Antimicrobial activity of fermented industrial soybean waste extracts against food spoilers and foodborne pathogens

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Abstract: The spoilage of food caused by the microorganism affects food safety and consider as important problem in food industry. Control of microbial growth with chemical preservatives have negative effect to consumers' health. Bio-preservatives, for example extract of fermented soybean meal, are improved antimicrobial activity by fermentation process. Therefore, this study aimed to study the antimicrobial properties of seven fermented soybean meal extracts including B001, B010G, LY4/9, LY4/11, LY7/6, LY9/5, and PB5/6 by agar well and diffusion assays against the following microorganisms: *Bacillus cereus* TISTR747, *Escherichia coli* TISTR117, *Micrococcus luteus* TISTR1918, *Pseudomonas aeruginosa* TISTR1287, *Salmonella enterica* TISTR1469, *Staphylococcus aureus* TISTR746, *Vibrio parahaemolyticus*, *Saccharomyces cerevisiae* wild-type BY4742 and the mutant strain Δpdr5. Bactericidal activity was observed in the PB5/6 and LY7/6 extracts against *E. coli* TISTR117 and *M. luteus* TISTR1918, respectively. The LY7/6 extract inhibited *S. enterica* TISTR1469 and *V. parahaemolyticus* at concentration 100 mg/ml with bacteriostatic activity. In summary, the fermented soybean meal extracts LY7/6 and PB5/6 were effective inhibit food spoilage microorganisms suggesting potential application as bio-preservatives for foods.

Keywords: Antimicrobial properties, Bio-preservatives, Food spoilage, Fermentation, Fermented soybean meal

1. Introduction

Food spoilage is mainly occurred by microbial contamination of bacteria, yeast, and fungi in food, as well as chemical and physical changes. Microbial food spoilage is the effect of bacterial contamination such as Lactic acid bacteria (LAB), as some strains containing important role in food spoilage and decay [1]. Yeast such as Saccharomyces cerevisiae can be producing carbon dioxide and ethanol, as well as compounds of ester, ketone, aldehyde, alcohol, and sulfur compounds. These compounds can be able to change the smell and taste of food [2]. Fungi such as Aspergillus flavus can produce aflatoxin during food storage [3]. Chemical food spoilage occurred when ingredients in food react with each other or containing components changing the sensory characteristics of food such as oxidation reaction which affects to lipids, proteins, and carbohydrates. Lipid oxidation reaction is the main cause of food quality deterioration. Which leads to rancidity and shorter shelf life [4]. Enzymatic browning reaction and non- enzymatic browning reaction. Physical food spoilage occurred when the food over losses or absorbs of moisture [5].

In order to prevent the growth of microbial spoilage of food, several preservation methods have been applied by food industries, ranging from chemical to physical preservation techniques. However, the popularity of chemical preservation is decreasing due to the customer's awareness about the health associated with accumulation of chemicals in

body which can affect the function of the liver and kidneys [6]. Consequently, natural healthy foods trended to be increasing.

During fermentation of soybean meal by microorganism, such as *Bacillus*, complex organic compounds are broken down into small molecules and enhancing various physiological functions in addition to nutritional properties. The main bioactive substances found in fermented soybean meal extracts such as phenol, flavonoid, isoflavones and peptides [7].

Common phenolic compounds in fermented soybean meal are isoflavone which contain antimicrobial activity. For example, berberine that has antimicrobial activity, and it can potentiate other antibiotics [8]. Phytoalexin can be used as insecticide and exhibits antioxidant activity, moreover, it can prolong shelf life of food products [9]. There are several biological activities of flavonoids such as antioxidant and antimicrobial activity against *E. coli* ATCC25922 [10]. Flavonoids exerts antimicrobial activity through inhibition of synthesis of nucleic acid and membrane damage. Additionally, fermentation process produces proteins, protein hydrolysates and amino acids exhibiting antioxidant, antibacterial, antifungal, antiviral, and hemolytic activity. Moreover, they can function as angiotensin-Converting Enzyme Inhibitor and binds to opioids as well as calcium [4]. The aim of this study was to study the antimicrobial properties of fermented soybean meal extract.

