# Lactic Acid Bacteria from Indigenous Fermented Foods and Their Antimicrobial Activity

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## ABSTRACT

*Twenty-eight lactic acid bacteria (LAB) strains were isolated from various indigenous fermented foods, i.e.,* ***asinan rebung*** *(bamboo shoot pickle),* ***asinan terong*** *(eggplant pickle),* ***gatot*** *(fermented dried cassava),* ***growol*** *(fermented raw cassava),* ***tape*** *(fermented steamed cassava tubers),* ***tempe*** *(fermented soybean),* ***tempoyak*** *(fermented pulp of durian fruit), and* ***moromi****. All strains found and identified belong to facultative hetero-fermentative group lactobacilli. They produced DL-lactic acid, and contained* ***meso****-diaminopimelic acid in their peptidoglycan, and were identified as* ***Lactobacillus plantarum*** *and L.* ***pentosus*** *complex. These strains were further determined for their antimicrobial activity against* ***Staphylococcus aureus*** *using disc assay and turbidimetric assay. Two among them,* ***Lactobacillus*** *TGR-2 (from growol) and Lactobacillus TMO-4 (from* ***moromi****) were able to increase the lag phase, and to suppress the final population of the S.* ***aureus*** *growth after 12 h incubation.*

## INTRODUCTION

The term of lactic acid bacteria includes a constellation of microorganisms which are best noted for their capabilities of producing lactic acid from a fermentable carbohydrate source (Stamer, 1979).

The lactic acid bacteria have been involved in the certain fermented foods, including milk, meat, fruits, and vegetable. These fermented foods, usually preserved well because of the souring effect that lactic acid bacteria convert the sugars to organic acids. However it has also been recognized that lactic acid bacteria are capable of producing inhibitory substances other than organic acids that antagonistic toward other microorganisms, such as bacteriocin, $H_2O_2$, diacetyl and other secondary metabolites (Daeschel, 1989). Antagonistic actions of lactic acid bacteria toward pathogenic and spoilage bacteria have been well documented (Gilliland and Speck, 1975; Motlagh *et. al.*, 1992).

Indonesia, like other tropical country has many kind of traditional fermented foods, which have been consumed for a long time. These fermented foods are mostly produced by traditional methods using spontaneous inocculant. For example *tempe*, a fermented, *Rhizopus* mold-covered cake produced of dehulled and cooked soybean. Tempe is found in all part of Indonesia but particularly important in Java and Bali. *Tape ketela* is a fermented steamed cassava tubers by *ragi tape* starter, which dominantly consisting Amylomyces rouxii and Endomycopsis (Steinkraus, 1983). *Growol* is fermented raw cassava tubers, whereas *gatot* is fermented dried cassava tubers. These products which only found in certain part of Java are produced by soaking the peeled raw cassava for growol and dried cassava for gatot for several days until the tubers becoming soft. *Tempoyak*, which only found in Sumatra is produced by mixed pulp of *durian fruit* (*Durio zibethinus*) with small amount of salt, placed in a sealed container and then fermented for 3 – 7 days. While asinan *terong* and asinan *rebung* are typical pickles produced from eggplant and bamboo shoot, respectively. According to the unpublished data, lactic acid bacteria could be found in almost all of these fermented foods. Rahayu *et al.*, (1986) also reported that lactic acid bacteria were found in moromi or brine fermentation of fungal fermented soybean, for *kecap* making.

Lactic acid bacteria originally isolated from fermented foods are probably the best candidates for improving the fermentation methods in the case of their product safety, since they are well adapted to the conditions during spontaneous fermentation incident. The purpose of this study were to isolate the lactic acid bacteria from fermented foods and to screen their capability in producing antimicrobial substance. Strains which exhibited antimicrobial substance, particularly against pathogenic bacteria, can be later used as starter culture.

## MATERIALS AND METHODS

### Isolation method

Lactid acid bacteria were isolated from asinan *terong, gatot, growol, tape ketela pohon*, and *tempe* obtained from Yogyakarta; *moromi* from kecap factory at Kediri, East Jawa; *tempoyak* and *asinan rebung* (Bengkulu, Sumatra). MRS agar (Oxoid) was used for isolation, and the cultures were selected after incubation at 30°C for 2-3 days according to the colonial appearance. Pure cultures were maintained as frozen stock held at - 80°C in MRS broth plus 10 % glycerol. Cultures were propagated at 30°C in MRS broth before use in experiment.

