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Screening and Characterization of Lactic Acid Bacteria Producing Antimicrobial Substance against *Staphylococcus aureus*

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ABSTRACT

One isolate of lactic acid bacteria (LAB) producing antimicrobial substance (AMS) against *Staphylococcus aureus* was selected and designed as LS2-30. Morphological, physical and biochemical tests, and 16S rRNA analysis were performed and revealed that it was *Pediococcus acidilactici*. Optimal conditions for growth and AMS production were determined. The optimum temperature and initial pH for both growth and AMS production were 45 °C and pH 6, respectively. Cell-free culture fluid of LS2-30 showed an inhibitory action against *Staphylococcus aureus, Salmonella* sp., *Bacillus* sp., and *E. coli* but no activity against all LAB tested. When mode of action was studied, the cell-free culture fluid exhibited a bactericidal mode of action but did not lyse its cells. After removing organic acids, partial purified antimicrobial substance at pH 3 showed inhibitory activity as bactericidal action. Hydrolytic enzyme treatment suggested that AMS structure was neither proteinaceous, starch nor lipid moiety in nature. Therefore, the nature of inhibitory compound secreted by *P. acidilactici* LS2-30 was still unclear.

Key words: antimicrobial substance, lactic acid bacteria, *Staphylococcus aureus*

INTRODUCTION

Staphylococcus aureus is an important opportunistic pathogen that causes a variety of diseases in humans and animals. It is widely recognized that antibiotic therapies formulated for intramammary use are generally unsuccessful in eliminating existing *S. aureus* infections or in preventing the establishment of chronic udder disease (Leitnera *et al*., 2003). Currently, more than 95% of infectant with *S. aureus* infections worldwide do not respond to first-line antibiotics such as penicillin or ampicillin (Rubin *et al*., 1999). Moreover, with increasing public concern over food safety, there is regulatory pressure to justify the use of therapeutic drugs in dairy cattle and to reduce the incidence of residues in milk. The emergence and spread of drug-resistant staphylococci underscores the need to find new modes of prevention and alternative non-antibiotic treatment.

In a recent *in* vitro study, various substances have been found and shown antimicrobial activity against *S. aureus*, like, lactose chitosan derivative against *S. aureus* CCRC 12657 (Chen and Chou, 2005), the extract of *Panax ginseng* root (ginsenoside) (Hu *et al.*, 2003) and lactoferrin with penicillin G (Diarra *et al.*, 2002). In addition, many anti-*S. aureus* substances producing LAB have been a great interest because most of the LAB are

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considered as "Generally Recognized as Safe" microorganism (Holzapfel *et al.*, 1995). Several LAB species were found to be a potent anti-*S. aureus* substance producer such as *Lactobacillus plantarum* BS, *Lb. salivarius, Lb. plantarum* 35d (Messi *et al.*, 2001), *P. acidilactici* F (Osmanagaoglu *et al.*, 1998) and *Lb. acidophilus* LA1 (Bernet-camard *et al.*, 1997). However, most of them produced the substances that inhibited themselves as well. Therefore, it is still important to isolate a novel anti-*S. aureus* substance with new properties and without significant inhibitory activity against its host.

The objective of this study was to characterize LAB producing antimicrobial substance against *S. aureus*. The antibacterial spectrum and their mode of action were described as well.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Antimicrobial substance (AMS) producing lactic acid bacteria isolated from silage and fermented pork (Nham) were obtained from the culture collection of Department of Biotechnology, Kasetsart University (Thailand). They were anaerobically grown in De Man Rogosa and Shape (MRS, Merck) at 45 °C. All strains of *S. aureus* isolated from mastitis cow milk from the National Institute of Animal Health (Thailand) and pathogenic bacteria used as indicator strains were grown in Nutrient broth at 37 °C while LAB was done in MRS at 37 °C. *E. coli* DH5α used as a host for cloning PCR-amplified 16S rDNA was grown in Luria broth at 37 °C. All strains were stored at -80 °C in desirable media containing 20% (v/v) glycerol, and propagated twice before experimental use.

Screening of antimicrobial substance producing LAB

Bacterial cells were removed from the overnight cultures by centrifugation at 10,956 g for 15 min at 4°C. Cell-free culture supernatant (CFS) obtained was filter-sterilized with a 0.2 µm pore size filter membrane and further assayed for antimicrobial activity.

