Antimicrobial Substance Produced by *Lactobacillus* sp. TGR-2 Isolated From *Growol*

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ABSTRAC

Lactic acid bacteria strain TGR-2 was isolated from ***growol*** *(fermented raw cassava) and then identified as* ***Lactobacillus*** *sp. TGR-2. According to results of the turbidimetric assay, the neutralized supernatant of* ***Lactobacillus*** *sp. TGR-2 were able to inhibit the growth of spoilage and pathogenic bacteria, i.e.,* ***Staphylococcus aureus*** *FNCC 0047,* ***Salmonella typhimurium*** *FNCC 0050,* ***Escherichia coli*** *FNCC 0091,* ***Bacillus cereus*** *FNCC 0057, and* ***Morganella morganii*** *FNCC 0122. The antimicrobial activity of neutralized supernatant of* ***Lactobacillus*** *sp. TGR-2 was stable at room temperature for 60 min, pH 3-8; heating 98 °C for 30 min, pH 3-8; 121 °C for 15 min, pH 3-8; storage at 4 °C for 21 days, pH 6.5. The third fraction obtained from purified of supernatant of* ***Lactobacillus*** *sp. TGR-2 by gel filtration which possessed the molecular weight 14,000 Dalton has a bactericidal effect on the growth of S.* ***aureus.***

INTRODUCTION

Lactic acid bacteria (LAB) is defined as bacteria with capabilities of producing lactic acid from fermentable carbohydrate sources (Stamer, 1979). They are Gram-positive rods or cocci, catalase negative, non spore-forming, non motile, microaerophilic to anaerobic and mesophilic. The genera of lactic acid bacteria is *Lactobacillus, Lactococcus* (group N *streptococci*), *Leuconostoc,* and *Pediococcus* (Daeschel, 1989).

Lactic acid bacteria are capable to inhibit the growth of various bacteria. Therefore, the LAB are potentially useful for food preservation agents. LAB are capable of producing inhibitory substance, namely lactic acid that are antagonistic toward other microorganisms. This bacteria are also capable of producing various antimicrobial substances potential as food preservative agents, i.e., hydrogen peroxide, diacetyl and other organic acid (Daeschel, 1989). Several strain of LAB are capable of producing protein substance, usually with small molecular weights, which are capable to inhibit growth of other bacteria known as bacteriocin (Bhunia, *et. al.,* 1988; Spelhaug and Harlander, 1989; Kojik *et. al.,* 1991; Lewus *et. al.,* 1991; Atrich *et. al.,* 1993; Cintas *et. al.,* 1995).

Bacteriocin are defined as protein with intraspecific antagonistic effects or possessing activity as bactericide with a narrow spectrum of activity (which differentiates them with antibiotics), and the synthesis of these proteins is encoded on plasmids (Daeschel, 1989; Eckner, 1992). Bacteriocin is connected with food safety because its inhibition to pathogenic bacteria is used as food preervation, controlling fermentations, and preventing or reducing spoilage.

Many species of bacteria are known to produce specific bacteriocin. Colicins are produced by *Escherichia coli* (Daeschel, 1989), certain bacilli produce subtilin or megacin, several different bacteriocins have been found in the genera *Lactobacillus, Listeria, Micrococcus, Staphylococcus, Streptococcus,* and *Pediococcus. Clostridium, Corynebacterium, Mycobacterium, Sarcina,* the enteric bacteria, and *Streptomyces* also have been noted to produce bacteriocin (Eckner, 1992). Bacteriocin also have been found in Carnobacteria (Schillinger and Holzaple, 1990; Stoffels, *et. al.,* 1992). However, when related with foods, only LAB possessed of greatest opportunity because this bacteria is traditionally involved in processing of food fermentation.

This study was conducted to characterize of antimicrobial substance produced by *Lactobacillus* sp. TGR-2 isolated from *growol* (fermented raw cassava).

MATERIALS AND METHODS

Lactic acid bacteria

The lactic acid bacteria strain used in this study is TGR-2 originated from *growol* (fermented raw cassava), which from previous study was identified as *Lactobacillus plantarum* and *pentosus* complex. Culture was stored at - 80 °C, and grown in TGE broth before used.