### Morphological, biochemical, and physiological characterization

Morphological characters including cell forms, cell size, cell arrangement, and spore formation were examined on the cells grown on MRS agar after 2 – 3 days incubation. Gram staining was determined by a general method. Production of gas from glucose was detected by a Durham tube on growing cells, using MRS as medium. Catalase reaction by pouring the young cultures of lactic acid bacteria with $H_2O_2$ solution, the reaction of catalase indicated by the production of gas $CO_2$ (bubble). Motility was detected by stabbed cultured of isolates on glucose-yeast extract-peptone (GYP) soft agar. Dextran from sucrose was detected by growing the culture on sucrose-yeast extract-peptone (SYP) agar, production of dextran shown by slimy appearance upon the colony. Effects of temperature (15°C and 45°C) and of different starting pHs (3.5, 4.0, 4.5, 5.0, 7.5, 8.0, 8.5, 9.0, and 9.6) were detected by growth in GYP broth. Acid formation from sugars was detected by growing the cultures in PGY broth, and titrated with 0.1 N NaOH using BTB and NR mixture ethanol as indicator.

### Isomer of lactic acid

D- and L-isomers of lactic acid from supernatant were separated by HPLC using an enantiomeric resolution column (TSK-gel Enantio L1, Tosoh, Tokyo, Otsuka *et al.*, 1994).

### Fermentation type

Culture was grown in 5 ml of GYP broth for 2 days. The amount of lactic acid accumulated in this culture was calculated from the titration value of 0.1 N NaOH solution. For the determination of ethanol culture was centrifuged, and the supernatant was diluted to 1/100 with distilled water and analyzed enzymatically by the use of F-kit ethanol (Cat no. 167 290, Boehringer Mannheim, Okada, *et al.*, 1991).

### Peptidoglycan type

Five ml of culture was centrifuged, and the pellet of cells after washing with distilled water was hydrolysis with 6N HCN 0.1 N at 100 °C for 2 h, and the hydrolysate was applied on cellulose TLC plate (Merck no. 5716). The TLC plate was developed for about 3 h with the solvent system of methanolpyridine-water-concentrated HCl (80:26:4:10 v/v). The spots were visualized by spraying with 0.2 % ninhidrin solution in *n*-butanol (Tokyo Kasei Kogyo Co.,Ltd,Tokyo,Japan) followed by heating at 100 °C for a few minutes (Komagata and Suzuki, 1987).

### Preparation of the antimicrobial substance

Production of the antimicrobial substances were done by growing the lactic acid bacteria in TGE broth (pH 6.5), incubating at 37°C for 96 h (Bar *et al.*,). Supernatant fluids were obtained by centrifuging the cultures. After adjusting the pH to 6.5 with NaOH, they were sterilized by filtration through a 0.2 µm Whatman filter paper.

### Detection of antimicrobial substance

Two methods were used to detect the antimicrobial substance produced by lactic acid bacteria, and *Staphylococcus aureus* was used as indicator. First method was disc assay (Bar, *et al.*, 1987; Bhunia, *et al.*, 1988). Indicator lawn for the detection of antimicrobial activity by this method was prepared by inoculation 10 ml of TSA with 24 h old culture of *S. aureus* and then poured into Petri dish. After the agar solidified, sterile filter papers (Whatman, diameter 18 mm) was put at the surface of agar, then 100 µl neutralized sterile supernatant was dropped in the paper. After the diffusion of supernatant into the agar (1 h, room temperature), then incubated at 37°C for 18 h. The inhibition was shown by clear zone surround the filter paper. Second method was turbidimetic assay (Davidson and Parish, 1989). One ml of neutralized supernatant sterile was added 1 ml of TSB (double strength) and then inoculated with 1% of *S. aureus*

culture ($OD_{600}$ = 1.2) and incubated at 37°C for 24 h. The growths were periodically determined by measurement of turbidity using spectrophotometer (600 nm).

## RESULTS AND DISCUSSIONS

### Characterization and identification of lactic acid bacteria

Lactic acid bacteria were isolated from several Indonesian fermented foods (*growol, gatot, tape, tempoyak, pickle*, and *tempe*) obtained from Java and Sumatra Islands. These lactic acid are listed in Table 1 and their characteristics are shown in Table 2.

