

Full Length Research Paper

Antagonistic properties of microorganisms associated with cassava (*Manihot esculenta* Crantz) products

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The antagonistic properties of indigenous microflora from cassava starch, flour and grated cassava were investigated using the conventional streak, novel ring and well diffusion methods. Antagonism was measured by zone of inhibition between the fungal plug and bacterial streak/ring. *Bacillus* species were more effective to inhibit the growth of *Aspergillus niger*, *A. fumigatus*, *Fusarium moniliforme* and *Rhizoctonia* sp. *Pseudomonas fluorescens*, *Escherichia coli* and *Saccharomyces* sp. inhibited the growth of *A. niger*, *A. flavus* and *F. moniliforme*. There were considerable variations in inhibitory activity. The zone of inhibition was more apparent in the novel ring method. Percentage inhibition increased to 78% in the Ring method and 62% in streak method after 168 h of incubation. *Lactobacillus brevis*, *L. acidophilus* and *Bacillus subtilis* inhibited the growth of *E. coli* and *Staphylococcus aureus*. Inhibition of the pathogenic microorganisms was probably due to the production of organic acids and bacteriocins.

Key words: Cassava product microflora, antagonism, bacteriocins, organic acids.

INTRODUCTION

Cassava (*Manihot esculenta*, Crantz) roots are processed into different products according to local customs and preferences. The products derived are high energy foods of excellent quality. Cassava, is however affected by fungi, bacteria, viral and virus-like and mycoplasma agent (IITA, 1993). Achievement of bio-control measure through microorganisms that occur naturally in the field has been made a reality via antibiotics, bacteriocins and bacteriophage (Moore, 1983). The anti-microbial properties of microbes have therefore led to the characterization of various anti-microbial substances as organics acids (lactic acid and formic acids), diacetyl, and hydrogen peroxide alone or in combination (Piard and Desmazeaud, 1992). Other anti-microbial substances with antagonistic properties include biocides, sterilants, and probiotics. The production of specific protein or protein complexes, bacteriocin that inhibits some Gram positive bacteria, mainly homologous

species, some sphaeroplast of Gram negative bacteria and antifungal antibiotics also confirmed the possibilities of bio-competitive exclusion of pathogenic organisms from food. Therefore, the objectives of this paper were to investigate the antagonistic properties of isolates from cassava products and to characterize the inhibitory substance(s) of the isolates.

MATERIALS AND METHODS

Microbial cultures

The microorganisms used in this investigation (*Bacillus cereus*, *B. subtilis*, *B. pumilus*, *Escherichia coli*, *Klebsiella aerogenes*, *Lactobacillus acidophilus*, *Leuconostoc mesenteroides*, *Pseudomonas aeruginosa*, *P. fluorescens*, *Salmonella* sp., *Shigella flexneri*, *Staphylococcus aureus*, *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *Candida* sp., *Fusarium moniliforme*, *F. nivale*, *Mucor mucedo*, *Neurospora* sp., *Penicillium* sp, *Rhizopus stolonifer*, *Trichoderma viridae*, *Rhizoctonia* sp. and *Saccharomyces* sp.) were isolated from grated cassava, cassava flour and starch (Olowoyo et al., 2001). The organisms were maintained by routine culture on agar slants, stored at 4°C between transfers. At least two additional subcultures (24 h, 25°C) were made in fresh medium before use in the experiment.

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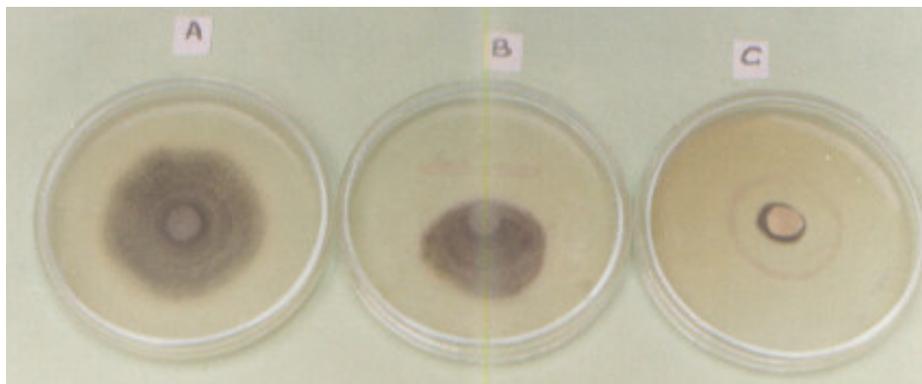


Figure 1. Antagonistic zone between *Bacillus pumilus* and *Aspergillus niger*. Photograph taken 5 days after inoculation. Codes: A, control; B, Fokkema/Conventional streak method; C, Concentric/Novel Ring bioassay.