2. Materials and Methods

2.1 Microorganism and culture media

Antimicrobial activity analysis was performed by using the following microorganisms, *Bacillus cereus* TISTR747, *Escherichia coli* TISTR117, *Pseudomonas aeruginosa* TISTR1287 and *Salmonella enterica* TISTR1469 were cultured at 37°C, 24 h and Micrococcus luteus TISTR1918 was cultured at 30°C, 48 h on Nutrient agar (NA*). Saccharomyces cerevisiae* BY4742 was cultured at 30°C, 48 h on Yeast extract peptone dextrose (YPD) agar. *Staphylococcus aureus* TISTR746 was cultured at 37°C, 48 h and *Vibrio parahaemolyticus* was cultured at 37°C, 24 h on Tryptic soy agar (TSA).

2.2 Preparation of crude extract

Eight of crude extracts derived from the fermented soybean meal were individually dissolved, where 0.1 g of crude extract was homogenized in 1000 µl of sterile distilled water at concentration 100 mg/ml. The homogenates were filtered through a syringe filter (Syringe plastic, Nipro). Thereafter, the stock solution store in Eppendorf tube at 2-8°C

2.3 Antimicrobial activity by agar diffusion assay

The determination of antimicrobial activity was done by agar diffusion assay as described by [11] with slight modifications. Overnight cultures of each strain were transferred to fresh culture media at OD600 of 0.1 and allowed to culture until exponential phase (OD600 of 0.6-0.8). Then, cultures were adjusted to OD600 of 0.1. 50 µl of each culture were spread on the agar and drying for 10 min. For agar diffusion assay, 20 µl of each crude extract was dropped on surface of agar. For agar well diffusion assay, a cork borer (5 mm diameter) was used to drilled on agar and 20 µl of crude extract was added into the wells. Sterile distilled water was used as control. After incubation, inhibition zones were examined, and clear zones diameter was measured.

3. Results

3.1. Antimicrobial diffusion assay

The evaluate antimicrobial activity of each of the eight-crude extract at concentration 100 mg/ml were test using the agar diffusion assay. As shown in Table 1, Results of antimicrobial activity of crude extract against microorganism strain. Four microorganism strain of *Bacillus cereus* TISTR747, *Pseudomonas aeruginosa* TISTR1287, *Saccharomyces cerevisiae* BY4742, and *Staphylococcus aureus* TISTR746 were found to be non-sensitive to crude

extracts. On the other hand, Escherichia coli TISTR117, *Salmonella enterica* TISTR1469 and *Vibrio parahaemolyticus* were found to be sensitive to the PB5/6 extract. The LY7/6 extract inhibited *Micrococcus luteus* TISTR1918. The diameters of the observed clear zones were indicated in figures 1 and 2.

Table 1 Growth inhibition of each microorganism strain by crude extracts from fermented soybean meal.
-, negative inhibition; +, positive inhibition (Bacteriostatic); ++, positive inhibition (Bactericidal)

Strains	Crude extracts								
	Control DW sterile	Control crude	B001	B010G	LY4/9	LY4/11	LY7/6	LY9/5	PB5/6
Bacillus cereus	-	-	-	-	-	-	-	-	-
Escherichia coli	-	-	-	-	-	-	-	-	++
Micrococcus luteus	-	-	-	-	-	-	++	-	-
Pseudomonas aeruginosa	-	-	-	-	-	-	-	-	-
Salmonella enterica	-	-	-	-	-	-	-	-	+
Staphylococcus aureus	-	-	-	-	-	-	-	-	-
Vibrio parahaemolyticus	-	-	-	-	-	-	-	-	+
Saccharomyces cerevisiae	-	-	-	-	-	-	-	-	-

The PB5/6 extract inhibited *Escherichia coli* TISTR117 with diameters of inhibition zones about 0.52 cm by the agar diffusion assay (Figure. 1-1A) and 1.2 cm by the agar well diffusion assay (Figure. 1-1B). *Micrococcus luteus* TISTR1918 that was treated with the LY7/6 extract was inhibited with diameters of inhibition zones of 1.9 cm by the agar diffusion assay (Figure. 1-2A) and 1.7 cm by the agar well diffusion assay (Figure. 1-2B). The PB5/6 extract and the LY7/6 extract showed bactericidal antimicrobial activity against *E. coli* TISTR117 and *M. luteus* TISTR1918, due to inhibition zones observed as clear cut without bacterial growth.

