

Identification of Antimicrobial Substance Producing Lactic Acid Bacteria Isolate KUB-KJ174 and Its Application as a Biopreservative Substance for Bakery Products

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ABSTRACT

The effective antimicrobial substance producing KUB-KJ174 was identified as *Lactobacillus plantarum*. The supernatant with a low pH of 3.6 showed 100% spectrum inhibitory activity against contaminant bacterial groups of 50 aerobic mesophiles, 10 *Bacillus cereus* and 53 coliform, but only 46% spectrum inhibitory activity against 13 lactic acid bacteria (LAB). However, when the pH was adjusted to 5, only low spectrum inhibitory activity against aerobic mesophilic bacteria, *Bacillus cereus*, coliform and LAB was shown with 26, 50, 47 and 0%, respectively. Partial purification by pH-mediated cell adsorption-desorption provided an active antimicrobial substance (the so-called PP-174) that displayed 100% growth inhibitory activity against microbial strains of aerobic mesophilic bacteria T6.14, *B. cereus* B6.2, coliform C4.1, LAB L2.2 and yeast Y5.1 showing low sensitivity to antimicrobial substances. However, a low concentration of 0.5% PP-174 could not inhibit the growth of LAB L2.2 within 24 h. A combination of 0.5% PP-174 with 17.42 mM lactic acid and 6.91 mM acetic acid (the so-called BAKE-SAFE-1) was able to inhibit the growth of LAB completely. Cream filling treated with BAKE-SAFE-1 completely inhibited the contaminant group of *B. cereus* and LAB at 15°C. When the temperature was increased to 25 and 37°C, only a low growth inhibitory efficiency of 11.68-41.45% against the contaminant group of aerobic mesophilic bacteria, *B. cereus* and LAB occurred. However, 100% growth inhibitory efficiency was displayed against the contaminant groups of coliform and yeast mold.

Key words: *Lactobacillus plantarum*, bakery product, antimicrobial substance, cream filling, *Bacillus cereus*, aerobic mesophilic bacteria, coliform

INTRODUCTION

Bakery products have become a convenient food in the changing lifestyle of the Thai people over recent decades. The high temperature (up to 170°C) used for the baking process means that baked products are generally considered microbiologically safe. However,

according to the Canadian Food Inspection Agency (CFIA), baked products do not pose a health risk unless they have cream or custard filling containing eggs or dairy products. This filling is the most likely source of serious food safety hazards due to post-baking microbial contamination. Another risk may come from climatic conditions, especially in a tropical country

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like Thailand, facilitating microbial growth. Phoonsawat *et al.* (2008) found that contamination of yeast, mold and *Escherichia coli* above standard levels from all of 10 bakery products came mainly from operators' hands and aprons. To solve these unavoidable problems, an effective, antimicrobial substance (AMS) is needed. So far, many kinds of chemical compounds of benzoate, propionate and potassium sorbate have been used as preservatives for bakery products (Guynot *et al.*, 2005). However, these compounds at permissible concentrations were not effective when applied to cake products with cream filling (Fustier *et al.*, 1998).

Lactic acid bacteria (LAB) are widely used in food production; they are becoming a major component as natural food preservatives, to improve food safety and stability. LAB can protect food from spoilage bacteria or fungi by producing AMS, such as organic acids (lactic acid, acetic acid, etc.), hydrogen peroxide, diacetyl, carbon dioxide, bacteriocin and other low molecular weight compounds. (Helander *et al.*, 1997; Ouwehand, 1998). In general, the primary inhibitory effect is achieved due to the production of organic acids, which lower the pH and directly inhibit microorganisms. These organic acids and other AMS may act synergistically and have broad-spectrum ability to inhibit bacteria, yeast and mold (Atanassova *et al.*, 2003). Although the antimicrobial action is difficult to explain due to the complex and commonly synergistic interactions between different compounds, there have been several reports on the use of antimicrobial properties and great interest because most of the LAB are considered as "generally recognized as safe" microorganisms (Holzapfel *et al.*, 1995). To date the authors' laboratory has screened AMS-producing LAB from many sources originating from both plants and animals. One strain, KUB-KJ174, was isolated from a fermented rice product (Khanomjeen) and showed growth-inhibiting ability against both *Penicillium* and *Aspergillus* species, which are reported to be

spoilage organisms in bakery products. To learn more about its efficacy, the present study, therefore, aimed to: (i) identify the strain KUB-KJ174; (ii) achieve microbial contaminant control by an AMS formulation consisting of partially purified AMS and acid; and (iii) determine its possible application as a biopreservative for cream filling.