Determination of antimicrobial activity

The spot-on-lawn method was used to determine antimicrobial activity. The desired medium agar plates were overlaid with 5 ml of soft medium agar containing 50 µl of an overnight culture of the indicator strain. 10 µl of sample was spotted onto the overlaid surface. The plates were incubated for 6 and 12 h at 37°C and determined for antimicrobial substance (AMS) production. The AMS titre was determined by the serial twofold dilution method previously described by Mayr-Harting *et al.* (1972). The activity was defined as the reciprocal of the dilution after the last serial dilution giving a zone of inhibition and expressed as activity units (AU) per milliliter.

Identification of the LAB

Sixteen-hour culture solution was determined for morphology, gram stain, catalase reaction and motility according to Axelsson (1998). Growth of LAB in MRS broth at the temperatures of 10, 45, 50 and 55 °C and NaCl concentration of 6% and 18% were determined by turbidity measurement at 600 nm. CO_2 production from glucose was examined by the method of Gerhardt *et al.* (1981). Carbohydrate fermentation patterns were determined by API CHL50 tests (API, BioMeriuex, France) as described by the manufacturer. 16S rRNA analysis was done by the modified method of Nitisinprasert *et al.* (2000). Primer 1407B (5'-GACGGGCGGTGTGTAC-3') and 8UA (5'-AGAGTTTGATCCTGGCTC AG-3') were used as forward and reverse primers, respectively. Amplification was carried out in a thermo-cycler (Hybaid, UK) with annealing for 2 min at 55 °C. The purified DNA fragment was ligated into pGEM-T® Easy vector (Promega,

USA) and then transformed into *E. coli* DH5α competent cells. The presence of the PCR product ligated to the plasmid was confirmed by whole cell PCR reaction (Nitisinprasert *et al*., 2000) and restriction analysis with *Eco*RI under standard condition. Nucleotide sequences were determined by Bioservice unit (BSU, Thailand). The sequences of 16S rDNA clone were aligned with data from GenBank by BLAST analysis.

Optimization of growth and AMS production

To determine the effect of temperature and pH on growth and AMS production, the culture solution in MRS medium was incubated at the temperatures of 30, 37, 45, 50 and 55°C, and the pH of 4, 5, 6, 7 and 8. Culture solution was sampled every 2 h until 24 h and then determined the values of turbidity at 600 nm, viable cell numbers, pH and AMS activity against *S. aureus* TM67.

Preparation of partial purified AMS (ppAMS)

Partially purified AMS (ppAMS) was prepared by vacuum evaporator and cation exchange chromatography. The CFS was concentrated to 10-fold by vacuum evaporator. One mililiter of 10-fold concentrated CFS was loaded onto a Sephadex fast flow column equilibrated with 20 mM sodium citrate buffer (pH 3.0). After initial washing with 20 mM sodium citrate buffer (pH 3.0), AMS was eluted with 1 M NaCl in the same buffer at a flow rate of 1 ml/min. The ppAMS obtained was free from lactic acid and acetic acid tested by HPLC with an Aminex HPX-87H cation exchanger. The acids were eluted with 8 mM sulphuric acid at the flow rate of 0.5 ml/min and tartaric acid was used as internal standard.

Mode of action

To determine the mode of action of CFS and ppAMS, 1% (v/v) of the overnight culture solution of *S. aureus* was inoculated into NB and cultivated at 37°C until the cells reached logarithmic phase. Then, CFS at the concentrations of 100, 200 and 300 AU/ml was added into the culture broth and further cultivated at 37°C. For control, equal amount of sterile distilled water was added instead of CFS. Viable cell numbers and turbidity at 600 nm were monitored at 1-h intervals.

For ppAMS, 100 AU/ml of ppAMS was directly added into 10^2 CFU/ml of *S. aureus* and the number of viable cell was determined at 1-h intervals. twenty mM sodium citrate buffer (pH 3.0) used instead of ppAMS was performed as a control.

Structural determination by enzyme treatment

A ppAMS was treated with various enzymes including pepsin, trypsin, proteinase K, protease, α-chymotrypsin, papain, α-amylase and lipase. Each enzyme was added to the ppAMS (100 AU/ml) to obtain a final concentration of 1 mg/ml. The mixture was incubated at the optimum temperature of each enzyme for 18 h. At the end of incubation, the residual activity of AMS was assayed.