Detection of Antagonistic Activity

For detection of antagonistic activity of bacterial isolates towards the growth of the fungal cultures, both Fokkema (1973) and Adetuyi and Cartwright (1985) methods were used. For the conventional streak method (Fokkema, 1973), 40 mm streak was made from 24 h – old culture of bacteria, 23 mm away from the centre of petridish. Using a 7 mm diameter sterile cork borer, the growing edge of a 4-day old fungal culture was aseptically cut and placed at the centre of the plate already inoculated with the test antagonist. Plates were incubated at 25°C and monitored for 7 days. Measurement of the percentage of inhibition and intercolony distance were taken daily.

For the Novel ring method of Adetuyi and Cartwright (1985), bacteria were cultured on nutrient agar plates into confluent growth. Prepared Malt Extract Agar plates were inoculated separately with concentric ring culture of the organism. The fungal plug (7 mm) was transferred aseptically into the centre of the plate already inoculated with the test antagonist. Plates were incubated at 25°C and monitored for 7 days. The measurement of the percentage of inhibition and intercolony distance were taken daily.

The well diffusion assay described by Schillinger and Lucke (1989) was used for the in-vitro test of antagonistic activities of bacterial isolates against other bacteria. Plates containing solidified Nutrient Agar (20 ml) were overlaid with 10ml of soft Nutrient Agar and inoculated with 0.05 ml of an overnight culture of test isolate. Four wells, three of the periphery and one at the centre, each 7 mm in diameter were made in the agar using cork borer and 0.1 ml of culture supernatant of test bacterium were transferred into each well. The plates were incubated for 24 h at 25°C, examined for clear inhibition zone around the well. The assay was carried out in duplicate for all the test organisms.

Preparation of culture supernatants

The test antagonist was grown in a Nutrient Broth (Oxoid) at 25°C for 24 h. Bacteria cells were removed by centrifuging the cultures at 3900 rpm for 30 min. The supernatant was used immediately.

Characterization of inhibitory substance(s)

To characterize the inhibitory compounds produced by the bacterial isolates, their culture supernatants were assayed for organic acids, bacteriocins and hydrogen peroxide by having their culture

supernatants' pH adjusted to 6.5, with the addition of 1 mol NaOH; treated (concentration 1 mg ml⁻¹) for 12 h at 37°C, or with catalase (concentration 0.5 mg ml⁻¹), respectively. Each treated supernatant (0.1 ml) was transferred into a well at the periphery of the agar and the untreated culture supernatant (0.1 ml) was transferred into the well at the centre. The presence or absence of inhibitory zones around the wells was determined after incubation for 24 h at 25°C. The assay was conducted in duplicates.

RESULTS AND DISCUSSION

Bacillus species were more effective in inhibiting the growth of *A. niger*, *A. fumigatus*, *F. moniliforme*, *Rhizoctonia* sp. and *E. coli* (Figures 1-4). Clear zones of inhibition were maintained throughout in both streak and ring bioassay. Antagonism was measured by zone of inhibition between the fungal plug and bacterial streak/ring. There were considerable variation in inhibitory activity. The zone of inhibition was more apparent in the novel ring method. Percentage inhibition increased to 78% in the ring method and 62% in the streak after 168 h of incubation (Figures 7-8). The antagonistic activities of *Bacillus* species could be attributed to the production of endospores, the antagonists are more adapted to environmental extremes than the pathogens.