Strains of *Salmonella enterica* TISTR1469 and *Vibrio parahaemolyticus* were found to be sensitive to the PB5/6 extract with diameters of inhibition zone of 1.3 cm and 1.2, respectively. Antimicrobial of the PB5/6 extract showed a bacteriostatic antimicrobial activity against both strains because bacterial growth was presented in the inhibition zones (Figure. 2.)

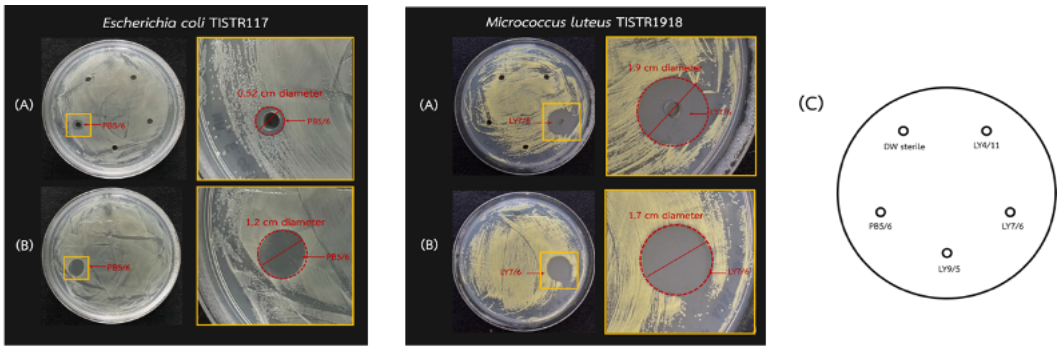

Figure 1. Bactericidal antimicrobial activity of fermented soybean extract by agar well (A) and diffusion assay (B) against *E. coli* TISTR117 (1) and *M. luteus* TISTR1918 (2). 20 µl of crude extracts were added into the well or dropped on the surface of agar. Extracts were individually tested on the position indicated in (C). Zone of inhibition was indicated with red dash line and magnified in yellow square.

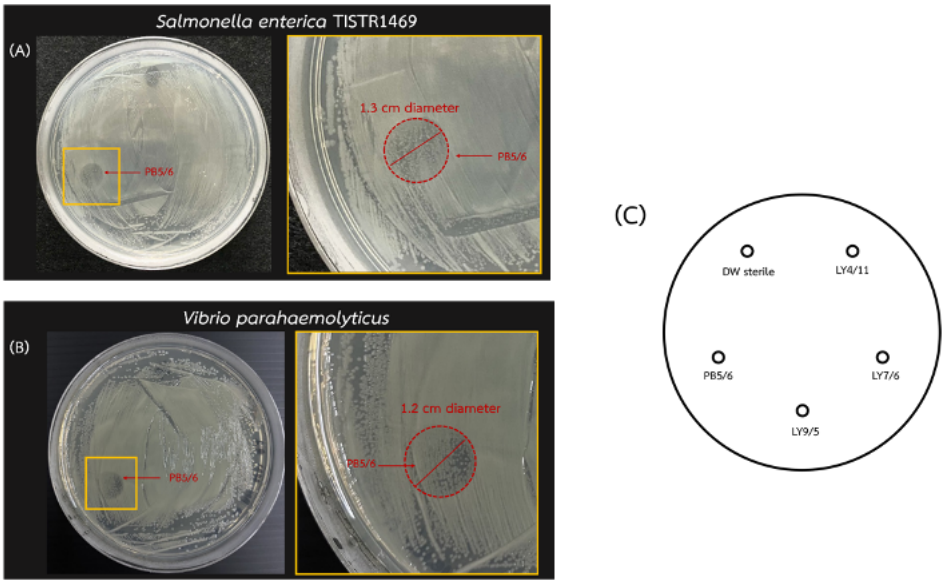

Figure 2. Bacteriostatic antimicrobial activity of fermented soybean extract by diffusion assay against *S. enterica* TISTR1469 (A) and *V. parahaemolyticus* (2). 20 µl of crude extracts were dropped on the surface of agar. Extracts were individually tested on the position indicated in (C). Zone of inhibition was indicated with red dash line and magnified in yellow square.