Production of antimicrobial substance

Production of the antimicrobial substance was done by growing *Lactobacillus* TGR-2 in TGE broth (pH 6.5), incubating at 37 °C for 96 h, according to the procedure of Bar *et al.*, 1987. The composition of TGE broth (tryptone-glucose-yeast extract) is 1% tryptone, 1% glucose, 1% yeast extract, 0.2% Tween 80, 0.033 mM Mn^{2+} and 0.02 mM Mg^{2+}, pH 6.5 (Biswas *et. al.*, 1991). Supernatant fluids were obtained by centrifuging the cultures at 20,000 rpm for 15 min, 4 °C to remove the cells. After adjusting the pH to 6.5 with sterile NaOH, they were sterilized by filtration through a 0.2 μm pore membrane (Whatman paper).

Detection of antimicrobial substance

The turbidimetric assay was used to determine the antimicrobial activity of neutralized supernatant of *Lactobacillus* sp. TGR-2. One ml of 5X concentrated supernatant was added to one ml of tryptic soy broth (TSB, Difco), double strength, pH 6.5, and then inoculated with 1 % of *Streptococcus aureus* (OD_{600} = 1.2) as indicator bacteria, incubated at 37 °C for 24 h. The growths were periodically determined by measurement of turbidity using spectrophotometer (600 nm).

Spectrum of antimicrobial activity

The inhibition activity of 5X concentrated supernatant was determined on several food spoilage and pathogenic bacteria, i.e., *Staphylococcus aureus* FNCC 0047, *Salmonella typhimurium* FNCC 0050, *Escherichia coli* FNCC 0091, *Bacillus cereus* FNCC 0057, and *Morganella morganii* FNCC 0122 as indicator bacteria.

Purification of antimicrobial substance

Purification of the antimicrobial substance was performed by precipitation with acetone 95 % vol/vol (Branen *et al.*, 1975) and further purified by gel filtration (Bhunia *et al.*, 1988). A 25X concentrated supernatant was added with acetone 95 % vol/vol with slow stirring for overnight at 0 °C, and then centrifuged at 45,000 rpm for 30 min, 0 °C to separate acetone and the precipitate. The precipitate was dissolved in phosphate buffer, pH 6.5 and then run through a Sephadex G-50 Fine (90 x 1.7 cm column). The elusion was performed with the same buffer at a flow rate of 12 ml/h. The fractions were collected on 5 ml and monitored at 280 nm with Spectronic 1201. Each fraction was assayed for antimicrobial activity by turbidimetric assay. Fraction with the highest inhibition activity was determined for its molecular weight. Standard molecular weight, namely aprotinine (6,500 Dalton), cythochrome (12,000 Dalton), carbonic anhydrase (29,000 Dalton) and albumin (66,000 Dalton) were used for comparison.

Effect of pH and temperature on antimicrobial activity (Bhunia *et al.*, 1988)

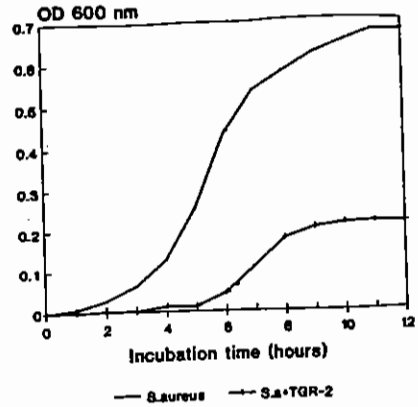
A 25X concentrated supernatant after precipitation using 95 % acetone vol/vol were adjusted their pH with sterile NaOH or HCl to pH 3; 4; 5; 6; 7; and 8. Supernatant with various pH was further treated as fallow: (1) storage at room temperature for 1 h; (2) heated at 98 °C for 30 min; (3) heated at 121 °C for 15 min. To eliminate the effect of acid/alkali, before performing the antimicrobial activity, supernatant after treated with several pH were adjusted to 6.5. The supernatant (pH 6.5) was also stored at 4 °C for 7, 14, and 21 days. Antimicrobial activity assay was done using *S. aureus* FNCC 0047 as an indicator bacteria.