P. fluorescens inhibited the growth of *A. niger* (Figure 5). Fluorescent *Pseudomonas* sp. have been suggested to be an important natural antagonist of plant pathogens (Klopper et al., 1980). *L. brevis* and *L. acidophilus* showed varying degrees of inhibitory ability against *E. coli* and *B. subtilis*, the radii of inhibition zones varied from 10.0 to 12.5 mm. *B. pumilus* was the least effective in inhibiting the growth of *E. coli* (8.6 mm). On the other hand, *Saccharomyces* sp. inhibited the growth of *E. coli* and *S. aureus*. The radii of their inhibition zones ranged from 8.0 to 8.9 mm. *Lactobacillus* sp. are not known to be strong inhibitors of Gram-negative bacteria (Tagg et al., 1976). This observation is in conformity

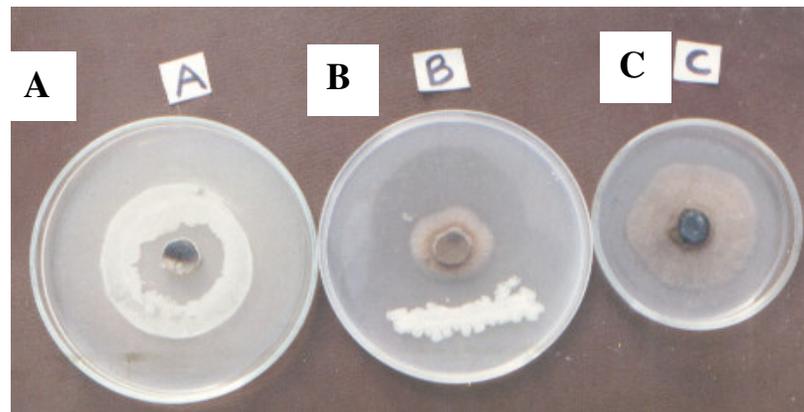


Figure 2. Antagonistic zone between *Bacillus pumilus* and *A. fumigatus*. Photograph taken 5 days after inoculation. Codes: A, Concentric/Novel Ring bioassay; B, Fokkema/Conventional streak method; C, control.



Figure 3. Antagonistic zone between *Bacillus pumilus* and *Fusarium moniliforme*. Photograph taken 5 days after inoculation. Codes: A, control; B, Fokkema/Conventional streak method; C, Concentric/Novel Ring bioassay.

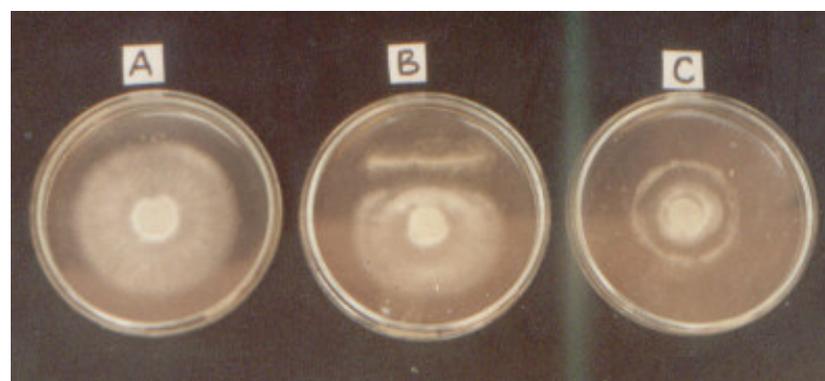


Figure 4. Antagonistic zone between *Bacillus pumilus* and *Fusarium nivale*. Photograph taken 5 days after inoculation. Codes: A, control; B, Fokkema/Conventional streak method; C, Concentric/Novel Ring bioassay.

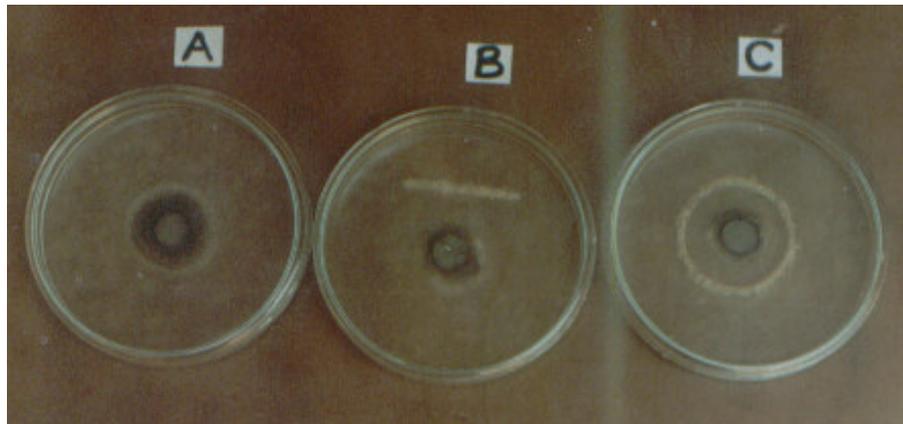


Figure 5. Antagonistic zone between *Pseudomonas fluorescens* and *A.niger*. Photograph taken 5 days after inoculation. Codes: A, control; B, Fokkema/Conventional streak method; C, Concentric/Novel Ring bioassay.

