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Utilization of Antibacterial Substances from Lactic Acid Bacteria for Extension Shelf-life of Chilled Crab Meat

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ABSTRACT

Six isolates screened from fifty samples of fermented fishery products including Pla-ra, Pla-som, Pla-chao, Pla-chom and Kung-chom, were described as KPL-1, KPL-2, KPL-3, KPL-4, KPL-5 and KPL-6 and found to inhibit indicator organisms such as *Lactobacillus plantarum* ATCC 8041, *Pediococcus pentosaceus* ATCC 33316 and *Listeria ivanovii* DSM 4553, of which the KPL-4 isolate showed a maximum activity of 1,600 AU/ml. The six isolates were identified as *Enterococcus faecalis* based on morphological and biochemical characteristics. The antibacterial performance from the KPL-4 was decreased 50 percent by trypsin and pepsin, stable after heat treatment at 60°C for 15 min and 100°C for 30 min, while inactive at 121°C for 15 min. The molecular weight of the antibacterial compound was 3,800 dalton. The KPL-4 crude extracts of various concentrations including undiluted ones exhibited significant difference ($P \leq 0.05$) in reduction of total microorganisms as well as *Listeria ivanovii* in crab meat, with the 10% crude extracts showing most effective. Chilled crab meat treated with 10% crude extracts and kept for 10 days at 0°C had lower total viable count of bacteria than the untreated ($P \leq 0.05$) between treatments. Using antibacterial substances from lactic acid bacteria could decrease total viable count of bacteria and extend shelf-life of chilled crab meat for at least 10 days.

Key words: lactic acid bacteria, chilled crab meat

INTRODUCTION

Lactic acid bacteria are very important to fishery industry. Bacteriocin is the most useful antibacterial agent produced from lactic acid bacteria. Among the antibacterial agents, nisin from *Lactococcus* is widely used for food preservation purpose in Swiss cheese, beer, meat patties, etc. Lactic acid bacteria were also significant for nutritional and therapeutic aspects. (Fernandes *et al*., 1987; Gurr, 1987; Gilliland, 1990)

Lactic acid bacteria possess several interesting properties of great economic importance. Many of these properties, such as lactose utilization, proteinase activity, bacteriophage defence mechanisms, bacteriocin production and immunity, etc, are genetically mediated by often unstable and naturally transferable plasmids (McKay,1985). Moreover, lactic acid bacteria may be successfully used as industrial microorganisms for the synthesis of fine chemicals, pharmaceuticals, and other products useful to humans, since they have several

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advantages in industrial fermentations. Among the Gram-positive bacteria, the lactic acid bacteria in particular produce a wide variety of antimicrobial proteins including peptide antibiotics, antibiotic-like substances, bacteriocins and bacteriocin-like substances. (De Vuyst and Vandamme, 1994)

The aim of this study was to extend shelf-life of chilled crab meat by using the substances from lactic acid bacteria to inhibit bacterial growth in chilled crab meat.

MATERIALS AND METHODS

1. Screening of lactic acid bacteria from fermented fish products

Fifty samples of fermented fish products such as Pla-ra, Pla-chom, Pla-chao, Pla-som, and Kung-chom were taken from markets in Bangkok and nearby. Twenty five grams of each sample was mixed in 225 ml. 0.1% peptone water and blended by using stomacher. One ml. of 10^{-6}-10^{-8} dilutions was dropped on MRS agar plate prepared by adding bromocresol purple 0.004 percent and calcium carbonate 0.5 percent. All samples were incubated at 30°C for 48 hours. Some colonies of lactic acid bacteria which changed the indicator from purple to yellow were picked and purified by cross streak method. Pure culture of lactic acid bacteria were kept at -20°C in TSB broth with 40 percent of glycerol added.

2. Isolation of bacteria which produced antibacterial substances

Two species of lactic acid bacteria, *Lactobacillus plantarum* ATCC 8014 and *Pediococcus pentosaceus* ATCC 33316, were used in this study as test organisms for producing antibacterial substances. Pathogenic bacteria namely, *Staphylococcus aureus* ATCC 25923, *Salmonella* Enteritidis ATCC 25923, *Salmonella* Enteritidis ATCC 14028, *Vibrio parahaemolyticus* DMST 5665, *Escherichia coli* ATCC 25922, and *Listeria ivanovii* DMST 4553 were used as test organisms against antibacterial substances produced from the selected lactic acid bacteria. Screening and isolation of lactic acid bacteria were performed according to Lewus *et al*. (1991) and subsequent screening for those produced antibacterial substances followed the method of flip streak by Spelhaug and Harlander (1989) as well as the method of agar spot test by Montville and Kaiser (1993).