RESULTS AND DISCUSSIONS

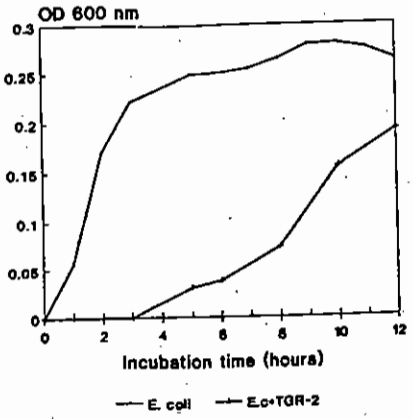
Spectrum of antimicrobial activity

The antimicrobial activity of *Lactobacillus* sp. TGR-2 on growth of several pathogenic and spoilage bacteria is shown at Fig. 1 to 5. Inhibition activity of supernatant containing antimicrobial substance is shown by a lag phase extension and suppression of final population at 12 h incubation. Figure 1 shows that supernatant was able to extent a lag phase of *S. aureus* FNCC 0047 culture from 2 h to 5 h, whereas lag phase of *Salmonella typhimurium* FNCC 0050 culture was extended from 1 h to 6 h (Fig. 3). Culture of *E. coli* FNCC 0091 grown on TSB media when added with supernatant will delay its lag phase for 3 h (Fig. 2). Thus, Fig. 4 and 5 show that supernatant was able to inhibit the growth and to

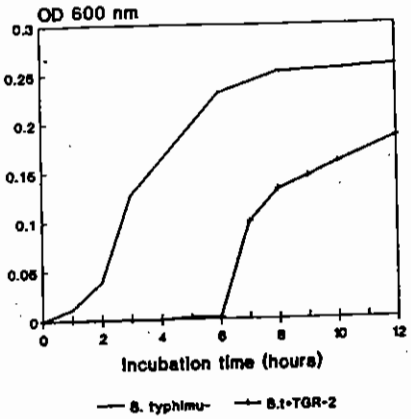
suppress of final population culture *Bacillus cereus* FNCC 0057 and *Morganella morganii* FNCC 0122.



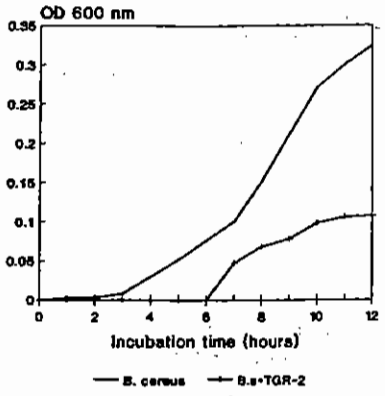
Figur 1. **Inhibition effect of neutralized supernatant of *Lactobacillus* TGR-2 on the growth of *S. aureus*.**



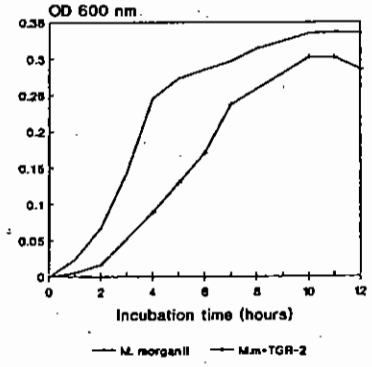
Figur 2. **Inhibition effect of neutralized supernatant of *Lactobacillus* TGR-2 on the growth of *E. coli*.**



Figur. 3. **Inhibition effect of neutralized supernatant of *Lactobacillus* TGR-2 on the growth of *S. typhimurium*.**



Figur 4. **Inhibition effect of neutralized supernatant of *Lactobacillus* TGR-2 on the growth of *B. cereus*.**