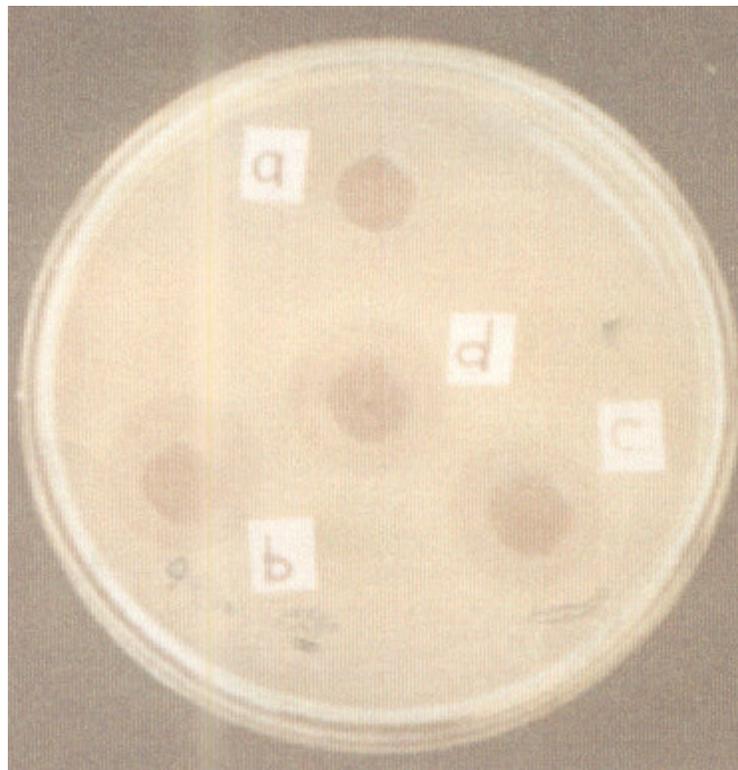


Figure 6. Antagonistic zones between *Escherichia coli* and treated culture supernatants of *Bacillus pulmilus* in agar well diffusion assay. **A**-well containing supernatant treated with 1 mg/ml trypsin. **B**-well containing supernatant treated with NaOH. **C**-well containing supernatant treated with 0.5 mg/ml catalase. **D**-well containing untreated culture supernatant (control).