MATERIALS AND METHODS

Microorganisms and growth conditions

LAB KUB-KJ174 samples that had been isolated from fermented rice noodles (known as Khanomjeen) were used as AMS sources. The samples were cultivated on Man Rogosa and Sharp (MRS, sourced from Merck, Germany) at 37°C for 18-24 h. Target test microorganisms were isolated from spoilage cream puffs, commonly called "eclairs". The microorganisms consisted of 30 yeast isolates grown on PDA (Potato dextrose agar) and 126 bacterial isolates consisting of 50 isolates of aerobic mesophilic bacteria grown on PCA (plate count agar), 10 isolates of *Bacillus cereus* grown on MYP (Mannitol-egg-yolk-polymyxine-agar), 53 isolates of coliform grown on Violet Red Bile Agar and 13 LAB isolates grown in MRS. Since all the bacterial target strains except LAB were able to be grown on a simple medium of nutrient broth (NB, Pronadisa, Spain) they were cultivated on either NB or NA at 37°C for 18-24 h, while the yeast isolates were grown on potato dextrose agar (PDA; Merck, Germany) at 30°C for 2 d. These indicator strains were stored at -80°C in desirable media containing 20% (v/v) glycerol, and propagated twice before experimental use.

Identification of the LAB

Identification of the isolate KUB-KJ174 was based on its cell morphology, a physiological test (Axelsson, 1998), carbohydrate fermentation using the API 50CHL test kit (Biomerieux, France) and 16S rDNA sequence analysis

(Nitisinprasert *et al.*, 2000). Primers 1407b (5'-GACGGGCGGTGTGTAC-3') and 8UA (5'-AGAGTTTGATCCTGGCTCAG-3') were used as forward and reverse primers, respectively. Amplification was carried out in a thermo-cycler (Hybrid, UK) with annealing for 2 min at 55°C. The purified PCR product was ligated into pGEM-T® Easy vector (Promega, USA) and then transformed into *E. coli* DH5 α competent cells to obtain the 16S rDNA clone. The sequence of amplified product 16S rDNA was analyzed by a commercial provider (Macrogen Inc., Korea). The resulting sequence was compared with the non-redundant nucleotide database from GenBank using the BLAST program.

Preparation of supernatant, intracellular fluid and cell-bound antimicrobial substances

One milliliter of supernatant was prepared from 24 h culture which was centrifuged at 10 900g at 4°C for 15 min. The intracellular fluid and cell-bound antimicrobial substance were prepared according to the modified method of Randazzo *et al.* (2002) and Zue *et al.* (2002). The cell pellet was washed once with TE buffer pH 7.6, suspended in 1 ml of the same buffer and later broken by 0.3 g of zirconium bead in a Mini-beadbeater (Biospec Products Inc., USA) at 4800 rpm for 50 sec, followed by 1min in ice and then 80 sec followed by 1 min in ice for the 2nd and 3rd rounds. The disintegrated solution obtained was centrifuged at 10 900g for 15 min to gain intracellular fluid and cell debris. Two hundred microliters of TE buffer pH 7.6 was used to suspend the cell debris. The suspended solution was defined as cell-bound antimicrobial substance.

Determination of antimicrobial activity

The spot-on-lawn-method was used to determine antimicrobial activity as previously described by Hoover and Harlender (1993). The agar plates containing either NA, MRS or PDA were overlaid with medium soft agar (0.7%) that contained the target test strains of approximately

10⁷ CFU/ml. Ten microliters of sample was spotted onto the overlaid surface and the plates were incubated for 18-24 h at 37°C. The antimicrobial substance titre was determined by serial twofold dilution. Activity was defined as the reciprocal of the last serial dilution that produced an inhibitory zone and was expressed as activity units per milliliter (AU/ml). To determine the antimicrobial activity of supernatant, intracellular fluid and cell-bound antimicrobial substances, *Salmonella* Enteritidis S003 was used as a target test strain.

Determination of spectrum inhibitory activity

Aerobic mesophilic bacteria, *Bacillus cereus*, coliform, LAB and yeast of 50, 10, 53, 13 and 30 isolates, respectively, contaminating spoilage cream puff were used as target test strains to determine antimicrobial activity as mentioned above. The spectrum inhibitory activity (SI activity) was defined as the percentage of isolate number inhibited by the prepared antimicrobial substance in all isolates tested.

