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Abstract. Tropical disease can be prevented by consumming fermented foods that have antimicrobial activity. One of them is *tempeh gembus* that has short shelf life. It can be overcome by processing it into hydrolyzate. This study aimed to determine antimicrobial activity of *tempeh gembus* hydrolyzate. *Tempeh gembus* was made of local soybean from Grobogan. They were added 5,000 ppm, 8,000 ppm, and 10,000 ppm of bromelain enzyme (TGH BE). Antimicrobial effects of TGH BE were tested against *Staphylococcus aureus, Escherichia coli, Bacillus subtilis,* and *Steptococcus mutans.* Antimicrobial test was carried out using *Kirby-Bauer Disc Diffussion* method. Soluble protein test used *Bradford* method. The largest inhibition zone against *S. aureus* and *S. mutans* were shown by TGH BE 8,000 ppm, 0.89±0.53 mm and 2.40±0.72 mm. The largest inhibition zone of *B. subtilis,* 7.33±2,25 mm, was shown by TGH BE 5,000 ppm. There wasn't antimicrobial effect of TGH BE against *E. coli.* There weren't significant differences of soluble protein (P=0.293) and the inhibition zones againt *S. aureus* (P= 0.967), *E. coli* (P= 1.000), *B. subtilis* (P= 0.645), *S. mutans* (P=0.817) of all treatments. There were antimicrobial activities of TGH BE against *S. aureus, B. subtilis,* and *S. mutans.*

Keywords : Antimicrobial activity, Hydrolyzate, *Tempeh Gembus,* Bromelain enzyme.

1. Introduction

Tropical disease is a common disease in tropics and subtropics. One of the causes of this disease is a bacterial infection. Some pathogenic microbes are *Staphylococcus aureus, Escherichia coli, Bacillus subtilis,* and *Streptococcus mutans. S. aureus* can lead to septicemia, endocarditis, cerebral abscess, puerperal sepsis, and pneumonia [1]. *E. coli* is a normal microflora in the gut that can cause disease. This microbe becomes pathogenic when its number in the digestive tract increase or it's in outside of the intestinal. Some diseases caused by *E. coli* are urinary tract infections, diarrhea, sepsis, and meningitis. *B. subtilis* can induce the insident of meningitis, diarrhea, endocarditis, eye infections, acute gastroenteritis, and immune dysfunction diseases [2]. Whereas, *S. mutans* can cause dental caries [3].

Foods that have antimicrobial function can be consumed to prevent infectious disease. *Tempeh gembus* is one of traditional foods in Indonesia. It is a fermented foods made of solid waste-tofu by *Rhizopus oligosporus* [4]. Eventhough *tempeh gembus* is made of by waste-tofu, it contains several nutrition contents, such as essential fatty acids (linoleic acid (21.51%), linolenic acid (1.81%), and oleic

unsaturated fatty acid (16.72%)), protein, carbohydrate, fiber, calcium, iron [5]. The fermentation process of soybean generates functional properties changes owing to hydrolysis process, e.g protein hydrolysis into amino acids and peptides by proteolytic enzymes which can act as antimicrobial compounds [6]. Additionally, *Rhizopus* used in this fermentation process can produce antibiotics against pathogenic microbes as well [7].

Tempeh gembus which generally can not be stored for a long time, has a large size and the limited availability in some areas, as well as a distinctive flavor that sometimes is not liked by some people, can be tackled by processing it into flour or hydrolyzate [8]. Therefore, in this research, *tempeh gembus* was processed into hydrolyzate by adding bromelain enzyme. Besides, adding bromelain can hydrolyze the protein or polypeptide into simple compounds, such as peptide which may have bioactive function [5].

Tempeh extract has been shown to inhibit *B. subtilis* and *S. aureus* [9]. Study of antimicrobial activity of tempeh has done by Kuligoswki et al [10] against *B. subtilis, Bifidobacterium, E. coli, Lactobacillus acidophilus,* and *Lactobacillus paracasei* in the simulation of human intestinal condition. Tempeh extract has been also shown to inhibit *Enterotoxigenic Escherichia coli* (ETEC) adhesion to Caco-2 cell in the intestinal epithelium [11]. Hence, The objective of the present work is to study the antimicrobial activity of *tempeh gembus* hydrolyzate.

