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Research Article

Screening and Identification of Fibrinolitic Bacteria from Tempeh

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ABSTRACT

Tempe is a food that is well known by indonesian people. The purpose of this study was to obtain bacteria with the largest fibrinolytic activity from Tempe. This research sample was obtained from four different markets in Surabaya area. Proteolytic activity of bacteria is examined using Skim Milk Agar media. Followed by fibrinolytic examination on the fibrin plate media. Identification of bacteria is carried out by gram staining method and 16S rRNA method. The sample is prepared by mixing the sample with a normal saline solution until 10-7 dilution is obtained. Then the bacterial suspension is spread on SMA media then incubated at 37°C for 24 hours. The bacterial suspension is spread onSMA media then incubated at 37°C for 24 hours. The selection of fibrinolytic protease producing bacteria was carried out using Skim Milk Agar (SMA) and Fibrin Agar (FA) are characterized by the presence of clear zones around them. Bacteria that produce fibrinolytic activity are then cultured in the Nutrient Agar medium. The bacteria was then tested for fibrinolytic activity on the Fibrin Plate media in incubation at 37°C for 24 hours. Positive bacteria are calculated by measuring the diameter of clear zones. Bacteria with sample code T2.2 produce the largest fibrinolytic index. T2.2 bacteria are then microscopically characterized, macroscopically, and bacterial isolates showing fibrinolytic activities were identified using Polymerase Chain Reaction (PCR) targeting their 16S rRNA gene. T2.2 bacteria is thought to be Stenotrophomonas maltophilia with a percentage similarity 96%.

Keywords: 16S rRNA, Fibrinolytic, Tempeh, Screening, Identification

Introduction

Functional foods in Asia such as natto, cungkook-jang, doen-jang, jeot-gal, and tempeh have been known to contain fibrinolytic enzymes. The enzyme is produced by microorganisms. This explains why not much Asians experience Cardio Vascular Disease (Kotb, 2012). One example of functional food originating from Indonesia is tempeh.

Tempeh is well known by Indonesian people. According to a survey conducted by Badan Pusat Statistik, the consumption per week of tempeh is quite large. Tempeh commonly known by the public is tempeh which comes from soy fermentation by Rhizopus sp. The yeast breaks down the protein contained in soybeans. Until now it has been known that some yeast for the manufacture of tempeh are mushrooms Rizhopus sp. Like R. oligosporus. R. Oryzae, R. Stolonifer. In addition to soybeans, these fungi can also be used to ferment other nuts (Astuti, Meliala, Dalais, & Wahlqvist, 2000). According to research that has been done, Rizhophus oligosoprus yeast has been proven to produce fibrinolytic enzymes. In addition to the report that extracts of tempeh water from Fusarium sp. showed strong thrombolytic activity (Sugimoto, Fujii, Morimiya, Johdo, & Nakamura, 2007). Fermentation that occurs in soybeans during the process of making tempeh causes the chemical content of the soybeans to change. Changes in the chemical content that occurs is the breakdown of chemicals so that they are more easily digested. Tempeh is not durable in storage because it basically contains mold. At 2 x 24 hours the mold found on tempeh will die and then the protein-degrading microbes will grow, as a result tempeh will rotten (Sarwono, 2005).

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Protease is an enzyme that can break down peptide bonds resulting simple amino acids and peptides. Protease is known can be isolated from plants, animals and microbes. Fibrinolytic enzyme is a protease enzyme that can degrade fibrin. Fibrinolysis is the process of breaking fibrin protein by the fibrinolytic enzyme. This fibrinolytic enzyme is commonly referred to as a thrombolytic agent. There have been many studies that prove that microbes from functional foods produced by microbes are a large contribution as a producer of fibrinolytic enzymes (Poernomo, Isnaeni, & Purwanto, 2014). Based on the mechanism of action, thrombolytic agents are divided into two. The first is plasminogen activator, such as tissue type plasminogen activator (t-PA) and urokinase which activates plasminogen to active plasmin to degrade fibrin. The second is protein-like plasmin which directly degrades fibrin in the bloodstream so it degrades the thrombus quickly and completely. (Zhang et al., 2005).

Several studies have been carried out, aiming to find fibrinolytic sources that can be obtained cheaply and quickly. One of the bacteria that is proven to produce fibrinolytic enzyme is Bacillus subtilis (Jeong, Park, Baek, Park, & Kong, 2001). Another study conducted by Choi et al., 2005 found that a 42kDa molecular mass DJ-2 bacillopeptidase obtained from Bacillus sp. The DJ-2 was isolated from Doen-Jang, a traditional Korean food made from fermented soybeans. Huy, Hao, & Hung, 2016 also perform screening soybean fermented foods from Vietnam. Seven of the sixteen samples studied were found to be positive for fibrinolytic enzymes.