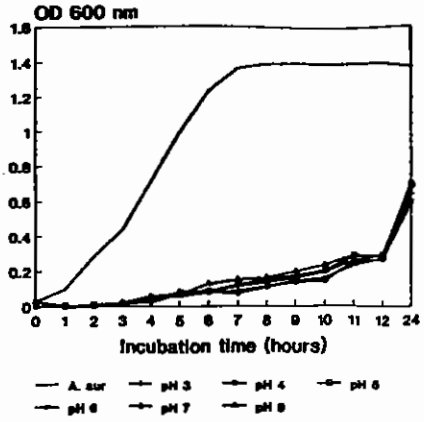


Figur 5. **Inhibition effect of neutralized supernatant of *Lactobacillus* TGR-2 on the growth of *M. morganii***

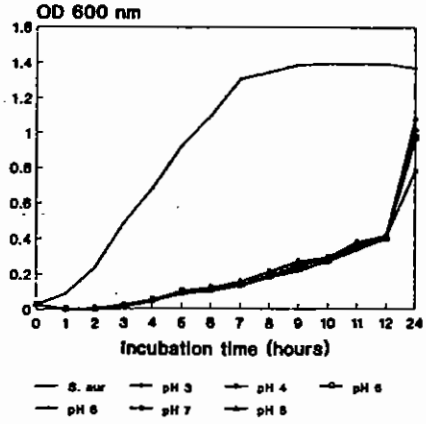
Antimicrobial substance from *Lactobacillus* has already identified as bacteriocin, i.e. plantaricin C 19 produced by *L. plantarum* C 19 (Atrih, *et. al.*, 1993), and sakacin A from *L. sake* (Schillinger and Lucke, 1989). Both of these bacteriocins have inhibition activities on pathogenic bacteria particularly *Listeria* and spoilage bacteria. In this study, inhibition activity of *Lactobacillus* sp. TGR-2 on *Listeria* has not yet been done.

Effect of pH and temperature on antimicrobial activity

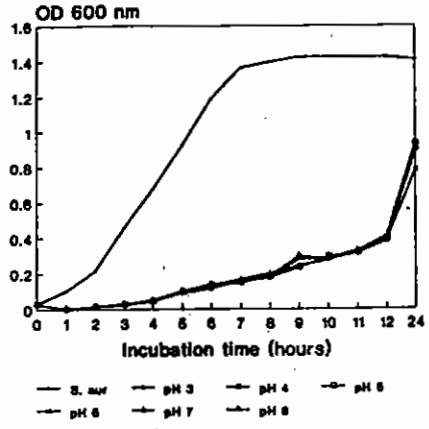
The effect of pH and temperature on antimicrobial activity is shown in Fig. 6, 7 & 8, and the effect of storage at 4 °C is shown Fig. 9. Figure 6, 7 & 8 show that pH and temperature do not influent its antimicrobial activity of *Lactobacillus* sp. TGR-2. After heating 98 °C for 30 min and 121 °C for 15 min, the antimicrobial activity of TGR-2 does not decreased.



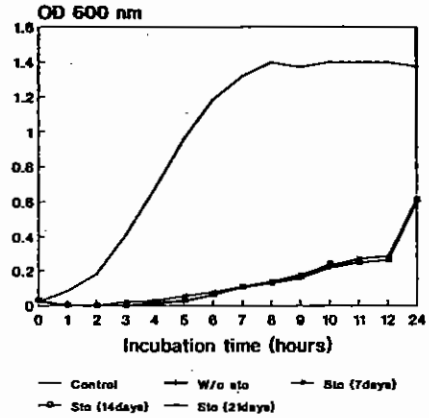
Figur 6. **Influence of pH and temperature (25C, 1h) on the inhibition activity of TGR-2 substance against *S. aureus*.**



Figur 7. **Influence of pH and temp (98C, 30 min) on the inhibition activity of TGR-2 substance against *S. aureus*.**



Figur 8. **Influence of pH and temp (121C – 15 min) on the inhibition activity of TGR-2 substance against *S. aureus*.**



Figur 9. **Influence of storage (4C for 21 days) on the inhibition activity of TGR-2 substance against *S. aureus*.**

Antimicrobial substance of *Lactobacillus* sp. TGR-2 was also stable on storage at 4 °C for 21 days. Figure 9 shows no difference of inhibition activity on growth of *S. aureus* FNCC 0047. Results have indicated that antimicrobial substance of TGR-2 has enough potential to be used as preservative agents because its ability to inhibit pathogenic and spoilage bacteria and stable on heating and storage.