Table 1. **Lactic acid bacteria isolates**

| Number | Source |
|---|---|
| TAR-1 | *Asinan rebung* (bamboo shoot pickle) |
| TAR-2 | *Asinan rebung* (bamboo shoot pickle) |
| TAR-3 | *Asinan rebung* (bamboo shoot pickle) |
| TAT-1 | *Asinan terong* (eggplant pickle) |
| TAT-2 | *Asinan terong* (eggplant pickle) |
| TAT-3 | *Asinan terong* (eggplant pickle) |
| TGA-1 | *Gatot* (fermented dried cassava) |
| TGA-2 | *Gatot* (fermented dried cassava) |
| TGA-3 | *Gatot* (fermented dried cassava) |
| TGR-1 | *Growol* (fermented raw cassava) |
| TGR-2 | *Growol* (fermented raw cassava) |
| TGR-21 | *Growol* (fermented raw cassava) |
| TGR-22 | *Growol* (fermented raw cassava) |
| TGR-23 | *Growol* (fermented raw cassava) |
| TGR-24 | *Growol* (fermented raw cassava) |
| TGR-25 | *Growol* (fermented raw cassava) |
| TTA-1 | *Tape* (fermented cooked cassava) |
| TTA-2 | *Tape* (fermented cooked cassava) |
| TTA-3 | *Tape* (fermented cooked cassava) |
| TTE-1 | *Tempe* (fermented soybean) |
| TTE-2 | *Tempe* (fermented soybean) |
| TTE-3 | *Tempe* (fermented soybean) |
| TTK-1 | *Tempoyak* (fermented pulp of durian fruit) |
| TTK-21 | *Tempoyak* (fermented pulp of durian fruit) |
| TMO-1 | *Moromi* |
| TMO-2 | *Moromi* |
| TMO-3 | *Moromi* |
| TMO-4 | *Moromi* |

All of the 28 isolates were Gram positive rods, (0.5 – 1.0) x (1.0 – 2.0) in size, and appeared singly or in pair. Cells were nonmotile and nonsporing. All isolates gave negative reaction for catalase, dextran formation from sucrose, and gas production from glucose. They grew at range temperature 15 – 45°C and initial pH from 3.5 to 9.0. Almost all of them produced acid from 18 sugars among 21 tested, only three sugars which can not be used, i.e., L-rhamnose, D-xylose, and starch. According to morphological, biochemical, and physiological characterization as listed in Table 1. all isolates were concluded in the member of the genus *Lactobacillus*. Kandler and Weiss (1986) have grouped the lactobacilli based on biochemical-physiological criteria, and Hammes and Vogel (1995), rearranged these groups based on the earlier criteria and phylogenetic relationship, becoming group A, B, and C correspond to the respective groups I, II, and III of Kandler and Weiss. The three groups according to Hammes and Vogel (1955) are followed.

Group A is obligate homofermentative lactobacilli. Hexoses are almost exclusively (> 85%) fermented to lactic acid by EMP pathway. The organisms possess fructose-1,6-bisphosphate-aldolase but lack of phosphoketolase, and therefore, neither gluconate nor pentoses are fermented.

Group B is facultative heterofermentative lactobacilli. Hexoses are almost exclusively fermented to lactic acid bacteria by EMP pathway. The organisms possess both aldolase and phosphoketolase, and therefore, not only ferment hexose but also pentoses (and often gluconate). In the presence of glucose, the enzymes of the phosphogluconate pathway are repressed.

Group C is obligate heterofermentative lactobacilli. Hexoses are fermented by the phosphogluconate pathway yielding lactate, ethanol (acetic acid) and $CO_2$ in. Pentoses enter this pathway and may be fermented.

The *lactobacillus* strains isolated from Indonesian fermented foods were able to ferment gluconate and some pentoses, and they do not produce ethanol in equimolar amount, therefore these strains were concluded to be a member of Group B, i.e., facultative heterofer-mentative. Among 17 species belong to Group B, according to their characters in carbohydrates fermented, these *Lactobacillus* strains are mostly close related to species *plantarum* and *pentosus*.

