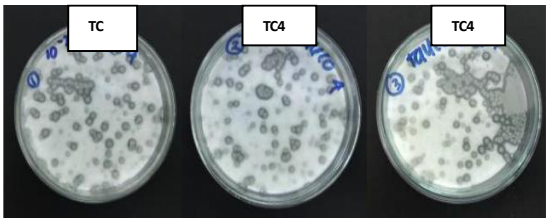

Figure 1. Proteolytic Activity Test Results show the bacterial isolate of tauco TC4a 10-7 dilution on Skim Milk media. Each sample is replicated 3x using the spread plate method.

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E-ISSN: 2808-1048

24 hours. The formation of clear zones around bacterial colonies indicates positive results.

Based on Figure 1 shows that the TC4a sample is the sample that produces the most considerable proteolytic activity in the white SMA media incubated for 24 hours and 10-7 dilution; it can be seen that there is a colony of proteolytic-producing bacteria indicated by a clear zone. The clear zone can be formed due to breaking the peptide bonds in casein to amino acids. Of the many clear zones formed, only a few bacterial colonies that have the largest size and are distinguished from other bacterial colonies are selected. Furthermore, bacteria found in the clear zone will be cultured on Nutrient media to be tilted.

Bacteria Culture

Bacteria that produce proteolytic activity are grown on Nutrient Agar (NA) slant media, incubated in an incubator at 37°C for 24 hours, then stored at 10-20ºC. The culture is used as a stock for further testing. Each culture is given a sample code with the TC format followed by the sample numbers of tauco (TC1a, TC1b, TC2a, TC2b, TC2c, TC3a, TC3b, TC3c, TC4a, TC4b, TC4c, TC5a, TC5b, TC5c,TC6a, TC6b, and TC6c).

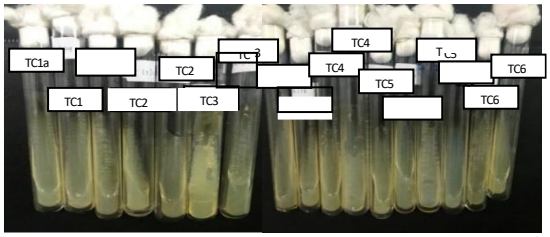

Figure 2. Bacterial Culture of Proteolytic Test Results from various Tauco samples on dilution of 10-7 NA slant media.

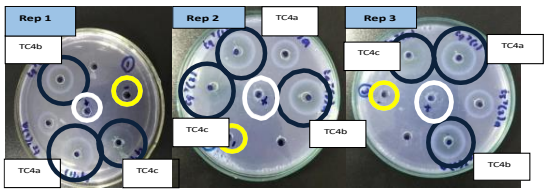

Figure 3. Fibrinolytic Activity Test Results of TC4a 10-7 dilution in SMA media with well diffusion method showed positive results indicated by clear zones with blue circles.
(-) = negative control (0,9% copy) (marked yellow circle);
(+)= nattokinase (marked white circle);
Number 1,2,3 =replication of bacterial inoculation.

Figure 2 Bacterial Culture of Proteolytic Test Results from various Tauco samples on dilution of 10-7 NA slant media.

A clear zone around the hole shows positive results, marked with a blue circle (Figure 3). The clear zone can be formed due to bacteria's breakdown of the fibrin substrate into amino acids. The fibrinolytic activity test uses positive and negative controls—a positive control using nattokinase and negative control using 0,9% NaCl. Nattocination is used as a positive control because it has fibrinolytic activity. Then the fibrinolytic index was determined, which was obtained by measuring the diameter of the clear zone using calipers with an accuracy of 0.05mm. The fibrinolytic index is obtained by calculating the diameter of the clear zone divided by the diameter of the hole. The results of the enzyme fibrinolytic index can be seen in table colony color, and these results can be determined, including Gram-positive or negative. Gram stain observation showed that the TC4a bacterial isolate was a Gram-negative form of *bacillus*. These results are supported by Rozirwan (2009), that Gram-negative indications will be red while the Gram-positive bacterial staining will be violet. The results of staining of TC4a bacterial isolates in the figure in Figure 4 show Gram-negative and bacillary forms.

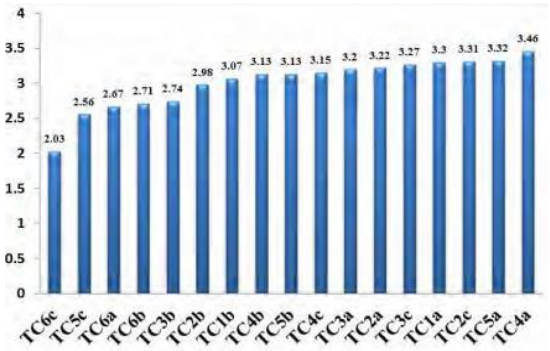

Figure 4. Chart of Fibrinolytic Activity Test Results of Tauco Bacterial Isolates on fibrin plate media by well diffusion method (with an incubation time for 24 hours at 37ºC).