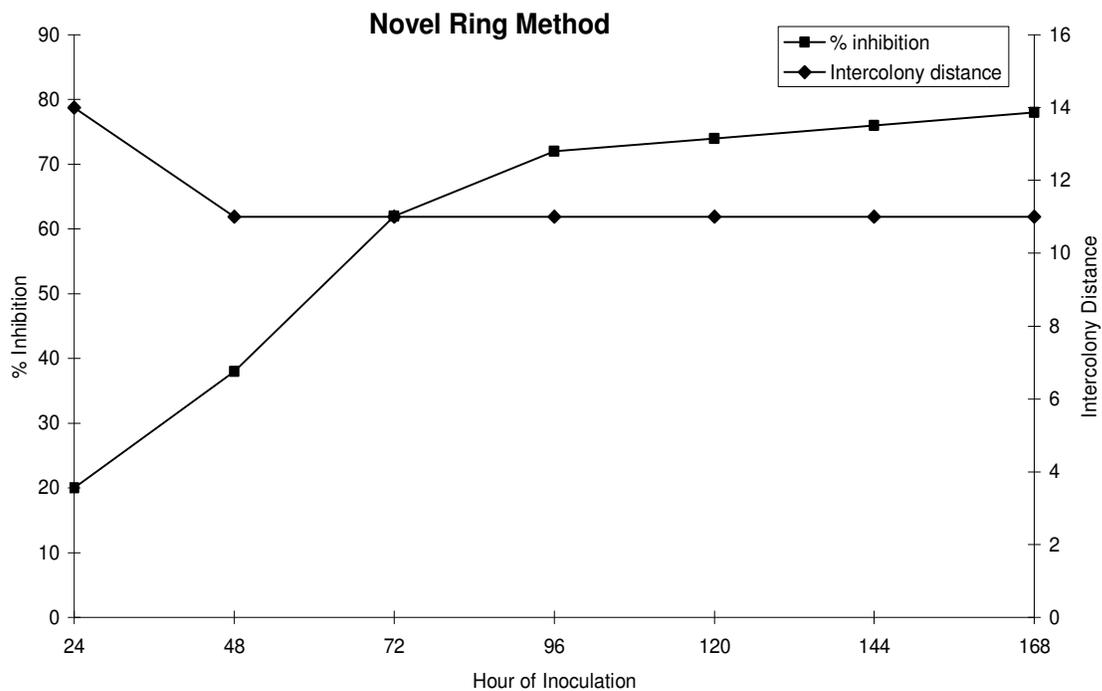


Figure 7. Effect of anatanogistic ability of *Bacillus pulmelus* on *Fusarium nivale* In terms of intercolony distance and percentage inhibiton of radial growth rate.

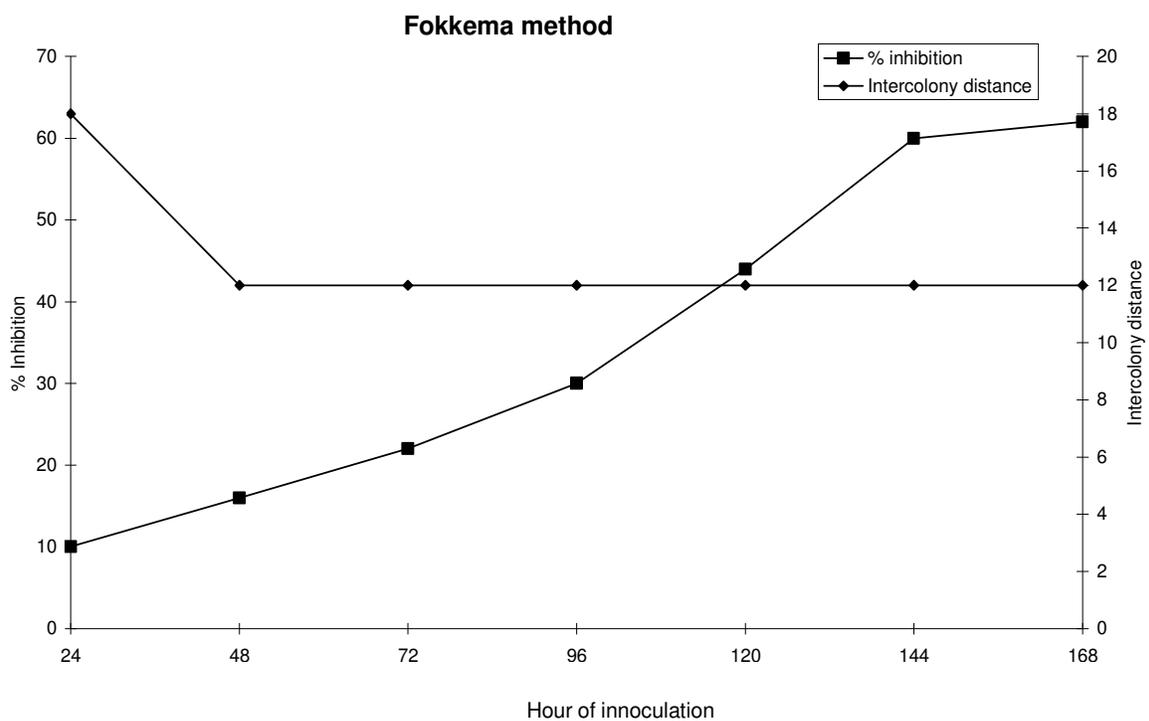


Figure 8. Effect of anatanogistic ability of *Bacillus Pulmelus* on *Fusarium nivale* in terms of intercolony distance and percentage inhibition of radial growth rate.