Bacillus sp. strain CK 11-4 produces a fibrinolytic enzyme, which is obtained from chungkook-jang screening results from fermented foods from Korea. Yoon et al., 2002 perform screening for microorganisms that produce fibrinolytic enzymes derived from traditional foods from various countries. From the screening, Staphylococcus sciuri, Citrobacter or Enterobacter, Enterococcus faecalis, and Bacillus subtilis were found to produce fibrinolytic enzymes. According to Kotb, 2013 Bacillus subtillis is one of the good fibrinolytic producers because it is not pathogenic and its ability to secrete functional extracellular proteins into culture media. Sher, Nadeem, Syed, Abass, & Hassan, 2011 also perform research on the ease of proteases produced from tempeh digested in vitro. The study mentioned that after experiencing protein fermentation in tempeh it becomes more easily digested up to twice that.

Tempeh is produced from fermented soybeans, the soybeans act as a medium for fungal growth. Research conducted by Peng, Huang, Zhang, & Zhang, 2003 purify and characterize fibrinolytic enzymes derived from traditional Chinese douchi foods. The douchi is also a fermented soy food. In addition, the researcher also did the same thing in traditional Japanese natto food. Natto is also a fermented soy food. From this study it can be seen that soybeans are the right medium to grow fibrinolytic enzyme-producing bacteria. Tempeh is also made from fermented soybeans. The fermentation process carried out by the usual tempeh producers may not apply aseptic techniques. So that the manufacturing process and also when the distribution of tempeh there are several bacteria that grow. So it can be assumed that tempeh contains fibrinolytic enzyme-producing bacteria. In this study screening of fibrinolytic-producing bacteria from tempeh will be conducted in the Surabaya market.

Materials And Method

The sample used in this study was tempeh which on sale in the market in Surabaya (T1, T2, T3, T4, T5, and T6). Samples of T1, T2, and T3 are tempeh with closed plastic packaging and are obtained from the Karamenjangan market. T4 and T5 samples are tempeh with plastic packaging but are not closed and obtained from the Gubeng market. T6 samples are tempeh with banana leaf packaging and obtained from Pucang market. In this study using 0.9% NaCl (Sodium Chloride)

Solution, high casein Skim Milk (SMA) Casein, fibrinplate. Other solutions and chemicals used are spirtus, 70% alcohol, distilled water, phosphate buffer, fatty cotton, methylene blue, crystal violet solution, iodine solution, safarin solution, and malachite green solutionSolution, high casein Skim Milk (SMA) Casein, fibrinplate. Other solutions and chemicals used are spirtus, 70% alcohol, distilled water, phosphate buffer, fatty cotton, methylene blue, crystal violet solution, iodine solution, safarin solution, and malachite green solution.

Sample Preparation

Samples of tempeh obtained were weighed then added with 0.9% NaCl then shaken. Dilution with saline solution is obtained so that dilution is 10^{-7}.

Proteolitic Activity Test

Measured 15 ml of Skim Milk Agar (SMA) media and then poured 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 called spread plate and then incubated at 37 °C for 24 hours. If there are bacteria producing proteolytic enzymes, clear zone will form. (Modification of Rani, 2013).

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Bacterial Culture

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

Fibrinolytic Activity Test

Bacterial isolates obtained from proteolytic assays were tested for fibrinolytic activity using well diffusion method. Made by using a sterile pit, fill in 50 µL of bacterial suspension of isolates in 0.9% NaCl solution. Incubated at 37 ° C for 48 hours. Positive results are indicated by the formation of clear zones around the wells. Fibrinolytic activity was determined by the Enzyme Activity Index which compares the diameter of the clear zone with the diameter of the hole divided by diameter of the well (modification of Ashipala & He, 2008).

Bacterial DNA Isolation

Pure bacterial isolates were inoculated into MRS broth medium. Inoculated at 37oC for 24 hours. Bacteria were centrifuged for 10 minutes 5000 g. The removed supernatant precipitate was added using gram-positive lysis buffer (20 mM Tris-HCL, 0.2 mM EDTA pH 8, 1.2% Triton X-100, and 200 mg/ml lysozyme) and incubated at 37oC for 30 minutes. Centrifuged for 2 minutes at 13000 – 16000 g then the supernatant was eliminated. The pellets were added to 60uL of lysis solution and incubated for 5 minutes at 80ºC then cooled to room temperature, then added 3 L of RNase mixed solution, incubated at 37ºC for 30-60 minutes. The protein precipitated with 200µL of protein precipitator solution was then incubated for 5 minutes. After that, 13000 g was centrifuged for 3 minutes and the supernatant was transferred to Eppendorf containing 600 µL isopropanol then centrifuged 13000 g for 3 minutes, supernatant eliminated then inserted 600 µL ethanol 70 % then centrifugedand ethanol evaporated. Pellets can be rehydrated using 100 µL TE buffer (Promega, n.d.).