RESULTS

Screening of AMS-producing lactic acid bacteria

Fifteen LAB strains isolated from silage and fermented pork were screened for AMS production as shown in Table 1. All CFS exhibited higher inhibition number activity than CFS adjusted pH to 5 and 6. It indicated a synergistic effect between antimicrobial substance and organic acids. The isolate designed as LS2-30 displayed the highest inhibition number against 9 *S. aureus* strains at different conditions of CFS, CFS adjusted to pH 5 (CFS-5) and CFS adjusted to pH 6 (CFS-6). Moreover, the AMS of LS2-30 was active against food-borne and pathogenic bacteria not only gram positive, *S. aureus* and *Bacillus sp.*, but also gram negative such as *E. coli* as shown in Table 2. The inhibition activity against those strains appeared at the range of 100 – 300 AU/ml (data not shown). The highest activity obtained

Table 1 The antimicrobial activity of LAB 15 isolates against *S. aureus* 9 strains.

LAB	%Inhibition number[1/]		
	CFS	*CFS-5*	*CFS-6*
CS1-10	100	0	22.2
CS1-11	100	55.5	0
CS4-10	100	66.7	0
CS4-11	100	22.2	11.1
LG1-1	100	11.1	0
LG1-2	100	55.5	0
LG2-1	100	0	0
LS2-30	100	77.8	55.6
LS2-31	100	22.2	0
LS2-32	100	44.4	11.1
N1-9	100	44.4	0
SG1-1	100	22.2	0
SG1-2	100	11.1	0
SK1-3	100	55.6	11.1
SK1-4	100	11.1	0

[1/] % Inhibition number = the number of inhibited *S. aureus* / total *S. aureus* tested

was against *S. aureus*, *Salmonella* sp., *Sal.* Typhimurium, *B. cereus*, and *B. subtilis*. Interestingly, its AMS was not active against all LAB, *M. luteus*, and *L. innocua*.

Identification of the strain LS2-30

Morphological and biochemical tests were used to identify this isolate. LS2-30 was gram-positive, coccus-shaped, tetrad formed, without catalase activity, non-motile and no spore forming. It grew at high temperatures of 45, 50 and 55°C, and did not produce gas indicated as homofermentative LAB. It also grew in MRS broth with the pH adjusted to 4.5 and 9.6, and NaCl concentration to 6% but not to 18%. Based on Bergey's Manual of systematic of bacteriology, this isolate was tentatively assigned to the genus *Pediococcus*. For species determination, carbohydrate fermentation using API 50 CHL was performed and analyzed by APILAB program. The results showed that LS2-30 was similar to *P. pentosaceus* at 92.8%. However, the difference in maltose fermentation was found as shown in Table 3. Therefore, 1,500 bp of the 16S rRNA of LS2-30 was analyzed and the result showed 97% identity to *P. acidilactici* B1104 (accession no. AJ 305322.1).

Optimization of growth and AMS production

The effects of temperature and the initial pH of medium on growth and AMS production were monitored at various conditions. The temperature effect was performed at 30, 37, 45, 50 and 55°C as shown in Figure 1. Similar growth profile was observed at 37 and 45°C, however, the maximum activity of 300 AU/ml obtained at 45 ° C was greater and even maintained for longer time of 24 h. At high temperature of 50 °C, both growth and AMS activity reached the maximum after 18 h and then dropped dramatically while the one of 55 °C was clearly inhibited after 6 h. It seems that AMS produced after 6 h actively killed all viable target cells at high temperature of 55 °C.

The initial pH of 4, 5, 6, 7 and 8 displayed a significant effect on AMS production but not on its growth as shown in Figure 2. At initial pH of 4, 5 and 6, AMS production started immediately after growth began and reached a maximum of 200-300 AU/ml during stationary phase. The maximum of AMS activity was observed after 8 h of cultivation at initial pH of 6. At the higher pH of 7, only low activity of 100 AU/ml was detected after 12 h while there was no AMS production found at the initial pH of 8.

Mode of action

To determine whether the AMS had bactericidal or bacteriostatic effect on the sensitive strains, the AMS treated condition of 100, 200 and 300 AU/ml and a control (without AMS) were performed as shown in Figure 3. Comparing to the control, the number of viable indicator cell from

Table 2 The antibacterial spectrum of LS2-30.