Partially purified antimicrobial substances (PP-AMS) by pH-mediated cell adsorption-desorption

The AMS was partially purified and concentrated using the pH-mediated cell adsorption-desorption method described by Yang *et al.* (1992). Five hundred milliliters of 24 h culture were heated at 70°C for 35 min to inactivate any native protease, cooled down to room temperature and then adjusted to pH 5.5-6.0 with 4N NaOH by stirring at room temperature for 2 h to allow AMS adsorption onto the cell. Cells were collected by centrifugation at 10 900g at 4°C for 15 min and washed once with pH 6.5 sodium phosphate buffer. Then, the AMS was released by re-suspending in 25 ml of 100 mM NaCl adjusted to pH 2 with 5% phosphoric acid (v/v) and stirred at 4°C for 18 h. The cells were removed by centrifugation to obtain partially purified AMS (PP-AMS), which was later determined for antimicrobial activity.

Analysis of organic acids from supernatant

Organic acids from 24 h supernatant were separated by high performance liquid chromatography (HPLC) with an Aminex HPX 87H column (Bio-rad, USA) and a mobile phase of 0.008 M sulfuric acid according to the modified method of Cheigh *et al.* (2002). The running conditions involved 45°C, a flow rate of 0.5 ml/min and 0.1% (w/v) tartaric acid used as an internal standard. The samples of 24-hour supernatant or standard markers, consisting of acetic acid, butyric acid, lactic acid and propionic acid, were mixed with 0.2% (w/v) tartaric acid at 1:1 ratio (v/v) and filtered through a 0.22 µm membrane. The acid peaks were detected by the ChromQuest Program version 2.51 (ThermoQuest, Inc., USA) at a wavelength of 230 nm. Qualitative acid analysis was determined by the retention time of the acid peaks, while quantitative analysis was carried out using a standard curve composed of the various acid concentrations versus the peak area ratio of the acid peaks and the internal standard.

Determination of growth inhibitory activity of PP-AMS against the microbial strains showing high resistant to antimicrobial substance for biopreservative compound formulation

Both PP-AMS alone and in combination with a mixture of organic acids (MOR) at different twofold dilutions with a 1:1 ratio were tested for growth inhibitory activity against five target strains showing high resistance to AMS: T6.14, B6.2, C4.1, L2.2 and Y5.1 from the microbial groups of aerobic mesophilic bacteria, *Bacillus cereus*, coliform, LAB and yeast, respectively. The growth inhibitory reaction composed of 800 µl of culture solution (approx. 10^{4-7} CFU) and 200 µl of AMS mixture solution was incubated at 37°C for 15 min and 24 h. The survival cells from each reaction were assayed by standard plate count using suitable media for each target strain. The growth inhibitory activity was evaluated using Equation 1:

$$\text{Growth inhibitory activity (GI)} = \frac{C_0 - C_t}{C_0} \times 100 \quad (1)$$

where: C_0 and C_t equal logCFU of target strain at 0 and 15 min or 24 h of incubation time, respectively.

Determination of growth inhibitory efficiency in cream filling during storage at 15, 25 and 37°C

Cream filling consisting of 20% water and 80% corn starch, sugar, egg, milk and butter was prepared and mixed well at 60-70°C for 10 min according to the bakery production recipe and was used as a control treatment. For the AMS treatment, 20% of the formulated AMS was added instead of water and prepared as above. Twenty-five grams of cream filling from each treatment were packed in sterilized polyethylene bags and then kept at 15, 25 and 37°C for 2 d. The cell number of aerobic mesophilic bacteria, *Bacillus cereus*, coliform, LAB and fungi were determined by standard plate assay using PCA, MYP, VRB, MRS+0.5% CaCO_3 and PDA, respectively. The growth inhibitory efficiency in cream filling (GIE) was evaluated using Equation 2:

$$\text{Growth inhibitory efficiency in cream filling (GIE)} = \frac{C_c - C_t}{C_c} \times 100 \quad (2)$$

where: C_c and C_t were logCFU of control and formulated AMS treatment, respectively.

RESULTS

Identification of the LAB

The isolate KUB-KJ174 had the following characteristics: gram-positive, rod shape, catalase-negative, non-motile, no gas production, no spore forming and good growth at temperatures of 37, 45 and 50°C, pH 4.5 and 6.5% NaCl. Thus, it was indicated to be a homofermentative *Lactobacillus*, as proposed by Salminen and Wright (1998). Based on

carbohydrate fermentation analyzed by the APILAB program version 4.0 (Table 1) and 1478 bp of 16S rDNA sequence analysis, the KUB-KJ174 was classified to be *Lactobacillus plantarum* with 99% similarity to accession no. DQ141558.1.