PCR Processes

PCR tube (0.1 ml PCR tube) contains 10x 30µL buffer reaction (100 mM Tris-HCl, pH 8.8 (at 25°C), 500 mM KCl, 1% Triton X-100, 15 mM MgCl2) added with 5 µL dNTPs (10 mM dATP, dCTP, dGTP, dTTP), 3 µL primary forward B27F for (5'-AGAGTTTGATCCTGGCTCAG-3') concentration 10 pmol, 3 µL primary reverse 1492R for (5'-ACGGCTACCTTGTTACGACT T-3') concentration of 10 pmol, 0.25 µl TaqPolymerase (2.5U/µL), 5µL DNA template, added ddH2O to ad final volume of 50 µL (Sujaya, 2015). Mixing is carried out in cold conditions. The Eppendorf tube is then divortex, and a 5-second spindown, then inserted into the PCR machine. The PCR machine was set at denaturation at 95°C for 30 seconds,annealing at 60°C for 30 seconds, and extended at 72°C for 1 minute for 35 cycles. Furthermore PCR products are then in electrophoresis using gel agarose 1 %.

Electrophoresis

Electrophoresis begins with the agarose gel prepared. Weighed 1% agarose gel as much as 0.3g put in Erlenmeyer, added with tae buffer up to 30 ml volume, then heated in the microwave until gel agarose dissolved perfectly then after the temperature drops added 1 µl Ethidium Bromide. Agarosa gel solution is then poured into the prepared electrophoresis mold along with the well mold. Agarosa waited until compacted then the mold was removed. Then agarose is soaked with a 100ml TAE solution. Furthermore, 5µL PCR products that have been mixed with loading buffer dye (6x) as much as 1µL and marker promega DNA ladder 1kb put in wells as much as 5 µL, then electrophoresis run with a voltage of 100 volts for 30 minutes until the color blue migrated 3/4 gel, further seen with UV transylluminator rays (Aini, Sarjono, & Aminin, 2011).

DNA purification

The DNA band around the 1500bp area was cut with a sterile cutter, then taken and inserted into the eppendorf. The pieces were weighed, every 10 mg of agarose gel was added 20 uL of membrane binding solution. Agarose was homogenized with a vortex and heated at a temperature of 50-65°C for 30 seconds or until the gel dissolved, then put into a collection tube. The fully soluble gel was poured into the mini column and then incubated at 25°C for 1 minute. The collection tube was then centrifuged 16.000g for 1 minute. After that, rinse with 700µL of membrane wash solution and centrifuged again 16.000g for 1 minute. The DNA was then transferred into a sterile eppendorf tube by adding 500µL of ddH2O (Wizard SV Gel and PC).

DNA and 16s rRNA sequencing

DNA is duplicated so that it can be used for sequencing. A 5 µL purification product DNA sample was added with EF-Taq SolGen Korea (SolGent™ 0.25 ul EF-Taq DNA Polymerase 2.5 U/µL, 10 mM dNTP of 5 µL, 10X EF-Taq Reaction Buffer of 30 µL), 3 primary primary primer forward 16S rRNA (5'AGAGTTTGATCCTGGCTCAG-3'), 3 µL Primer reverse 16S rRNA (5'-CGGTTACCTTGTTACGACTT-3'), added ddH2O to ad 50 µL final volume. PCR is performed at denaturation conditions of 95°C for 1 minute, annealing at 55°C for 30 seconds, and extended at 72°C for 1 minute (Macrogen Korea Guide).

Sequencing was carried out by Macrogen Korea using the ABI PRISM 3730XL Analyzer machine. The stationary phase used in the ABI PRISM 3730XL Analyzer machine is acrylamide in a

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capillary tube with TBE buffered mobile phase (3730/3730xl DNA Analyzer Guide.