2. Materials and Method

2.1. Preparation of tempeh gembus

Tempeh gembus was made of soybean from Grobogan. Soybean was sorted and soaked for 24 h. Furthermore, the epidermis was stripped and washed. Soybean which is already clean was milled using hot water, with a ratio of soybean : water (1:8). Subsquently, the soy bean was boiled for 15 min and be filtered using filter cloth to separate the filtrate and the pulp. The obtained pulp was steamed for 30 min and be cooled. The fermentation process was carried out by adding 1% of yeast, then it was wrapped using polypropylene plastic and allowed to stand for 24 h.

2.2. *Preparation of tempeh gembus hydrolyzate (TGH BE)*

Tempeh gembus hydrolyzate was made by hydrolyzing *tempeh gembus* using bromelain enzyme. Firstly, *tempeh gembus* was weighed 300 g, and be steamed for 10 min. The steamed *tempeh gembus* was divided into three parts, each weighing 100 g. Afterwards, they were blended and added water to each 100 mL. Bromelain enzyme (Bromelyn, Yogyakarta,Indonesia) was added to each concentration of 5,000 ppm, 8,000 ppm, and 10,000 ppm using Phosphat Buffer Saline as the solvent. The mixture was incubated at 55 °C in water bath incubator for 1.5 h. *Tempeh gembus* hydrolyzate was heated by adding dextrin and NaCl (each 0.5 g/sample). Furthermore, *tempeh gembus* hydrolyzate added bromelain enzyme (TGH BE) condensed materials were dried using freezedryer [8].

2.3. Preparation of microbes

Stock culture was made by scratching *S. aureus, B. subtilis, E. coli,* and *S. mutans* on Nutrient Agar slant, After that, they were incubated in incubator at a temperature of 37 °C for 24 h. The colonies of microbes taken from the stock cultures using loopful and be suspended in a sterile tube containing 10 mL of NaCl solution.

2.4. Antibacterial assay using disk diffusion method

The sterilized Mueller Hinton Agar (MHA) was poured in a petri dish. After the media solified, microbial suspension was leveled at media surface using a sterile swab. Media containing microbes was allowed to stand about 10 min and put the paper discs dipped to TGH BE 5,000 ppm, 8,000 ppm, and 10,000 ppm, that have been diluted using sterilized distilled water with a concentration of 30%, for the comparison there was positive control using amoxycilin 2%, and negative control using sterilized distilled water. Subsquently, they were incubated in an incubator at a temperature 37 °C for 24 h [12].

2.5. Soluble protein assay using Bradford method

Preparation of bradford reagent was done by dissolving 10 mg of coomassie brilliant blue G-250 in 5 mL of 95% ethanol. Then, 10 mL of 85% phosphoric acid was added. The solution was diluted using distilled water to a volume of 100 mL. The samples (each 0.1 g) were added bradford reagent, then it was incubated for an hour. The solution was homogenized using a vortex and it was incubated at room temperature for an hour. Afterwards, each sample was measured using a spectrophotometer (Shimadzu UV-1280,Kyoto, Japan) at wavelength of 595 nm to measure the absorbance [13].

2.6. Statistical analysis

The results were presented as mean ± standard deviation. Antimicrobial activity (inhibition zone) of TGH BE was expressed in mm and soluble protein was expressed in percentage. The data were analyzed using non-parametric statistical computer program. The univariate analysis was conducted by calculating a mean of TGH BE inhibition zone. The bivariate analysis was conducted using one way ANOVA (Analysis of Variance) statistical test to find out the difference of inhibition zone and soluble protein [14].

3. Results

3.1. Antimicrobial activity

All three TGH BE were tested on selected Gram-positive and Gram-negative bacteria which is presented in Table 1. The largest diameter of inhibition zone against *S. aureus* and *S. mutans* was indicated by TGH BE 8,000 ppm that is respectively 0.89±0.53 mm and 2.40±0.72 mm. Whereas, the smallest diameter of inhibition zone by TGH BE 5,000 ppm against *S. aureus* that is 0.82±0.53 mm, and TGH BE against *S. mutans* that is 1.93±0.76 mm. While, TGH BE 5,000 ppm showed the largest diameter of inhibition zone against *B. subtilis* (7.33±2.25 mm) and TGH BE 10,000 ppm showed the smallest diameter of inhibition (5.37±4.09 mm).