4. Discussion

The antimicrobial ability of fermented soybean meal extract is playing by variouse compositions including peptide and bioactive compounds. Antimicrobial peptides is an important component and it has mechanism of action through the ability of peptides to penetrate cell membrane associated by attraction between positively charged peptides and negative charge on surface of bacteral cell membrane [4]. Gram-positive bacteria contain teichoic acid on the membrain, therefore, gram- positive bacterial call membrane are stronger than gram- negative bacteria. Cell membrane of gram-negative bacteria compose of phosphate groups and lipopolysaccharides providing anion. The affinity of electric charges creates an attraction between the positive charge of the peptide and the negative charge of bacterial cell membrane allowing peptide to penetrate the cell wall and react with the peptidoglycan of gram-positive bacteria or outer membrane of gram-negative bacteria [12]. However, seven fermented soybean meal extracts were unable to inhibit the growth of gram-positive bacteria including Bacillus cereus TISTR747 and *Staphylococcus aureus* TISTR74 and gram-negative bacterial including *Pseudomonas aeruginosa* TISTR1287, suggesting that fermented soybean meal extract was not specific to gram-positive bacterial cells and no interaction between the electric charge of the peptide and the microbial cells. According to result, it this suggested that the peptides cannot penetrate the peptidoglycan layer of gram-positive bacteria and the outer membrane of gram-negative bacteria. Besides, bacterial used in this study may able to resist the antibacterial activity such as reducing negative ions at the surface of gram-positive bacteria, D-Ala Nielation process (D-alanylation) which causes an anti-inhibition of fermented soybean meal extract or the expresstion of dlt operon [13, 14].

LY7/6 extract has the best antibacterial against of gram-positive bacteria, *M. luteus*, which was indicated as bactericidal activity because of the clear inhibitory zone without

microbial growth suggesting all microorganisms are died without an irreversible inhibition of microbial growth. The bactericidal activity is good to prevent drug resistance in microorganism [15]. In this study used different inhibitory testing methods, agar well and diffusion assays. However, diameter of inhibition zone observed in agar well assay was smaller than agar diffusion assay suggesting in concern of diffusion efficiency of fermented soybean meal extracts through media [16]. PB5/6 extract showed bactericidal activity against gram-negative *E. coli* suggesting that the antibacterial activity involved in cell membrane meaning gram--negative bacteria contains thinner peptidoglycan layer than gram-positive bacteria [17]. However, the determination of minimal inhibitory concentration (MIC) of fermented soybean meal extracts should be performed. Furthermore, a study by Abutheraa et al, 2017 indicates that phenolic compounds such as anthocyanidins, pro anthocyanidins and isoflavones are found in different types of soybean meal cell. These compounds present biological activities that interfere with phenolic compounds [18]. Villalobos Mdel et al., 2016 states that the antimicrobial activity of a phenolic compound is induced by various compounds resulted to a synergistic effect that can inhibit nucleic acid synthesis, enzyme activity and cytoplasmic membranes [9]. Isoflavones found in soybeans inhibit the nucleic acid synthesis of S. aureus [19]. Phenolic compounds such as coumaric, ferulic and vanillic acid have antimicrobial activity against gram-positive and gram-negative bacteria [16]. Growth inhibition may be the result of various constituents in the extract having a synergistic effect on the inhibition of microorganisms [9]. Therefore, phenolic extracts should be further study to identify their antimicrobial activity and the cell viability test (Cytotoxicity) for future development in the food system.