Inhibitory activity of supernatant, intracellular fluid and cell-bound antimicrobial substance from *L. plantarum* KUB- KJ174 against food spoilage microorganisms from eclairs

The 24-hour supernatant was determined for pH and SI activity against 126 contaminant target strains as shown in Figure 1. The supernatant with a low pH of 3.6 exhibited SI activity of 100%

against aerobic mesophilic bacteria, *Bacillus cereus* and coliform, while it showed only 46% SI activity against LAB. When the pH of supernatant was adjusted to 5 and 6, the pH5-treated supernatant displayed a low SI activity of 26, 50, 47 and 0% against aerobic mesophilic bacteria, *Bacillus cereus*, coliform and LAB, respectively, while the pH6-treated CFS did not show any SI activity against any of the target strains. It was possible that the high SI activity was produced by organic acids or by both organic acids and other compounds, but this requires further study.

It was noticed that no inhibition occurred against 30 yeast isolates. By previous direct screening assay (data not shown), *L. plantarum*

Table 1 Carbohydrate fermentation pattern of the isolate KJ174.

Test	Results	Test	Results
1. Glycerol	-	26. Salicin	+
2. Erythritol	-	27. Cellubiose	+
3. D-Arabinose	-	28. Maltose	+
4. L-Arabinose	-	29. Lactose	+
5. Ribose	+	30. Melibiose	+
6. D-Xylose	-	31. Sucrose	+
7. L-Xylose	-	32. Trehalose	+
8. Adonitol	-	33. Inulin	-
9. Methyl-D-xyloside	-	34. Melezitose	+
10. Galactose	+	35. Raffinose	+
11. Glucose	+	36. Starch	-
12. Fructose	+	37. Glycogen	-
13. Mannose	+	38. Xylitol	-
14. Sorbose	-	39. Gentiobiose	+
15. Rhamnose	-	40. D-Turanose	+
16. Dulcitol	-	41. D-Lyxose	-
17. Inositol	-	42. D-Tagatose	-
18. Mannitol	+	43. D-Fucose	-
19. Sorbitol	+	44. L-Fucose	-
20. Methyl-D-Mannoside	+	45. D-Arabitol	-
21. Methyl-D-Glucoside	-	46. L- Arabitol	-
22. N-Acetyl-Glucosamine	+	47. Gluconate	+
23. Amygdalin	+	48. 2-keto-gluconate	-
24. Arbutin	+	49. 5-keto-gluconate	-
25. Esculin	+		

+ = acid production (positive); - = no acid production (negative).

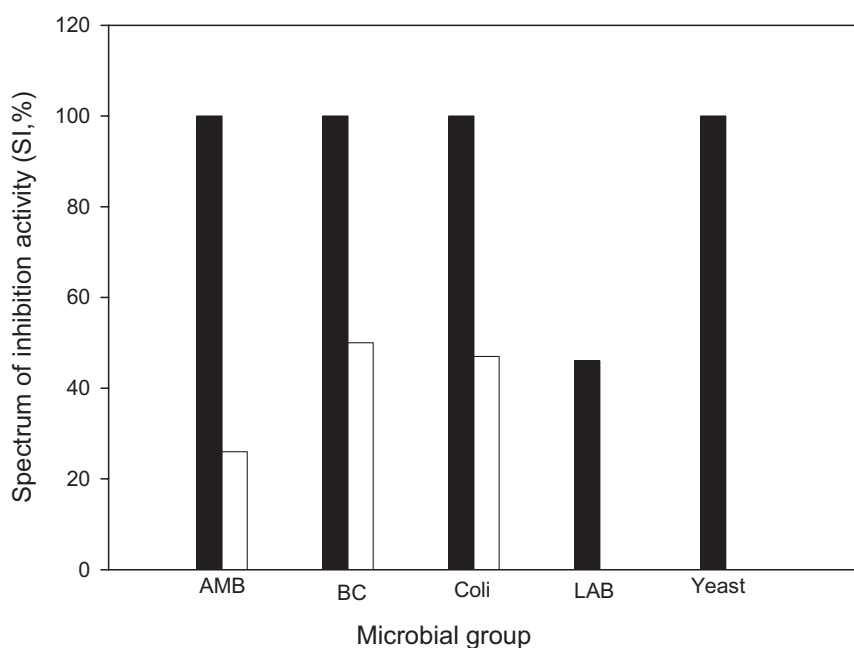


Figure 1 Spectrum of inhibitory activity of supernatant from *Lactobacillus plantarum* KUB-KJ174 against aerobic mesophilic bacteria (AMB), *Bacillus cereus* (BC), coliform (Coli), lactic acid bacteria (LAB) and yeast. Supernatant, ■ and supernatant at pH 5, □.