Table 1. Mean of Inhibition Zone Diameter of TGH BE 5,000 ppm, 8,000 ppm, 10,000 ppm, Negative Control, and Amoxycilin

Treatment	Mean of Inhibition Zone (mm) ± SD[a]			
	S. aureus	*E. coli*	*B. subtilis*	*S. mutans*
TGH BE 5,000 ppm	0.82 ± 0.53	0.00 ± 0.00	7.33 ± 2.25	2.10 ± 1.40
TGH BE 8,000 ppm	0.89 ± 0.53	0.00 ± 0.00	5.60 ± 0.46	2.40 ± 0.72
TGH BE 10,000 ppm	0.88 ± 0.33	0.00 ± 0.00	5.37 ± 4.09	1.93 ± 0.76
Kontrol (-)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Amoxycilin 2%	18.33 ± 0.23	13.00 ± 1.00	17.47 ± 0.80	7.53 ± 0.92

[a]SD = Standard Deviation; TGH = *Tempeh Gembus* Hydrolyzate; BE = Bromelain Enzyme.

Table 2. showed that the largest diameter of inbition zone of TGH BE 5,000 ppm, 8,000 ppm, and 10,000 ppm were shown to against *B. subtilis*,that is 7.33±2.25 mm, 5.60±0.46 mm, and 5.37±4.09 mm, respectively. However, all samples of TGH BE did not indicate any inhibiton zone against *E. coli*.They were served in Figure 1. There was not significant difference in the inhibition zone against *S. aureus* (P= 0.967), *E. coli* (P= 1.000), *B. subtilis* (P= 0.645), *S. mutans* (P=0.817) of all treatments.

Table 2. Mean of Inhibition Zone Diameter of TGH BE 5,000 ppm, 8,000 ppm, and 10,000 ppm.

Microbes	Mean of Inhibition Zone (mm) ± SD[a)]			P value
	TGH BE 5.000 ppm	TGH BE 8.000 ppm	TGH BE 10.000 ppm	
S. aureus	0.82 ± 0.53	0.89 ± 0.53	0.88 ± 0.33	0.976
E. coli	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.000
B. subtilis	7.33 ± 2.25	5.60 ± 0.46	5.37 ± 4.09	0.645
S. mutans	2.10 ± 1.40	2.40 ± 0.72	1.93 ± 0.76	0.817

[a)]SD = Standard Deviation; TGH = *Tempeh Gembus* Hydrolyzate; BE = Bromelain Enzyme.

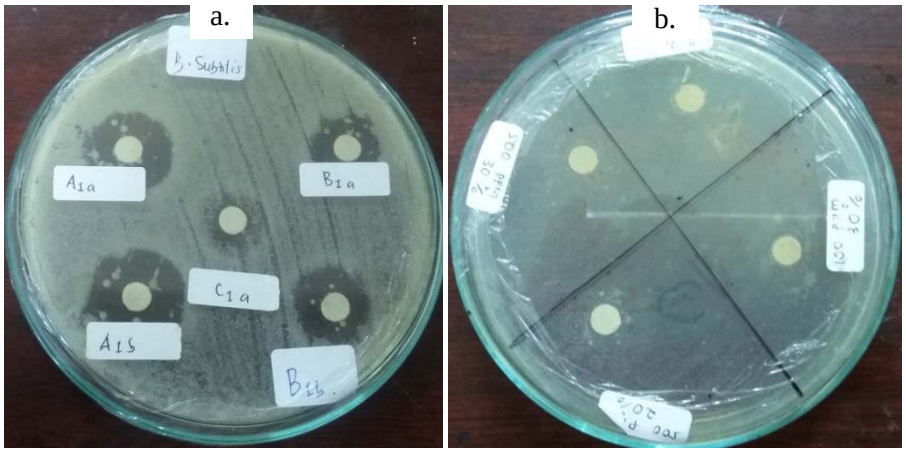

Figure 1. The largest inhibition zones were shown by TGH BE against a. *Bacillus subtilis;* and there wasn't inhibition zone of TGH BE against b. *Escherichia coli*

3.2. Soluble Protein

Table 3. presented the soluble protein of TGH BE. The highest content of soluble protein contained in TGH BE 5,000 ppm. While, TGH BE 10,000 ppm contained the lowest soluble protein. There was not significant difference in soluble protein content of TGH BE 5,000 ppm, 8,000 ppm, and 10,000 ppm (P= 0.293).

Table 3. Mean of Soluble Protein Percentage of TGH BE 5,000 ppm, 8,000 ppm, and 10,000 ppm.[a]

Treatment	% Mean of Soluble Protein	P value
TGH BE 5,000 ppm	0.73 ± 0.08	
TGH BE 8,000 ppm	0.65 ± 0.12	0.293
TGH BE 10,000 ppm	0.60 ± 0.03	

[a]TGH = *Tempeh Gembus* Hydrolyzate; BE = Bromelain Enzyme.