Presence of *meso*-diaminopimelic acid (*meso*-DAP) is one of the most important pieces of information concerning the cell wall peptidoglycan of Gram positive bacteria included lactic acid bacteria (Komagata and Suzuki, 1987). In the Group B, only three species possess DAP as peptidoglycan type, the other species have characteristic Lys-dAsp type. In this result, the *lacto-bacillus* strains tested contained *meso*-diaminopimelic acid in the peptidoglycan. This character gives more suggestion that these 28 strains of

*Lactobacillus* mostly close related to species *plantarum* and *pentosus*.

Table 2. **Characteristis of all lactic acid bacteria isolates**

| Characteristics | All isolates |
|---|---|
| Cell form | Rods |
| Cell size (um) | (0.5 – 1.0) x (1.0 – 2.0) |
| Cell arrangement | Single and pair |
| Spore formation | – |
| Gram staining | + |
| Production of gas | – |
| Catalase reaction | – |
| Motility | – |
| Dextran from sucrose | ND |
| Fermentation type | Homo-ferm |
| Lactic acid produced | DL |
| Growth at 15°C | + |
| Growth at 45°C | + |
| Growth at pH 3.5 | + |
| Growth at pH 9.0 | +/(1–) |
| Peptidoglycan type | DAP |
| Acid formation from carbodhydrates | |
| L-Arabinose | + |
| D-Cellobiose | + |
| D-Fructose | + |
| D-Galactose | + |
| Glucose | + |
| Gluconate | + |
| Lactose | + |
| D-Maltose | + |
| D-Mannitol | + |
| D-Mannose | + |
| D-Melibiose | + |
| D-Melezitose | + |
| Raffinose | +(2–) |
| L-Rhamnose | – |
| D-Ribose | + |
| Salicin | + |
| D-Sorbitol | +(2–) |
| Starch | – |
| Sucrose | + |
| D-Trehalose | + |
| D-Xylose | – |
| Free | – |
| Suspected species | *Lactobacillus plantarum* and *pentosus* complex |

*Lactobacillus plantarum* and *L. pentosus* are very similar in phenotypic characteristics. The production of acid from glycerol, D-melezitose and D-xylose, and the amount of cyclopropane acid of $C_{19}$ are useful for differentiating these two species, but these characteristics are not consistent for the separation of them (Tanasupawat, *et al.*, 1992). To distinguish these two species, more characters particularly which express their genetic properties are needed. DNA-DNA homology will be very important to classified these strains into two species.

Lactobacilli usually grow under anaerobic condition or at least under reduced oxygen tension in all habitats providing ample carbohydrates, breakdown products of protein and nucleic acids, and vitamins. Eventhough a mesophilic to slightly thermophilic range is favorable, however strain of some species grow - although slowly - even at low temperature (Klander and Weiss, 1986). Further description of Bergey's Manual of Systematic Bacteriology and Hammes and Vogel (1995), *Lactobacillus plantarum* and *L. pentosus* show no growth at 45°C. In the case of this result, all the isolated strains grow well at 45°C, may be this is related to their originally habitat, tropical fermented foods.

## Antimicrobial activity

A total of 28 strains of *Lactobacillus* from 8 kinds of fermented foods were selected according their antimicrobial activity using pathogenic *S. aureus* as indicator bacteria. Reason using *S. aureus* in this study, many cases of the occurrence of contamination in Indonesia, are due to the presence of this bacteria (unpublished data). *Staphyloccus aureus* has been used as indicator by Abdel-Bar *et al.*, (1987) in their study using *L. bulgaricus* for antimicrobial substance selection. Lewus *et al.*, (1991) also used *S. aureus* in their study for bacteriocin-producing *Lactobacillus*.

According to the result of an antimicrobial activity determination against *Staphylococcus aureus* using disc method, almost all neutralized supernatant fluids of lactic acid bacteria did not produce a zone of inhibition. Only two of them give slightly clear zone, i.e. strains TGR-2 and TMO-4. This may be due to the small level of antimicrobial produced by these strains.

In this report, antimicrobial activity was also determined using turbidimetric method. Davidson and Parish (1980) concluded that there are for possibilities for the antimicrobial activity test using turbidimetric method: First is growth level suppression, or sometimes called percentage growth inhibition; Second is a lag phase increase; Third is decreased in the growth rate with little effect on lag time; Fourth is a lethal effect.