The previous study done by Bhunia *et. al.*, (1988) also indicates that bacteriocin pediocin AcH produced by *Pediococcus acidilactici* H was stable at heating 90 or 121 °C and pH between 2.5 to 9.0. Bar *et. al.*, (1978) concluded that antimicrobial substance produced by *Lactobacillus bulgaricus* with optimum pH 4.0 was stable at storage in 6 °C for 18 days and heating 100 °C for 1 h.

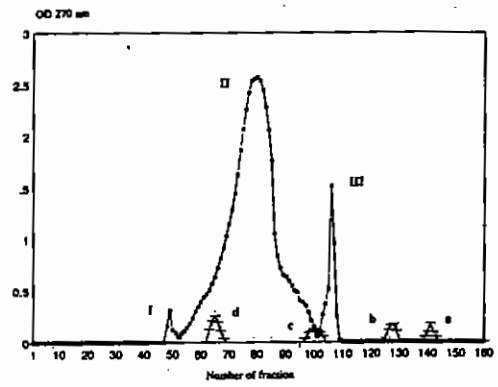
Purification and molecular weight of antimicrobial substance

The first step of purification is precipitation of proteins with saturated acetone 95 %. The organic solvent is capable in reducing water activity and there is interaction between protein part and hydrophobic parts of organic solvent to pass van der Waals band which resulted to protein precipitation. Acetone is selected because its lesser tendency to cause protein denaturation than the other solvents and also more volatile, which enables it to be removed easily from redissolved precipitates under reduced pressure (Scopes, 1987).

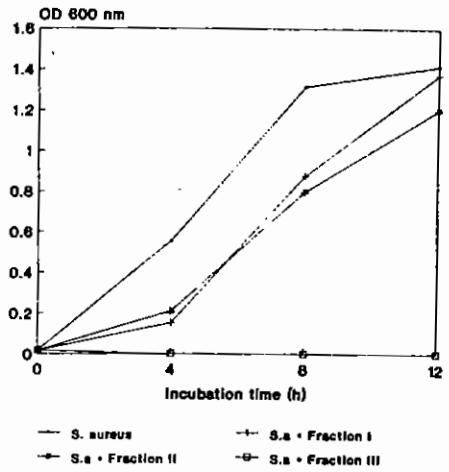
At second step, the extract was then separated through chromatography column. Gel filtration method was employed to separate proteins based on their

molecular weights. Sephadex G-50 Fine which able to separate protein with molecular weight 1,500 – 30,000 was chosen. The choice is made based on previous studies suggesting that the molecular weight of antimicrobial substance produced by lactic acid bacteria is between 1,700 – 100,000 Dalton (Bhunia *et. al.*, 1987; Muriana and Luchansky, 1993).

There were three fractions (Fig. 10) and an antimicrobial activity from the third fraction shown on Fig. 11 resulted from this experiment. The fraction which give inhibition activity of the most potential is of fraction III, to the extend of 12 h without growth of *S. aureus* FNCC 0047. From polypeptide band test or Nynhidrine test indicated that this fraction represent polypeptide. The estimate of molecular weight (Fig. 10) of polypeptide fraction (III) containing polypeptide antimicrobial activity or bacteriocin-like type is about 14,700 Dalton.



Figur 10. **Fractionation of antimicrobial substance of *Lactobacillus* TGR-2 protein molecule standards using Sephadex G-50 Fine. a = Aprotinine (6.500 D); b = Cytochrome C (12.400 D); c = Carbonic anhydrase (29.000 D); d = Albumin (66.000 D).**



Figur 11. **Inhibition effect of antimicrobial substance purified by Sephadex G-50 on the growth of S. aureus.**

CONCLUSION

Antimicrobial substance of *Lactobacillus* sp. TGR-2 suggested to be bacteriocin-like type, stable on pH 3-8 and high temperature, possesses molecular weight 14,700 Dalton has a bactericidal effect on the growth of *Staphylococcus aureus.*

ACKNOWLEDGEMENT

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