4. Discussion

This study showed that TGH BE 5,000 ppm, 8,000 ppm, and 10,000 ppm had antimicrobial activity against *S. aureus, B. subtilis,* and *S. mutans* which include Gram-positive bacteria. Similarly Bintari et al [15] has reported that antibacterial agents such as glycoproteins were produced during fermentation

by *Rhizopus*, so that the growth of Gram-positive bacteria, *Micrococcus luteus*, which is one of the contaminants in tempeh was inhibited.

Bioactive compounds that have antibacterial activity are formed during soybean fermentation [16]. Saraswaty et al [16] revealed that glycoprotein compounds, protein groups (amines) which have been isolated from media that has inoculated *R.oligosporus*, and flavonoids (isoflavones) are several antimicrobial compounds contained in tempeh. According to Jawetz et al [17], the microbe growth inhibition or the death of microbes due to antimicrobial agents can be through four ways, that is cell wall synthesis inhibition, cell membrane dysfunction, protein synthesis inhibition, nucleic acid synthesis inhibition.

Glycoprotein is one of the antimicrobial compound that can bind to iron in bacteria. Most microorganisms require iron for growth and glycoproteins have the potential to inhibit the growth of bacteria, and even kill them by depriving them of iron. Iron is an essential nutrient for growth and metabolism in virtually all microorganisms and an important cofactor in many metabolic and enzymatic processes. Iron is a component of cytochromes (electron bearer) on the respiratory system [18].

Antimicrobial peptide compounds are capable to regulate the cell target to modify the outside structure of the cell in order to be more sensitive to antimicrobial agents, resistance prevention, non-competitive working to other antimicrobial agents. The working mechanism of antimicrobial peptides are causing the membrane dysfunction (permeabilization, depolarization, and other membrane malfunctioning), the biopolymer extracellular synthesis inhibition, and intracellular function inhibition (protein, RNA, and DNA synthesis inhibition) [19]. Whereas, flavonoids have the ability to bind to extracellular proteins and integral proteins that join to the bacterial cell wall, and disrupt cell wall permeability [20]. Flavonoids are phenolic compounds having glycoside bounds. Phenolic compounds will interact with the bacterial cell membrane proteins through adsorption process by bounding to the hydrophilic part of the cell membrane. Afterwards, Phenolic compounds will enter into the cell membrane and induce cell protein precipitation. It interferes cell membrane permeability, therefore cell membrane can go through lysis [20].

Based on the mechanism of antimicrobial action contained in soybean tempeh that could be also contained in TGH BE, TGH BE have antimicrobial function through four ways, namely cell wall synthesis, cell membrane dysfunction, protein synthesis inhibition, and nucleic acid synthesis inhibition.

According to the David Stout method, antimicrobial activity is very strong if the diameter of inhibition zone is more than 20 mm, antimicrobial activity is strong if the diameter of inhibiton zone is 10-20 mm, antimicrobial activity is medium if the diameter of inhibiton zone is 5-10 mm, and antimicrobial activity is weak if the diameter of inhibition zone is less than 5 mm [21]. Hence, Antimicrobial activities of TGH BE 5,000 ppm, 8,000 ppm, and 10,000 ppm against *S. aureus* and *S. mutans* were included in weak antimicrobial as the diameters were less than 5 mm. While, they were included in medium antimicrobial against *B. subtilis* because the diameters were in the range of 5-10 mm. However, TGH BE of various concentrations didn't have antimicrobial effect against *E. coli* because there wasn't any inhibition zone.

The largest inhibition zone of TGH BE of various concentrations were against *B. subtilis*. *B. subtilis* included in Gram-positive bacteria is more easily inhibited than *E. coli* which is included in Gram-negative bacteria. The cell stucture of Gram-positive microbe is relatively simpler and it makes antimicrobial substances easier to enter the cell and find a target to work [22]. This cell wall is thick, about 25-30 μm, only has one layer with the largest components which consists of peptidoglycan, and the low content of lipid (1-4%) [23]. There are several factors that can affect microbial inhibition, these are the antimicrobial agent concentration, the number of microorganisms, the species of microorganisms, the temperature, and the presence of other organic microorganism spesies [23]. According those factors, *B. subtilis* was known to be one spesies of microbes that was more sensitive to antimicrobial compound contained in TGH BE than *S. aureus* and *S. mutans* which were also Gram-positive microbes.