Based on the results of the fibrinolytic activity test obtained from tauco bacteria isolates on fibrin plate media with the excellent diffusion method, the most significant fibrinolytic index results were found in TC4a samples.

Table 1. Tauco Bacterial Fibrinolytic Isolate Index Activity Test. TC4a samples are bacteria with the most extensive fibrinolytic index on fibrin plate media with well diffusion method.

Sample Code	Average fibriolytic index ±SD
TC1a	3.30±0,12
TC1b	3.07±0,07
TC2a	3.22±0,16
TC2b	2.98±0,14
TC2c	3.31±0,03
TC3a	3.20±0,10
TC3b	2.74±0,08
TC3c	3.27±0,10
TC4a*	3.46±0,07
TC4b	3.13±0,45
TC4c	3.15±0,20
TC5a	3.32±0,06
TC5b	3.13±0,05
TC5c	2.56±0,20
TC6a	2.67±0,11
TC6	2.71±0,17
TC6c	2.03±0,14

Phenotype identification of bacteria (Table 1).

Characterization of bacteria by microscopy is conducted by Gram staining

The colony's shape and colour are observed under a microscope, and the Gram-positive or negative status can be determined based on the results. Gram stain observation showed that the TC4a bacterial isolate was a Gram-negative form of *bacillus*. These results are supported by Rozirwan (2009), that Gram-negative

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E-ISSN: 2808-1048

indications will be red while the Gram-positive bacterial staining will be violet. Figure 4 shows the outcomes of staining TC4a bacterial isolates. Display the bacillary and gram-negative forms.

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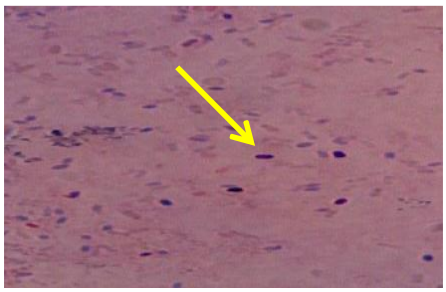

Figure 5. Characterization of Bacterial Staining.

staining TC4a bacterial isolates. Display the bacillary and gram-negative forms.

Macroscopic characterization

Subsequent phenotypic identification, namely macroscopic characterization, can be observed visually. These results can be observed in the form of the colony, colony colour, shape of the edge of the colony, and elevation. The appearance of TC4a bacterial isolates is round, dull-white, with flat edges and uneven elevation. All colonies in growing media showed that the colour and shape were homogeneous, so it can be concluded that the TC4a bacterial isolate in Figure 6 grew.

Figure 6. Macroscopic results of bacterial samples of TC4a on Nutrient media In order to be caught by petri.

Genotype Bacteria Identification
Amplification with PCR and electrophoresis.

The first stage of TC4a DNA isolation was carried out using the boiling lysis method, after which the DNA obtained was amplified by PCR using a universal primer resulting from PCR and then viewed under UV light The PCR DNA results of TC4a bacteria in Figure 6 obtained DNA fragments in the form of a band measuring 1,500 bp and appeared parallel to the DNA marker band size of 1,500 bp. This is consistent with the statement that the length of the 16s RNA is around 1,500-1,600 bp (Figure 7).

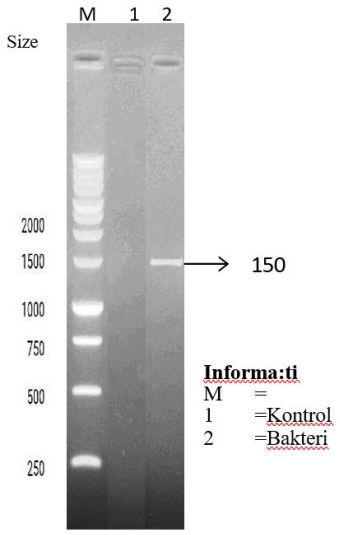

Figure 7. Bacterial DNA PCR results in Agarosa media.