Phylogenetic Tree Analysis

The phylogenetic tree analysis can be carried out after obtaining the bacterial nucleotide sequence from 16S rRNA sequencing, then analysis using the Basic Local Alignment Search Tool database tracking program, Alignment with the website address http://blast.ncbi.nlm.nih.gov/Blast.cgi . Alignment was visualized using the ClustalW program. The formation of a phylogenetic tree was carried out using the MEGA7 program. Comparison of the 16S rRNA coding gene sequences of tempeh bacteria with the 16S rRNA encoding gene sequences of other bacteria in GenBank.

The phylogenetic tree was designed using the MEGA7 program.

Result And Discussion

Results from the proteolytic activity test obtained 22 isolates producing proteolytic enzymes. Bacteria with proteolytic activity will be found in the clear zone around them, can be seen in table 5.1. This can happen because in skim milk there is white casein protein. The protein is hydrolysed by enzyme from bacteria to amino acids so that it becomes clear (Hwang et al., 2008). Colonies of bacteria that had clear zones around them were taken using Ose then scratched on the media to be oblique to be incubated for 24 hours at 36 ℃.

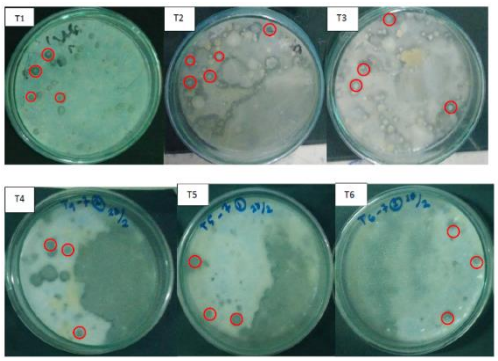

Figure 1 Result of proteolytic activity test

Bacteria are cultured in nutrient broth media so that fibrinolytic activity is further tested on fibrin media. From the Fibrinolytic Activity Test, 15 positive isolates were obtained to produce fibrinolytic enzymes. Each of these bacterial suspensions measured the number of colonies using a spectrophotometer with a transmitting range of 24-26% at a wavelength of 580 nm to ensure all bacterial suspensions had the same number of colonies. Each fibrin plate is given positive Nattokinase control and negative control of normal saline solution. Bacteria that have fibrinolytic activity produce clear zones around them.

Table 1 Fibrinolytic Index

Sample Code	Fibrinolytic Index
T1.1	2,51 ± 0,06
T1.2	2,72 ± 0,07
T1.3	2,15 ± 0,04
T1.4	2,60 ± 0,01
T2.1	2,44 ± 0,18
T2.2*	**3,46 ± 0,12**
T2.3	2,73 ± 0,09
T2.4	2,19 ± 0,07
T3.1	2,11 ± 0,05
T3.2	2,24 ± 0,04
T3.3	2,01 ± 0,09
T4.1	2,89 ± 0,07
T5.2	2,72 ± 0,03
T5.3	2,08 ± 0,01
T6.3	2,15 ± 0,09

Bacterial isolate that produces the largest fibrinolytic activity are T2.2. Isolate T2.2 was then identified with gram staining, macroscopic characteristics, and using 16S rRNA.

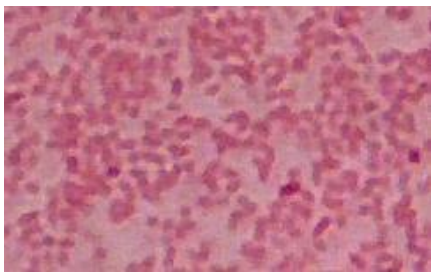

Figure 2 Isolate of T2.2 are Gram negative cocus

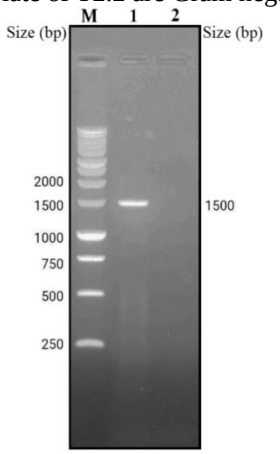

Figure 3 Electrophoregram of T2.2 isolate of 16S rRNA gene detected by 1.5% agarose gel electrophoresis, M: marker, 1: DNA, 2: negative control

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Table 2 BLAST results of sequence of 16S rRNA gene of T2.2 isolate to 16S rRNA base data

NCBI code	Species Name	Sequence legh (bp)	Homology Percentage
NR_041577	*Stenotrophomonas maltophilia* strain IAM 12423	1538	96%
NR_024708	*Pseudomonas geniculata* strain ATCC 19374	1497	96%
NR_116366	*Stenotrophomonas chelatiphaga* strain LPM-5	1454	95%
NR_041957	*Pseudomonas pictorum* strain LMG 981	1502	94%
NR_074936	*Xanthomonas campestris* strain ATCC 33913	1547	93%
NR_117146	*Xanthomonas citri* subsp. malvacearum strain DSM 3849	1545	93%
NR_113972	*Pseudoxanthomonas japonensis* strain NBRC 101033	1470	93%
NR_116864	*Luteimonas terricola* strain BZ92r	1537	92%
NR_041783	*Xylella fastidiosa* subsp. multiplex strain PL.788	1533	92%