Indicator strain	Strain tested/inhibited
Staphylococcus aureus	*9/9*
Escherichia coli	13/13
Salmonella *sp.*	3/3
Salmonella *Typhimurium TISTR 292 (ATCC 13311)*	1/1
Bacillus cereus *(isolated from silage)*	1/1
Bacillus subtilis *TISTR 025*	1/1
Bacillus coagulans *JCM2257*	1/1
Bacillus subtilis *JCM1465*	1/1
Bacillus circulans *JCM2504*	1/0
Micrococcus luteus *IFO12708*	1/0
Listeria innocua *(ATCC33090)*	1/0
Pediococcus pentosaceus *JCM5885*	1/0
Enterococcus faecalis *JCM5803*	1/0
Lactococcus lactis *ssp.* lactis *(ATCC19435)*	1/0
Lactococcus lactis *ssp.* cremoris *TUA1344L*	1/0
Lactobacillus plantarum *(ATCC14917)*	1/0
Lactobacillus sakei *subsp.* sakei *JCM1157*	1/0
Leuconostoc mesenteroides *ssp.* mesenteroides *JCM6124*	1/0

the treated condition decreased immediately while the optical density of cell suspension still remained. This indicated that CFS of LS2-30 was bactericidal without cell lysis.

For ppAMS (Figure 4), the viable cell of 2.5 log CFU/ml decreased to less than 10 cfu/ml after 1 h incubation while the control declined slowly. This confirmed bactericidal effect against *S. aureus* TM67 while low pH of 3 displayed slight inhibition of its growth.

Determination of compound structure by enzyme treatment

The structure of ppAMS produced from LS2-30 was examined by various enzymes as shown in Table 4. The antimicrobial activity of ppAMS was not affected after treatment with proteinase K, protease type XIII, papain, pepsin A, trypsin type I, α-chymotrypsin type II, α-amylase type X-A and lipase indicating that the ppAMS was neither proteinaceous, starch nor lipid moiety in nature.

DISCUSSION

Isolate LS2-30, obtained from silage and identified as *P. acidilactici*, was selected to study as an AMS producing strain. Recently, a number of antagonistic substances from *P. acidilactici* were reported, for example, lactic acid (Stiles, 1996), pediocin PA-1, pediocin AcH, pediocin JD, pediocin SJ-1 (Schved *et al*., 1994), pediocin 5, pediocin L50, pediocin F (Osmanagaoglu *et al*., 1998), pediocin AcM (Elegado *et al*., 1997). They all, except lactic acid, had proteinaceous structure. However, the AMS of LS2-30 seems to be different from the results of various enzyme treatments, it didn't show the structure of either protein or complex compounds of protein, starch or lipid belonging to bacteriocin group IV. Therefore, it

Table 3 Carbohydrate fermentation pattern of LS2-30 compared with *P. pentosaceus*.

Carbohydrate	*P. pentosaceus*	LS2-30
Glycerol	±	-
Arabinose	+	+
Ribose	+	+
Xylose	±	+
Galactose	+	+
Glucose	+	+
Fructose	+	+
Mannose	+	+
Rhamnose	±	?
Mannitol	-	-
Sorbitol	-	-
Amygdalin	+	+
Salicin	+	+
Cellubiose	+	+
Maltose	+	+
Lactose	+	?
Melibiose	±	-
Trehalose	+	+
Inulin	±	-
Melezitose	-	-
Raffinose	±	-

+ = positive; - = negative; ? = doubtful

Table 4 Factors affecting antimicrobial activity of ppAMS.

Enzyme	Activity (AU/ml)
Proteinase K	100
Protease type XIII	100
Papain	100
Pepsin A	100
trypsin type I	100
α-Chymotrypsin type II	100
α-Amylase type X-A	100
Lipase	100
Control	100

could be either tolerate to enzyme or new compound structure.

Considering the inhibition ability, LS2-30 has shown the ability to secrete broad spectrum AMS against different non-related genera, including food-borne pathogens such as *S. aureus*, *Bacillus* sp., *Salmonella* sp., and *E. coli*. Interestingly, no inhibitory activity of LS2-30 was observed against LAB tested. Most bacteriocins produced by *P. acidilactici* displayed inhibitory effect to both LAB and some pathogenic microorganisms for example nisin, pediocin AcH (Kalchayanand *et al.*, 1992), salivacin 140 and plantaricin 35d (Messi *et al.*, 2001). Since the definition of bacteriocin indicated that it will inhibit closely related species (Klaenhammer, 1993), therefore, it was possible that the AMS found was not bacteriocin which correspond to above result of structure determination. This was the first report of non-bacteriocin substance excluding organic acid produced by homofermentative lactic acid bacteria. This property is an advantage to use this compound as a biological preservative in the dairy product and fermented food without any effect to LAB used as a starter culture.