KUB-KJ174 exhibited AMS activity of 14.5-15.8 (diameter ratio of inhibition zone and colony) against *Saccharomyces cerevisiae* SG2-1Y and *Penicillium* sp. S-C1 contaminating grass silage. Therefore, it was hypothesized that the AMS might still adsorb onto the cell. Therefore, the AMS activities of supernatant, intracellular fluid and cell-bound fractions were determined for their antimicrobial activities. The results showed that only cell-bound fractions exhibited activity of 100 AU/ml against *S. cerevisiae* SG2-1Y and *Penicillium* sp. S-C1. It could be concluded that the AMS inhibiting fungal contaminant appeared to be cell bound.

Preparation of partial purification of antimicrobial substances (PP-AMS) and their growth inhibitory activity (GI)

L. plantarum KUB-KJ174 exerted AMS against microbial contaminants from supernatant and cell-bound fluid as mentioned above.

Therefore, in order to effectively remove the AMS from both the supernatant and cell-bound fraction, a purification technique of pH-mediated adsorption and desorption was performed and resulted in the PP-AMS of 3200 AU/ml designated as PP-174. The representative microbial groups of aerobic mesophilic bacteria T6.14, *Bacillus cereus* B6.2, coliform C4.1, LAB L2.2 and yeast Y5.1, showing high resistance to supernatant, were selected as target test strains. The PP-174 displayed 100% GI against all five test strains within 15 min. However, when PP-174 was diluted to 0.5, 1.0, 5.0 and 10.0% (V/V), only 5-10% of PP-174 showed 100% GI against coliform C4.1 as shown in Figure 2, with the lower concentrations of 0.5 and 1.0% exhibiting lower activities of 6.41 and 27.66%, respectively. Only low GI values of 2.56-55.33% were obtained from 0.5-10% PP-174 treatment against the other test strains of aerobic mesophilic T6.14, *B. cereus* B6.2, LAB L2.2 and yeast Y5.1.

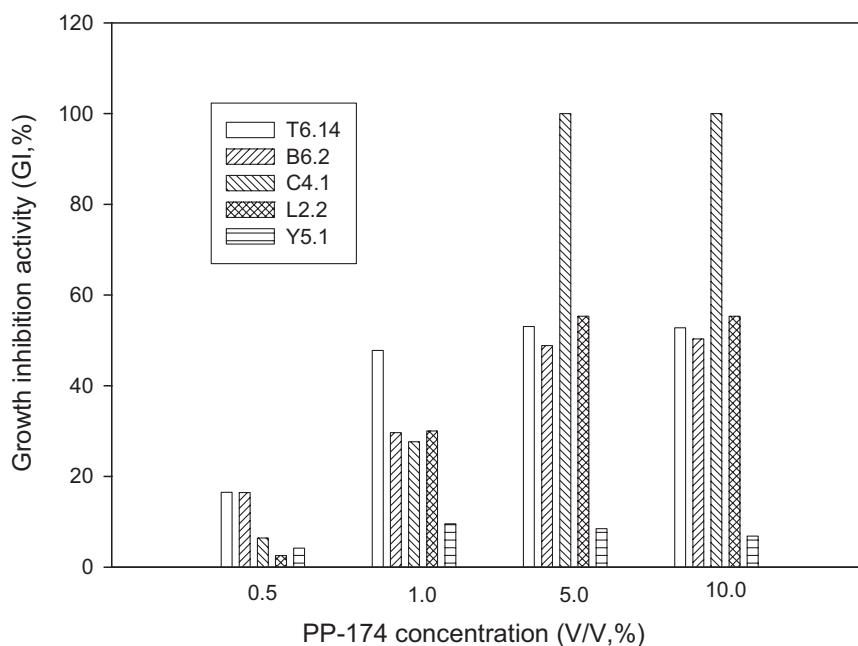


Figure 2 Effect of PP-174 at various concentrations to inhibit the growth of aerobic mesophilic T6.14, *Bacillus cereus* B6.2, Coliform C4.1, LAB L2.2 and yeast Y5.1 at 15 min.

The inhibitory activity also depended on the contact time, with 15 min expected to be too short. Therefore, only low concentration of 0.5 and 1% PP-174 were assayed against all five target strains for 24 h in a second test. Interestingly, 100% GI occurred with all target strains except LAB, which showed no inhibition.