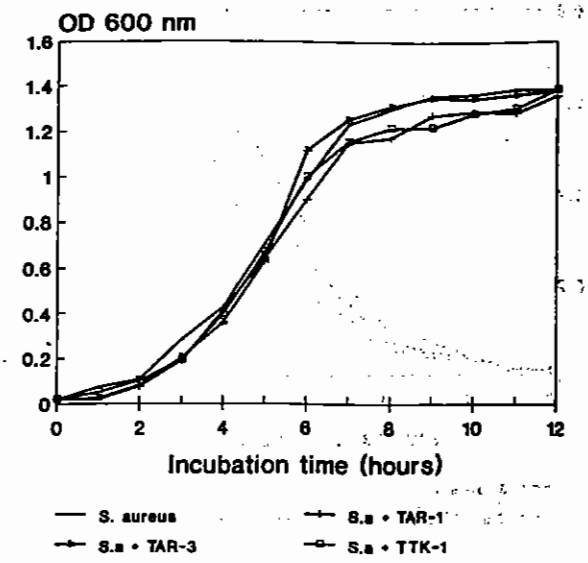


Figure 1. Inhibition effect of neutralized supernatant of lactic acid bacteria strains on the growth of S. aureus

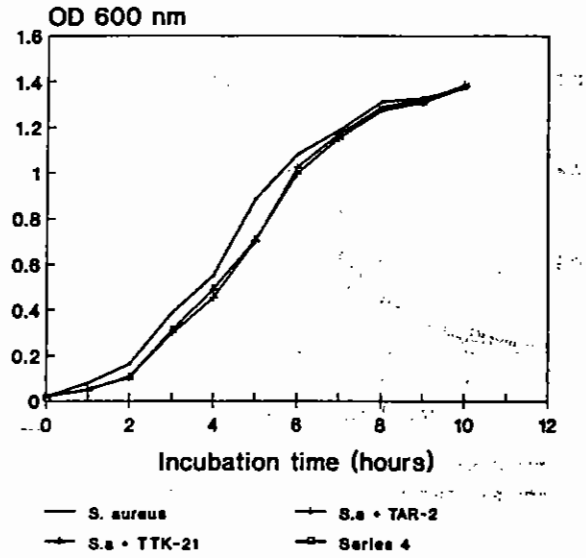


Figure 2. Inhibition effect of neutralized supernatant of lactic acid bacteria strains on the growth of S. aureus

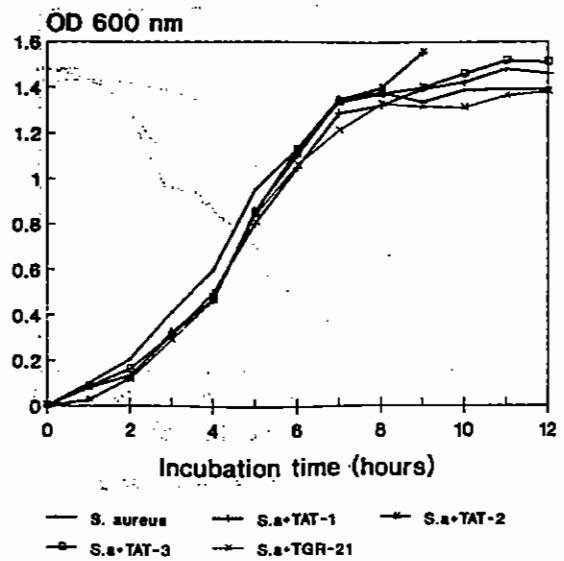


Figure 3. Inhibition effect of neutralized supernatant of lactic acid bacteria strains on the growth of S. aureus

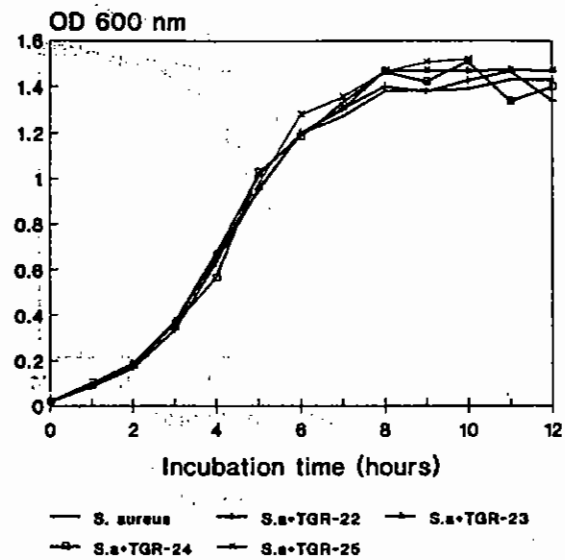


Figure 4. Inhibition effect of neutralized supernatant of lactic acid bacteria strains on the growth of S. aureus