The AMS from this isolate were secreted into culture medium at the beginning of the logarithmic growth phase and reached the maximum level during the early stationary phase at 45°C. Thus, the production of AMS from LS2-30 was growth-associated. The same pattern occurred with most of active compound produced by other LAB, such as carnocin U149, leucocin S and plancitaricin S. The initial optimum pH of AMS production was pH 6 which was similar to the previous reports (Mortvedt-Abildgaard *et al.*, 1995). In addition, this strain clearly showed that the inhibitory activity remained stable although the pH was down to pH 4 after prolonging incubation, suggesting that the antimicrobial substance was unaffected under acidic conditions. This could be an important asset allowing its use under acidic conditions such as fermented food.

Both AMS production and cell growth were observed at the same pH of 6.0 which was similar pattern to pediocin PD-1 production from *P. damnosus* NCFB 1832 (Nel *et al*., 2001). This should be beneficial to gain the high cell number and its products during fermentation.

The decline in number of living cells of *S. aureus* recorded after the addition of antimicrobial substance suggested that the mode of action was bactericidal activity without cell lysis. Its action was similar to plantaricin 35d properties as reported by Messi *et al.* (2001). When high cell concentration of target strain (10^8 cfu/ml) were applied, the CFC of 100, 200 and 300 AU/ml led to a decrease in viability of only ca. 50% in 7, 4 and 4 h, respectively. However, when low cell concentration of target strain 10^2 cfu/ml was applied, only low dose of ppAMS (100 AU/ml) could inhibit its growth. This corresponded to the work done by Boucabeille *et al*. (1997). It was found that linenscin OC2 of 10,520 AU/ml exhibited bactericidal against 10^8 cfu/ml of *L. innocua* while low dose of 850 AU/ml did only bacteriostatic action. Therefore, the inhibition efficiency would depend on the