The effect of PP-174 and the mixture of organic acid (MOR) on growth inhibitory activity (GI) against LAB

Since the supernatant was able to inhibit the growth of some LAB isolates, the organic acids produced might have some inhibitory activity by synergistic action with PP-174. Therefore, the organic acid content in the supernatant sample was analyzed by HPLC, which resulted in a high concentration of 139.37mM lactic and 55.25 mM acetic acid defined as a mixture of organic acids (MOR). Then, the mixture of 0.5% PP-174 and MOR at various multiple dilutions of 2, 4, 8, 16, 32, 64, 128 and 256 tested for GI against the

growth of LAB L2.2 were investigated as shown in Figure 3. The mixtures containing MOR with a twofold to eightfold dilution were able to inhibit the growth of L2.2 for 100% GI, while the remaining mixtures with the greater dilution factors of 16 and more only had a low GI value of 0-13.3%. In addition, the MOR with eightfold dilution alone, containing 17.42 mM lactic acid and 6.91 mM acetic acid, had no inhibitory activity against L2.2. Therefore, it was clearly shown that MOR (at eightfold dilution) combined with 0.5% PP-174, completely inhibited the growth of LAB. This formula, defined as BAKE-SAFE-1, was used for further applications in the experiment.

Effect of BAKE-SAFE-1 on microbial contaminants in cream filling during 2 d storage

A type of cream puff (the so-called eclair) is a bakery product that can spoil easily within 6 h at a room temperature of more than 30°C due to its cream filling enriched with egg, milk, starch and sugar supporting good growth of

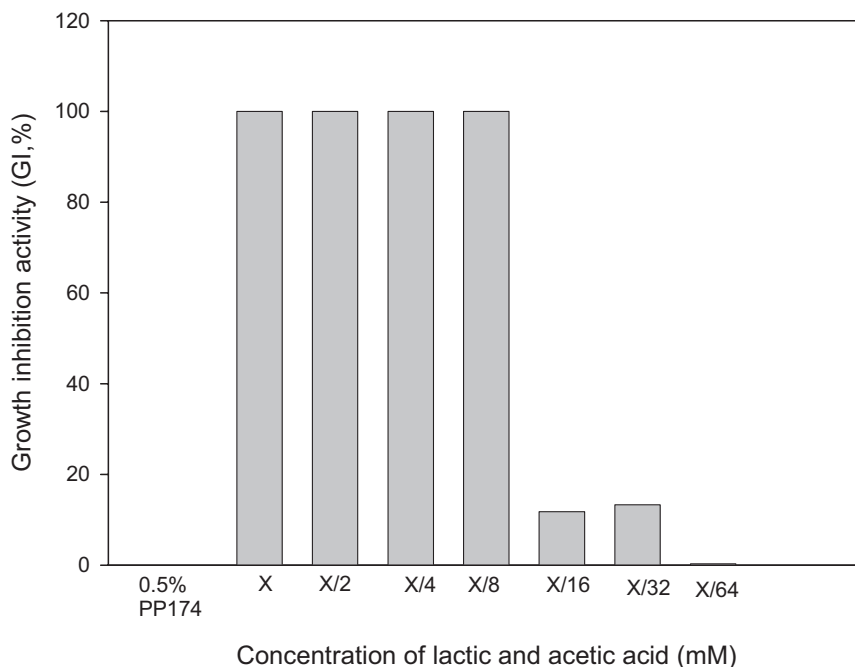


Figure 3 Effect on the growth of lactic acid bacteria L2.2 of 0.5% PP-174 alone and in combination with a mixture of organic acids (MOR) at various dilutions. X = the mixture of lactic acid and acetic acid at the concentration of 139 and 55 mM, respectively.

microorganisms. The efficiency of BAKE-SAFE-1 was tested to prolong the shelf life of cream filling under various possible conditions of storage (15°C), selling (25°C) and handling (37°C) for 2 d as shown in Figure 4. At 15°C, various cell concentrations of aerobic mesophilic bacteria, *Bacillus cereus* and LAB from the control treatment were detected for 4.73 ± 0.06 , 3.42 ± 0.01 and 4.48 ± 0.03 logCFU/g, respectively. Neither coliform nor yeast mold could be found (<logCFU/g). When the BAKE-SAFE-1 was added to the cream filling, lower cell concentrations of aerobic mesophilic bacteria, *Bacillus cereus* and LAB were found and resulted in a GIE of 26.8 ± 1.87 , 100 and 100%, respectively. It was clearly shown that cream filling kept at a low temperature of 15°C could delay all microbial growth except aerobic mesophilic bacteria.