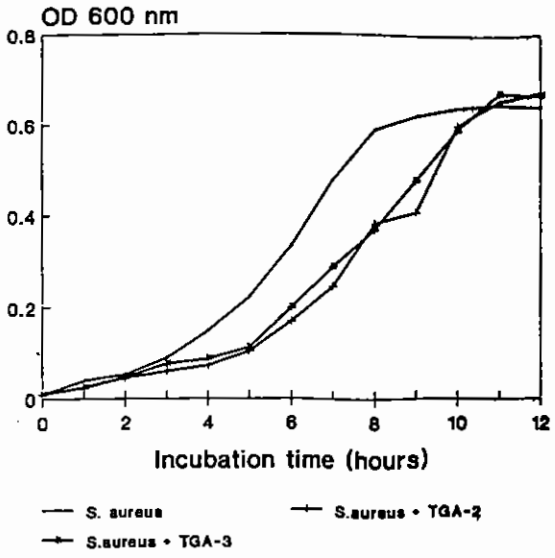


Figure 5. **Inhibition effect of neutralized supernantant of lactic acid bacteria strains on the growth of S. aureus**

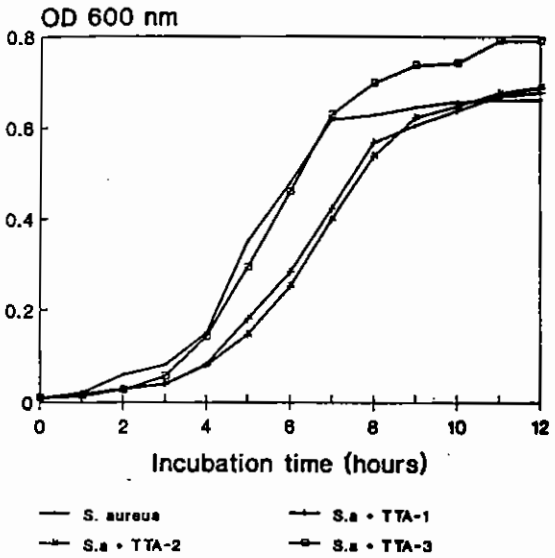


Figure 6. **Inhibition effect of neutralized supernatant of lactic acid bacteria strains on the growth of S. aureus**

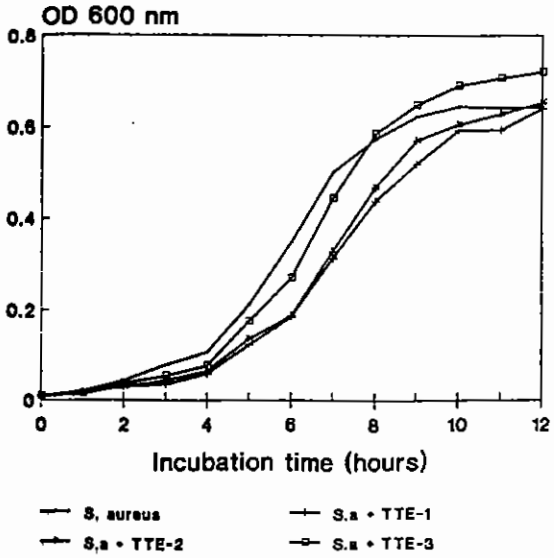


Figure 7. **Inhibition effect of neutralized supernatant of lactic acid bacteria strains on the growth of S. aureus**

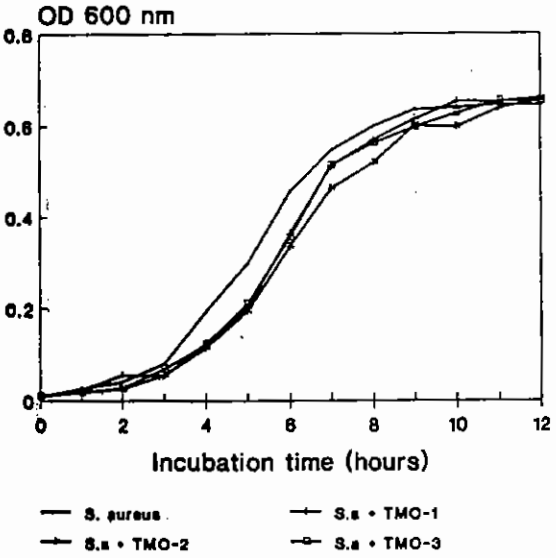


Figure 8. **Inhibition effect of neutralized supernatant of lactic acid bacteria strains on the growth of S. aureus**