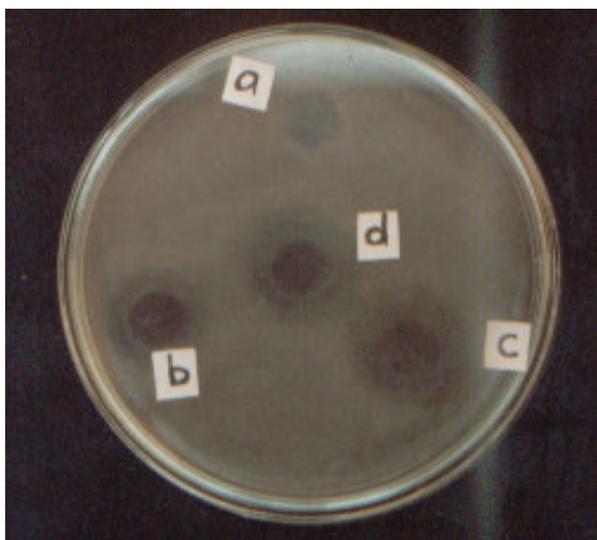


Figure 9. Inhibition of *Staphylococcus aureus* by treated supernatant of *Bacillus pumilus* in well diffusion assay. **A**-well containing supernatant treated with 1 mg/ml trypsin. **B**-well containing supernatant treated with NaOH. **C**-well containing supernatant treated with 0.5 mg/ml catalase. **D**-well containing untreated culture supernatant (control).

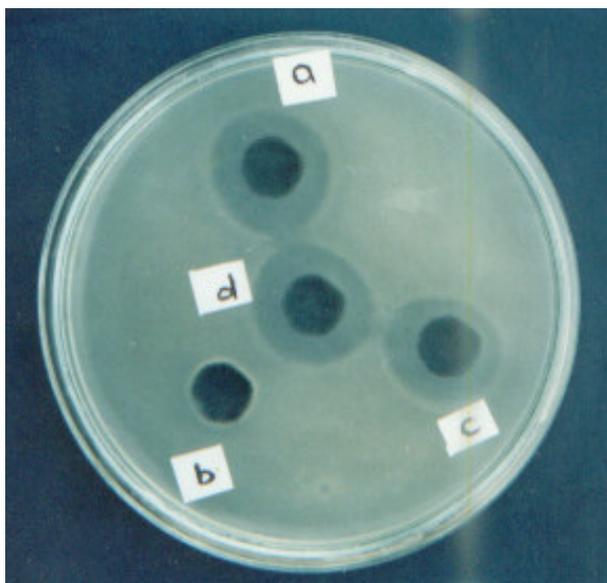


Figure 10. Inhibition of *Escherichia coli* by treated supernatant of *Bacillus pumilus* in an agar well diffusion assay. **A**-well containing supernatant treated with 1 mg/ml trypsin. **B**-well containing supernatant treated with NaOH. **C**-well containing supernatant treated with 0.5 mg/ml catalase. **D**-well containing untreated culture supernatant (control).

with the reports of Gilliland and Speck (1977) that *lactobacilli* showed stronger antibacterial properties against Gram-positive bacteria (such as *S. aureus*). It has been suggested that *lactobacilli* block the

adhering sites where pathogenic bacteria would attach. Their ability to inhibit the growth of pathogenic *E. coli* in the present investigation implies that the *Lactobacillus* strains not only attach to where the pathogens would adhere, but also produces some substance(s) to inhibit the growth of the pathogens.

The inhibitory zones remain unchanged when the culture supernatants were treated with catalase. This shows that inhibition was not caused by the action of hydrogen peroxide. Treatment of *L. brevis* and *Saccharomyces* sp. culture supernatants with trypsin also did not affect their inhibition activities. This suggests that inhibition was not due to the production of bacteriocin. No inhibitory zones were observed when *L. brevis* and *Saccharomyces* sp. culture supernatants were adjusted to pH 6.5 which indicates that inhibition was due to the production of organic acids. Jin et al. (1996) reported that *Lactobacillus* sp. isolated from chicken intestine were able to inhibit the growth of pathogenic bacteria such as *Salmonella* and *E. coli* with the inhibitory activities probably due to the production of organic acids. The inhibiting ability of *B. pumilus* against *S. aureus* and *E. coli* was attributed to the production of bacteriocin (a polypeptide); there was no inhibitory zone observed when supernatants were treated with trypsin (Figures 9-10).

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