AACGTACACGCGTATCACGTGAGAGCAGTTTAG
CGAGCGAATGGATTAAGAGCTTGCTACATTATG
AAGTTAGCGGCGGACGGGTGAGTAACACGTGG
GTAACCTGCCCATAAGACTGGGATAACTCCGGG
AAACCGGGGCTAATACCGGATAACATTTTGAAC
CGCATGGTTCGAAATTGAAAGGCGGCTTCGGCT
GTCACTTATGGATGGACCCGCGTCGCATTAGCT
AGTTGGTGAGGTAACGGCTCACCAAGGCAACG
ATGCGTAGCCGACCTGAGAGGGTGATCGGCCAC
ACTGGGACTGAACACGGCCCAGACTCCTACGGG
AGGCAGCAGTAGGGAATCTTCCGCAATGGACG
AAAGTCTGACGGAGCAACGCCGCGTGAGTGAT
GAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAG
GGAAGAACAAGTGCTAGTTGAATAAGCTGGCA
CCTTGACGGTACCTAACCAGAAAGCCACGGCTA
ACTACGTGCCAGCAGCCGCGGTAATACGTGGTG
GCAAGCGTTATCCGGAATTATTGGGCGTAAAGC
GCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAA
GCCCACGGCTCAACCGTGGGGTCATTGGAAACT
GGGAGACTTGAGTGCAGAAGAGGAAAGTGGAA
TTCCATGTGTAGCGGTGAAATGCGTAGAGATAT
GGAGGAACACCAGTGGCGAAGGCGACTTTCTG
GTCTGTAACTGACACTGAGGCGCGAAAGCGTGG
GGAGCAAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGAGTGCTAAGTGTTAGAGGG
TTTCCGCCCTTTAGCTGAAGTTAACGCATTAAGC
ACTCCGCCTGGGGAGTACGGCCGCAAGGTTGAA
ACTCAAAGGAATTGACGGGGGCCCACAAGCGG
TGGAGCATGTGGTTTAATTCGAAGCAACGCGAA
GAACCTTACCAGGTCTTGACATCCTCTGAAAAC
CCTAGAGATAGGGCTTCCCCTTCGGGAGCAGAG
TACAAGTGGTGCATGGTTGTCGTCCGCCCCGGG
CCGGGAAAAGTTGGGTTAAGTCCCGCAACGAG
GCCAACCCTTGATCTTAGTTGCCATCATTAAGTT
GGGCACTCTAAGGGGACTGCCGGTGACAAACC
GGAGGAAGGTGGGGATAACGCCAAATCTCCAT
GCCCCTTATGACCGGGGGCAACCACGGGCTCCA
ATGGGAGGGGCAAAAAGTTCCAAAACCGCGGG
GGGGGGGGATTCCTCTAAAAACGTTTCCCAGTT
CGGCATTTTGGGTCGGCACCCCCCCCACTGGAA
AGGGGAAACCCGGGATAACCCGGGAAAAAAGG
CCCGGGGGAAAAATTTCCCGGCCTTGTCAACCCC
GCCTCCACCCCCCGAGGTGAATACAAACGTCCG
GGGTACCTTTTCGACCGTTAGGCATTGCGGTTTG
GGAAGATGAGGTAGACGGAAACACATTAAACG
AAATGCAATTCGTGAA
CCTGTACACAATCTGAACTCTA

Figure 8. The gene encoding 16S rRNA ofTC4a Bacteria.

The results are the sequence of nucleotide bases encoding the 16S rRNA gene of bacterial isolate TC4a (Figure 8). The 16S rRNA gene encoding sequence of bacterial isolate TC4a was then tracked for homology to the 16S rRNA sequence of other bacteria in GenBank through the BLAST program with the website address

Tabel 2. BLAST analysis results of TC4a samples

NCBI code	Species Name	Sequence Length (BP)	Percentage of Homology
	Bakteri TC4a	1520	100%
LCI89362.1	Bacillus cereus	1482	95%
KY750686.1	Bacillus toyonensis strain DF-2	1501	94%
LC146717.1	Bacillus thuringiensis	1443	96%
LT545679.1	Bacillus Sp SK 1	1531	94%
JX847615.1	Bacillus Sp MML 1	1371	95%
MF662416.1	Bacillus siamensis strain HBUM06690	1414	96%
MF682017.1	Bacillys wiedmannii strain F46	1407	96%

http://blast.ncbi.nlm.nih.gov/.BLAST results were obtain ed as follows (Table 2).

KESIMPULAN

Obtained from the East Surabaya market, it can be seen that all bacterial isolates from the tauco sample can produce fibrinolytic enzymes. TC4a samples showed bacterial isolates that had the greatest fibrinolytic activity with a fibrinolytic index of 3,46. Based on the results of sequencing identification analysis with the 16S rRNA and phylogenetic tree methods of isolation of bacteria found in tauco, it was suspected that fibrinolytic bacteria isolates from TC4a samples were *Bacillus cereus* TC4a.

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P-ISSN: 2302-8270
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