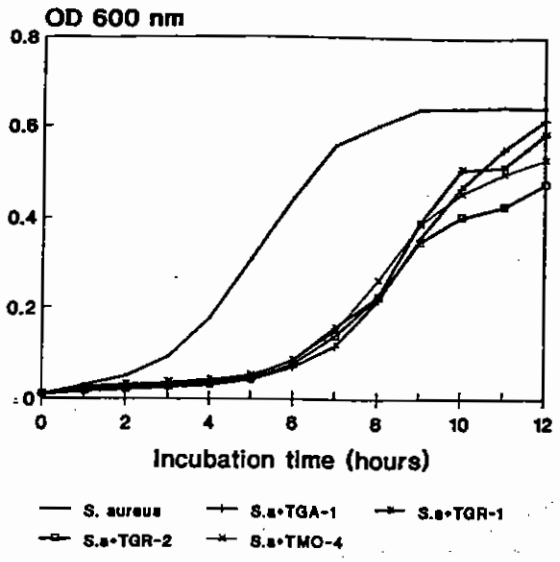


Figure 9. **Inhibition effect of neutralized supernatant of lactic acid bacteria strains on the growth of S. aureus**

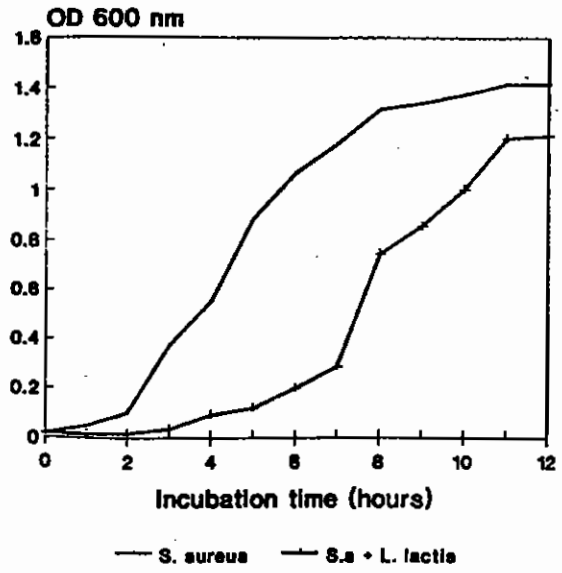


Figure 10. **Inhibition effect of neutralized supernatant of Lactococcus lactis on the growth of S. aureus**

Figure 1 to Figure 4, supernatant of the strains tested give no effect at all on the growth of *S. aureus*. These strains were isolated from *asinan rebung, asinan terong, tempoyak* and *growol*. Three of these fermented foods were fermented using additional salt, therefore it seem that antimicrobial producers lactic acid bacteria is hardly found in the salting fermented foods.

Figure 5 to Figure 8, there is slightly lag phase increases due to the inhibition effect from supernatant of several strains, particularly shown by TGA-2; TGA-3 (from *gatot*) - Fig. 5; TTA-1, TTA-2 (from *tape*), eventhough there is no final population suppression caused by this effect after 12 h incubation.

Figure 9, clearly shows, that supernatant of the four strains tested, i.e., TGA-1 (from *gatot*), TGR-1 and TGR-2 (*growol*), and TMO-4 (*moromi*) increased the lag phase of the growth of *S. aureus*. Even strains TGR-2 and TMO-4 showed the final population suppression after 12 h incubation, the same result also shown by the disc assay which only these two strains gave clear zone.

According to the result, Lactobacillus TGR-2 and Lactobacillus TMO-4 could be classified as a lactic acid bacteria which have antimicrobial activity with effects to increase the lag phase and to suppress the final population.

## CONCLUSION

Lactic acid bacteria which isolated from fermented foods dominated by *Lactobacillus plantarum* and L. *pentosus* complex. Strains TGR-2 obtained from *growol* and TMO-4 from *moromi* suspected to have an antimicrobial substance.

## ACKNOWLEDGEMENT

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