When the temperature increased to 25°C, the cell numbers of aerobic mesophilic bacteria, *Bacillus cereus*, coliform and LAB from the

control treatment increased to 8.96 ± 0.23 , 3.78 ± 0.03 , 3.55 ± 0.29 and 6.49 ± 0.13 logCFU/g, respectively. No growth of fungi occurred at <logCFU/g. The BAKE-SAFE-1 treatment increased the GIE against both aerobic mesophilic bacteria and coliform increased to $41.45 \pm 1.14\%$ and 100%, respectively, while the samples with *Bacillus cereus* and LAB decreased to $11.68 \pm 7.80\%$ and $17.49 \pm 9.27\%$, respectively. When the temperature increased to the higher temperature of 37°C, high cell numbers of aerobic mesophilic bacteria, *Bacillus cereus*, and LAB of 9.44 ± 0.07 , 4.56 ± 0.02 and 5.71 ± 0.08 logCFU/g, respectively were detected from the BAKE-SAFE-1 treatment showing low GIE values of 24.59 ± 0.06 , 11.53 ± 2.62 and 0%, respectively. This high temperature also enhanced the growth of coliform and mold with 6.65 ± 0.39 and 1.43 ± 0.0 logCFU/g, respectively. However, the BAKE-SAFE-1 treatment still exhibited a GIE of up to 100% against both coliform and mold.

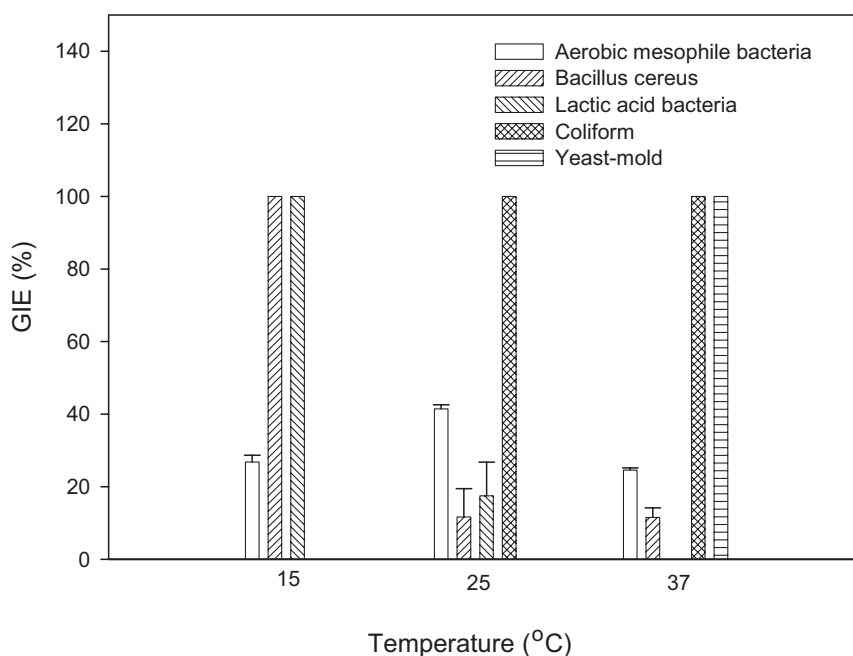


Figure 4 Growth inhibitory efficiency of cream filling treated with BAKE-SAFE-1 to total aerobic mesophiles, *Bacillus cereus*, lactic acid bacteria coliform and yeast-mold at 15, 25 and 37°C.

DISCUSSION

According to morphological, physiological, biochemical and genetic analysis, the strain KUB-KJ174 was classified as *Lactobacillus plantarum*. Its inhibitory activity against yeast growth was detected in only solid media but not in supernatant. It was different from other *L. plantarum* strains, for which bacteriocin activity was detected in both solid and liquid media when the target strain was used as an inducer (West and Warner, 1988; Fricourt *et al.*, 1994; Rojo-Bezares *et al.*, 2007). Therefore, the production of AMS from *L. plantarum* KUB-KJ174 was different from the other previous strains. Some AMS may be lost when the cells are separated from their supernatant, due to adsorption to the producer cells or formation of spontaneous aggregates. Like bacteriocins, amylovorin L471 and Lactocin S being antifungal substance producers, had highly hydrophobic molecules and rapidly adsorbed to the producer cells or formed spontaneous

aggregates (Magnusson and Schnürer, 2001). Therefore, the structure of PP-174 may be similar to these bacteriocins, which need further study.