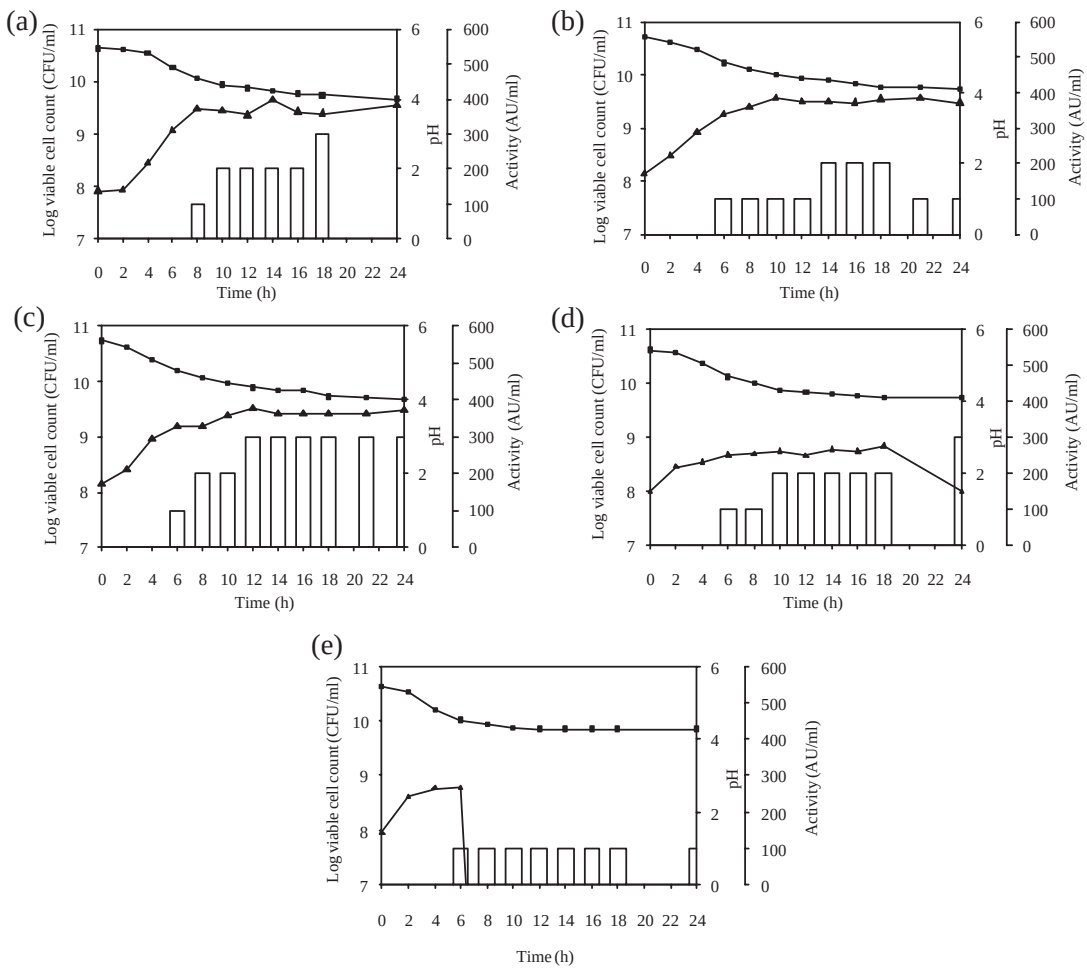


Figure 1 Influence of temperature on the cell growth (▲), pH (■) and antimicrobial substance production (❑) by *P. acidilactici* LS2-30 at 30 °C (a), 37 °C (b), 45 °C (c), 50 °C (d) and 55 °C (e).

concentration of antimicrobial substance and the cell concentration of the indicator strain as also supported by de Vuyst and Vandamme (1994).

The ambiguous structure of ppAMS still remained. Further study on purification and characterization will explain the inhibition mechanism for application uses in food, feed and pharmaceutical industries.

CONCLUSION

Fifteen LAB isolates have been screened according to AMS production against 9 *Staphylococcus* sp. strain. One of them designed as LS2-30 was classified as *Pediococcus acidilactici* by morphological, physical and biochemical tests, and 16S rRNA. The optimum growth temperature and initial pH were 45 °C and pH 6, respectively. CFC of LS2-30 showed an inhibitory action against some pathogens and