5. Conclusions

This study indicated that fermented soybean meal extract LY7/6 and PB5/6 at concentration 100 mg/ml killed to *M. luteus* and *E. coli*, respectively. The PB5/6 extract inhibited growth of *S. enterica* and *V. parahaemolyticus*. Therefore, fermented soybean meal extracts are good candidates for natural additives for application in food and bioactive food packaging.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Siddiqui, M. and M. Rahman, Minimally Processed Foods: Technologies for Safety, Quality, and Convenience. 2015.
2. Howell, K., Spoilage: Yeast Spoilage of Food and Beverages, in Encyclopedia of Food and Health, B. Caballero, P.M. Finglas, and F. Toldrá, Editors. 2016, Academic Press: Oxford. p. 113-117.
3. Muhialdin, B.J., N.F. Abdul Rani, and A.S. Meor Hussin, Identification of antioxidant and antibacterial activities for the bioactive peptides generated from bitter beans (Parkia speciosa) via boiling and fermentation processes. LWT, 2020. 131: p. 109776.
4. Tkaczewska, J., Peptides and protein hydrolysates as food preservatives and bioactive components of edible films and coatings - A review. Trends in Food Science & Technology, 2020. 106: p. 298-311.
5. Benner, R.A., Organisms of Concern but not Foodborne or Confirmed Foodborne: Spoilage Microorganisms. 2013. p. 245-250.
6. Zhang, F., et al., Antimicrobial, anti-biofilm properties of three naturally occurring antimicrobial peptides against spoilage bacteria, and their synergistic effect with chemical preservatives in food storage. Food Control, 2020: p. 107729.
7. Dai, C., et al., Improvement of nutritional value and bioactivity of soybean meal by solid-state fermentation with Bacillus subtilis. LWT, 2017. 86: p. 1-7.
8. Freitas, C.S., et al., Encrypted antimicrobial and antitumoral peptides recovered from a protein-rich soybean (Glycine max) by-product. Journal of Functional Foods, 2019. 54: p. 187-198.
9. Villalobos Mdel, C., et al., Antioxidant and antimicrobial activity of natural phenolic extract from defatted soybean flour by-product for stone fruit postharvest application. J Sci Food Agric, 2016. 96(6): p. 2116-24.
10. He, M., et al., Antimicrobial mechanism of flavonoids against Escherichia coli ATCC 25922 by model membrane study. Applied Surface Science, 2014. 305: p. 515-521.

11. Gosset-Erard, C., et al., Identification of punicalagin as the bioactive compound behind the antimicrobial activity of pomegranate (Punica granatum L.) peels. Food Chemistry, 2021. 352: p. 129396.
12. Karnchanatat, A. and N. Tiengburanatam, Antimicrobial Peptides. Thaksin.J, 2553. 13: p. 101-107.
13. Espeche, J.C., et al., Unravelling the mechanism of action of "de novo" designed peptide P1 with model membranes and gram-positive and gram-negative bacteria. Archives of Biochemistry and Biophysics, 2020. 693: p. 108549.
14. Assoni, L., et al., Resistance Mechanisms to Antimicrobial Peptides in Gram-Positive Bacteria. Front Microbiol, 2020. 11: p. 593215.
15. Svetlov, M.S., N. Vázquez-Laslop, and A.S. Mankin, Kinetics of drug-ribosome interactions defines the cidality of macrolide antibiotics. Proc Natl Acad Sci U S A, 2017. 114(52): p. 13673-13678.
16. Saviano, A.M. and F.R. Lourenço, Measurement uncertainty estimation based on multiple regression analysis (MRA) and Monte Carlo (MC) simulations – Application to agar diffusion method. Measurement, 2018. 115: p. 269-278.
17. Liu, W., et al., Development of fluorescent probes targeting the cell wall of pathogenic bacteria. Coordination Chemistry Reviews, 2021. 429: p. 213646.
18. Abutheraa, R., et al., Antimicrobial Activities of Phenolic Extracts Derived from Seed Coats of Selected Soybean Varieties. J Food Sci, 2017. 82(3): p. 731-737.
19. Wang, Q., H. Wang, and M. Xie, Antibacterial mechanism of soybean isoflavone on Staphylococcus aureus. Arch Microbiol, 2010. 192(11): p. 893-8.