De Vuyst *et al.* (1996) found that bacteriocin inactivation, ascribed to protein aggregation and adsorption, could be overcome by switching the pH to 2.0 after it had reached the activity peak during fermentation. By decreasing the pH to 2.0, the current study was successful in obtaining a high activity of 3200 AU/ml of PP-174 against all five microbial groups studied. When the minimum inhibitory concentration of PP-174 was investigated at different concentrations of 0.5, 1.0, 5 and 10%, a concentration of 5-10% resulted in the most active coliform bacteria levels, with only low GI activity against aerobic mesophilic bacteria, *B. cereus*, LAB and yeast. However when the contact time was increased to 24 h, the minimum concentration of 0.5% PP-174 could completely inhibit all microbial groups studied, except LAB. With the addition of MOR (17.42 mM lactic acid and 6.91

mM acetic acid), LAB could be completely inhibited. Therefore, a mixture of PP-174 and diluted MOR, designated as BAKE-SAFE-1, could be a potential biopreservative substance, depending on concentration and contact time, and would have a safety level guaranteed according to the Thai Industrial Standard Institute as well.

When BAKE-SAFE-1 was applied to cream filling, it showed effective inhibition of microbial groups of *B. cereus* and LAB at a low temperature of 15°C but not at high temperatures of 25 and 37°C. It was possible that the synergistic activity of BAKE-SAFE-1 and a low temperature of 15°C could provide specific growth inhibition against the contaminant isolates from these two groups. On the other hand, high temperature could enhance the growth of *B. cereus* and LAB, which exceeded the activity of BAKE-SAFE-1. A higher concentration of BAKE-SAFE-1 may be needed for optimal results.

BAKE-SAFE-1 in test media exhibited complete inhibition against all five microbial groups. However, when BAKE-SAFE-1 was applied to cream filling, only coliform bacteria and yeast were completely inhibited at high temperatures of 25 and 37°C but it showed low activity with regard to the aerobic mesophilic bacteria, *Bacillus cereus* and LAB. The difference between the test media and cream filling was the pH condition. The pH of the test media with BAKE-SAFE-1 was about 5.5-5.7, which was lower than the pH of cream filling with BAKE-SAFE-1 (about 6-6.5). Lind *et al.* (2004) reported that the inhibitory activity of organic acids depended on their pH. Inhibition at pH 7 generally required concentrations above 500 mM for both lactic and acetic acid, while at pH 5, the MIC values for lactic acid and acetic acid were above 500 and 20-200 mM, respectively. At a low pH of 3, the MIC value was above 160 mM for lactic acid and below 10 mM for acetic acid. Therefore, the high pH of cream filling might need a higher acid concentration to work effectively with PP-

174 to provide the same inhibitory activity as under the test media conditions. An investigation of suitable concentrations of AMS under normal application conditions would be needed to achieve successful preservation.

CONCLUSION

The effective antifungal substance producing KUB-KJ174 was identified as *L. plantarum*. The supernatant with a low pH of 3.6 showed SI activity of 100% against aerobic mesophilic bacteria, *Bacillus cereus* and coliform, while it showed only 46% SI activity against LAB. When the supernatant was adjusted to pH 5, the resultant supernatant displayed a low SI activity of 26, 50, 47 and 0% against aerobic mesophilic bacteria, *Bacillus cereus*, coliform and LAB, respectively. Partial purification by pH-mediated cell adsorption-desorption provided an active AMS PP-174 showing 100% inhibitory activity against the AMS-resistant strains of the aerobic mesophilic bacteria T6.14, *B. cereus* B6.2, coliform C4.1, LAB L2.2 and yeast Y5.1. However, a low concentration of 0.5-1% PP-174 displayed lower GI against all test strains at 15 min reaction time. When the incubation time was increased to 24 h, 100% GI against all test strains except LAB L2.2 was obtained. A combination of 0.5% PP-174 with 17.42 mM lactic acid and 6.91 mM acetic acid, the so-called BAKE-SAFE-1, was able to inhibit the growth of LAB completely. Cream filling treated with BAKE-SAFE-1 completely inhibited the contaminant group of *B. cereus* and LAB at 15°C but displayed a low GIE of 26.8% to aerobic mesophilic bacteria. When the temperature was increased to 25 and 37°C, only a low GIE of 11.68-41.45% against the contaminant groups of aerobic mesophilic bacteria, *Bacillus cereus* and LAB resulted. However, it displayed 100% GIE against the contaminant microbial group of coliform and mold.

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