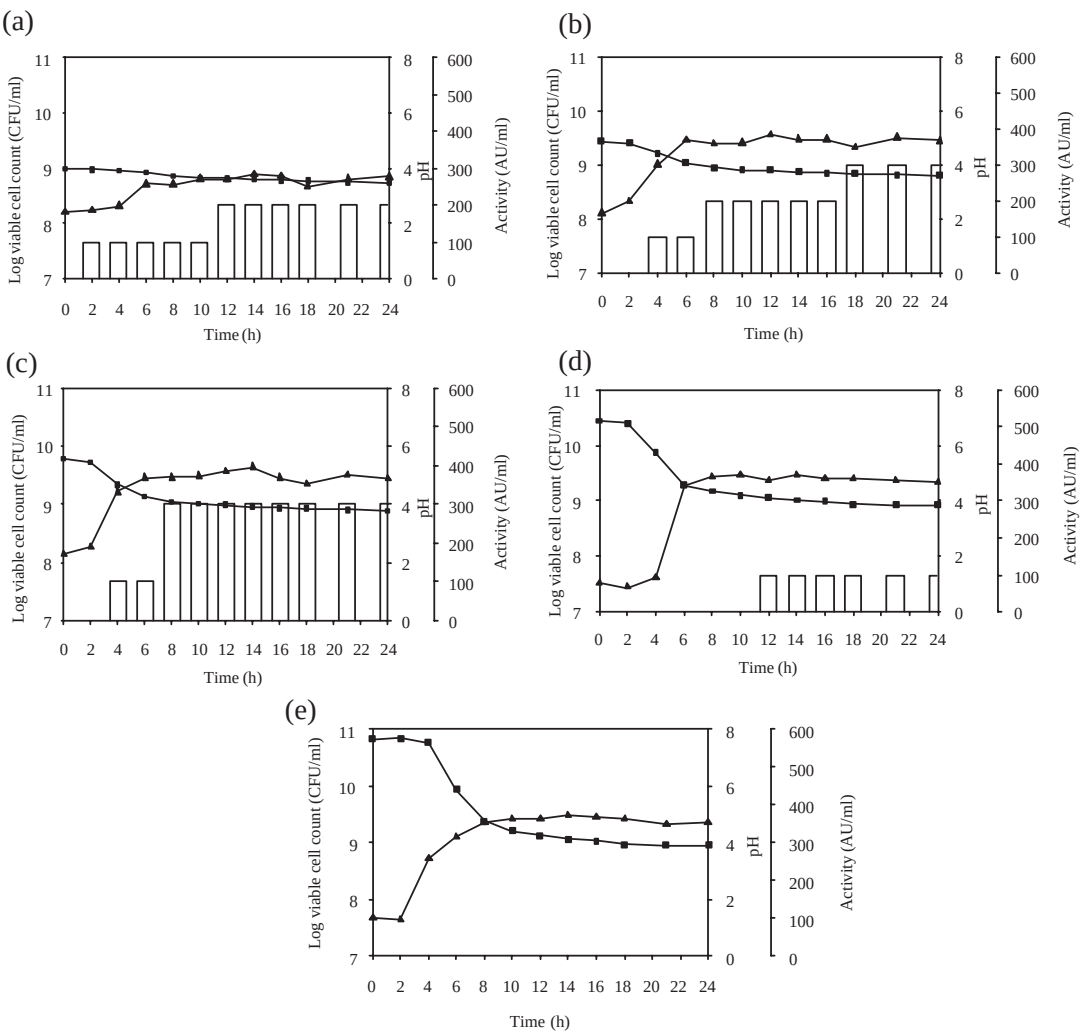


Figure 2 Influence of the initial pH of MRS broth on the cell growth (▲), pH (■) and antimicrobial substance production (❑) by *P. acidilactici* LS2-30. pH of 4 (a), pH 5 (b), pH 6 (c), pH 7 (d) and pH 8 (e).

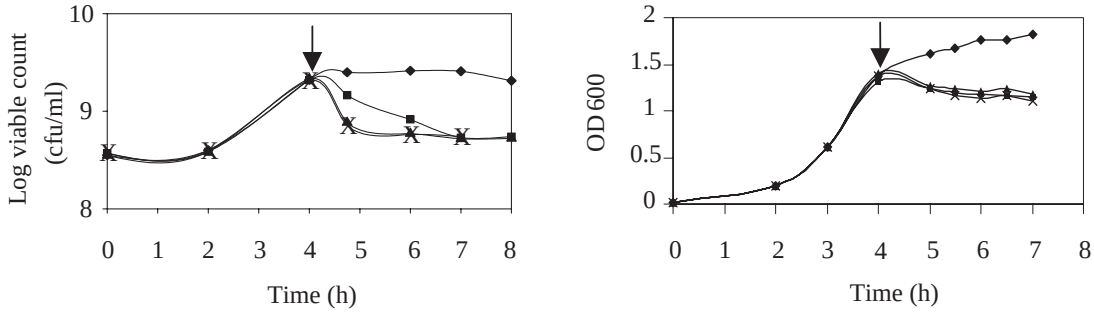


Figure 3 Effect of LS2-30 CFS on the viability (a) and absorbance at 600 nm (b) of *S. aureus*. CFS was added (arrow) at a final dose of 100 AU/ml (■), 200 AU/ml (▲), 300 AU/ml (X) and control (◆).

foodborne bacteria such as *S. aureus, Salmonella* sp., *Bacillus* sp., and *E. coli* . However, it didn't exhibit activity against all LAB tested. When mode of action was studied, the CFS displayed a bactericidal mode of action without cell lysis. The ppAMS at pH 3.0 showed inhibitory activity as bactericidal action at low concentration of 100 AU/ml against 10^2 CFU/ml of target strain. By various hydrolytic enzymes treatment, it suggested that the AMS was neither proteinaceous, starch nor lipid moiety in nature. Therefore, the nature of inhibitory compound secreted by *P. acidilactici* LS2-30 is still unclear.

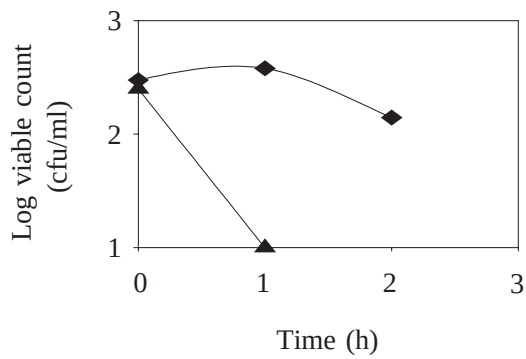


Figure 4 Effect of ppAMS of LS2-30 on the viability of *S. aureus* at 10^2 cfu/ml. ppAMS was added at 100 AU/ml (▲) and 20 mM sodium citrate buffer (pH 3.0) as control (◆).

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