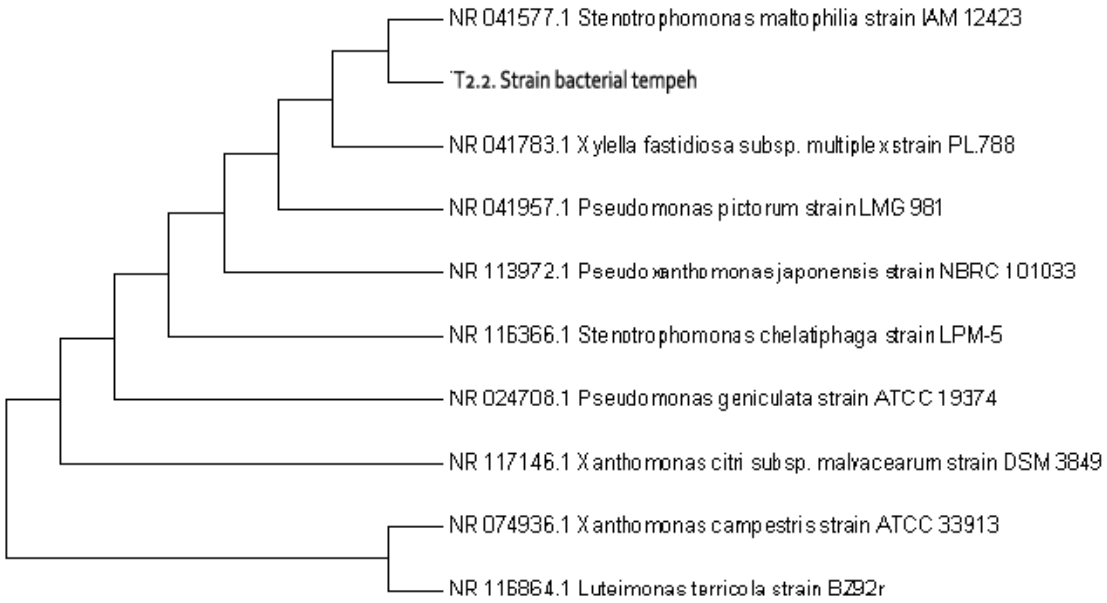

Fig 5 Phylogenetic relationships of Strain T2.2 and some related Pseudo It is noticeable that a tempeh with some what alteromonas species on the basis of 16S rRNA gene sequence analysis.

The identification was continued using the 16S rRNA method and phylogenetic tree to determine the relationship of T2.2 bacteria. The 16S rRNA method is known as an accurate identification method because in the 16S rRNA cipher gene there are conservative genes and hypervariable region genes. Conservative areas of the gene can be used to build the primer used for PCR. While the hypervariable region is located at about 500bp from the end of the sequence (Rinanda, 2011). The identification of 16S rRNA was carried out by Macrogen of Korea. The initial stage of identification by the 16S rRNA method is the isolation of DNA by boiling lysis method after which the DNA obtained is then amplified by the PCR method using a universal primer. then DNA

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PCR results dilektroforesis on gel agarosa with marker Promega DNA Ladder 1kb. Can be seen in Figure 3 PCR results of bacterial DNA produces DNA with a length of about 1500bp.

DNA which has a length of between 1400-1600bp is then purified from the agarosa so that it can be sequenced and the nucleotide base sequence of the gene is obtained. The results of the sequencing were then in BLAST to get bacteria that have similar nucleotide composition with T2.2 bacteria.

BLAST obtained it is known that T2.2 isolate adjacent to *Stenotrophomonas maltophilia* which has homology of 96%. Then in the design of phylogenetic trees based on the results of aligment BLAST results using the program MEGA 7. The results of the phylogenetic tree can be seen in the following figure 5.

Conclusion

There are bacteria that producing fibrinolytic activity in tempeh, the largest of which is T2.2.

Isolate with code T2.2 are thought to be Stenotrophomonas maltophilia because gram staining results, macroscopic observations, and the 16S rRNA method mention supportive results.

isolate image of T2.2 bacteria closely related to Stenotrophomonas maltophilia because it is on the same branch.

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