Tempeh gembus hydrolyzate with all treatments did not show inhibition zone against *E. coli*. This result was similar to study by Kiers et al [24] using disc method, recorded there was antibacterial activity

in soybean tempeh against *Bacillus stearothermophilus* and *B. subtilis*, but soybean extract did not show antimicrobial activity against *E. coli.* This is because *E. coli* is included in Gram-negative bacteria that has a better resistance to antimicrobial compounds. The Gram-negative microbes have a selection system against foreign substances that is in the lipopolysaccharide layer. The structure of Gram-negative microbe cell wall is relatively more complex, it has three layers namely the outer layer in the form of lipoproteins, the middle layer in the form of lipopolysaccharide and peptidoglycan layer [17]. The outer membrane contains protein molecules called porin that is hydrophilic. Porin contained in the outer membrane can cause antimicrobial components more difficult to get into the bacterial cell. Porin leads to the passive diffusion of hydrophilic compounds which have low molecular weight such as glucose and amino acids, while the high molecular weight molecules such as antimicrobial molecules are difficult to penetrate to the membrane. The differences in the structure and components of the cell wall causes *E. coli* as Gram-negative microbe more resistant because one of the mechanisms of antimicrobial work is damaging of the cell wall structure in the plasma membrane, therefore, the plasma membrane ability of bacterial cell as osmosis barrier is decrease and disturbing of the number of biosynthesis process required by the membrane [25].

The study by Kiers et al [24] showed that the percentage of piglets excreting *Enterotoxigenic Escherichia coli* (ETEC) K88 was lower in the group fed tempeh or cooked soy compared to a group fed roasted soy. This was due to several factors such as lactic acid bacteria contained in cooked soybeans and *Rhizopus* fermented soybeans. *Rhizopus microsporus* LU 573 fermented soybean could inhibit adhesion of ETEC K88 *in vitro* and it was similarly to the result of .the ETEC K88 colony decrease observed in piglets fed *Rhizopus* fermented soybean [25]. In addition, the mean incidence of diarrhea was lower in piglets fed *Rhizopus* fermented soybean [26].

The diameters of inhibition zone against *B. subtilis* showed by TGH BE 8,000 ppm and 10,000 ppm were lower than TGH BE 5,000 ppm. Whereas, TGH BE 5,000 ppm and 10.000 ppm showed the lower inhibition zones than TGH BE 8,000 ppm. One of the factors that influence this antimicrobial activity is antimicrobial peptide content within each sample. The process of thermal can deactivate antimicrobial agents. This result was similar to a study by Nowak and Steinkraus [27] which showed that peas tempeh cooked for 10 min did not show antimicrobial properties against *Clostridium perfringens*. Roubus-van den Hil et al [7] also observed the antimicrobial properties of peptides isolated from tempeh were reduce after heating to 60^0C. The heating over 60^0C caused the lost of the antimicrobial activity approximately 20-60% in the 24-h fermented tempe extract. Antibacterial agents isolated by Roubus-van den Hil et al [7] were susceptible to protease, their research was using pronase E or proteinase K. Thus, these enzymes can degrade the antimicrobial peptides found in soybean tempeh. The high temperature of heating and the increasing of enzyme concentration (bromelain enzyme) were factors that might contribute to the decline of antimicrobial activity of the TGH BE in this study.

High soluble protein content does not mean that the antimicrobial activity which is demonstrated also high. The highest diameters of inhibition zone against *S. aureus* and *S. mutans* in this study were shown by TGH BE 8,000 ppm, eventhough the results showed that the highest protein soluble content was in TGH BE 5,000 ppm, instead. It was because the high soluble protein content of substance does not mean it contains high antimicrobial peptides as well. Research by Kusumaningtyas et al [28] about antimicrobial and antioxidant activity in goat milk hydrolyzate showed that peptides that have high antimicrobial activities were peptides having a molecular weight less than 3 kDa. In this study, the test to determine antimicrobial peptide content of TGH BE did not conducted.

5. Conclusion

Tempeh gembus hydrolyzate added 5,000 ppm, 8,000 ppm, and 10,000 ppm of bromelain enzyme showed their antimicrobial activities against *S. aureus, B. subtilis,* and *S. mutans.* However, there was not antimicrobial activity against *E. coli.* There was not difference in soluble protein and the diameter of inhibition zones against *S. aureus, E. coli, B. subtilis,* and *S. mutans* of all three treatments.

6. Acknowledgement

The authors would like to acknowledge Diponegoro University.

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