3. Confirmed test of antibacterial substances produced from lactic acid bacteria.

The agar well diffusion method was used to confirm the bactericidal substances produced in this work. Isolatedly purified colonies of lactic acid bacteria were inoculated in to 5 ml. of MRS broth, incubated at 30°C for 24 hours, then transferred to 10 percent of Modified MRS broth, incubated at 25°C for 24 hours, and centrifuged at 27,950 gravitational force at 4°C for 20 minutes. Clear supernatant was adjusted to pH 6.5 and enzyme catalase was added to make the final concentration to 500 unit per ml, incubated at 25°C for 30 minutes, and then filtered through 0.2 μm millipore membrane. The supernatant was kept for test organisms by transferring each type of test organisms into 5 ml. of TSB (Trypticase soy broth), then incubated at 37°C for 18 hours. After that, the test culture of 1×10^6 CFU/ml. was mixed into Muller Hinton agar. The hole of agar was made by a cork borer and 50 microliter of the clear supernatant was dropped into the holes and incubated at 4°C for 4 hours followed by incubating at 37°C for 16 hours. The result was observed by clear zone observation and the colony showing clear zone >0.1-0.5 cm width was selected. Lactic acid bacteria species were classified by the method of Snealth *et al*. (1986). The ability of antibacterial substances was tested following Barefoot and Klaenhammer (1983).

4. The properties of antibacterial substances

The properties of antibacterial substances such as thermal stability was measured following the method of Ali *et al*. (1995) and the stability of antibacterial substance to enzyme by the method of Garriga *et al*. (1993). The molecular weights of antibacterial substances were determined by the SDS-PAGE method (Laemmli, 1970). The effects of temperature and time to produce antibacterial were studied by the method of Coventry *et al*. (1996).

5. Shelf-life of chilled crab meat

Chilled crab meat kept at 0°C were divided into 6 portions of 250 gm each. The samples were in 10% crude extracts of antibacterial substance for 30 minutes and the temperature during processing was 0°C. After soaking, the samples were left to dry at room temperature for 5 minutes. Chilled crab meat were transferred into plastic bags and kept at 4°C. Total viable count and sensory evaluation were carried on within 0-10 days. Chilled crab meat in the absence of crude extract of antimicrobial substance was compared. A 2^{10} factorial experimental design with three replicates were employed in this study.

RESULTS

Fifty samples of fermented fish products such as Pla-ra, Pla-chom, Pla-chao and Pla-som were used for the isolation of lactic acid bacteria, which were found between 30-2.25×10^8 CFU/gm and some showed clear zone in the MRS media of 10-8.0×10^7 CFU/gm.

Three hundred and thirty five isolates of Gram positive cocci and three hundred and nineteen isolates of Gram positive rod were chosen from lactic acid bacteria showing clear zone. Antibacterial substances from lactic acid bacteria were studied by spot test agar method and flip streak method for the first isolation. The agar well diffusion method was used for the confirmation of the isolates of lactic acid bacteria. From this study it was found that the antibacterial substances from strain KPL-4 showed the best activity in inhibiting *Listeria ivanovii* DMST 4553 and total bacteria. Inhibitation results of lactic acid bacteria by spot test agar method is shown in Table 1 and the efficiency of inhibition to test organisms by lactic acid bacteria are shown in Table 2. The suitability of pH and temperature for emzymes are shown in Table 3. Table 4 shows the influence of antibacterial agents in crude extract in lowering the total viable count of chilled crab meat.

DISSUSSION

Seven test microorganisms including *Salmonella* Enteritidis ATCC 14028, *Vibrio parahaemolyticus* DMST 5665, *Escherichia coli* ATCC 2592, *Staphylococcus aureus* ATCC 25923, *Listeria ivanovii* DMST 4593, *Lactobacillus plantarum* ATCC 8014 and *Pediococcus pentosaceus* ATCC 33316 were chosen for flip streak method to isolate lactic acid bacteria which produced antibacterial substances. Fourteen isolates had ability to inhibit test microorganisms as described. These isolates, coded KLC could inhibit *Lactobacillus plantarum* ATCC 8014 and *Pediococcus pentosaceus* ATCC 33316; while the isolates PS-1, KSS-4, PS-5.1 and SS-4.1 could also inhibit *V. parahaemolyticus* DMST 5665. The isolates KPL-1, KPL-2, KPL-3, KPL-4, KPL-5, KPL-6, PLD-18 and ZZ-2 showed the ability to inhibit *Listeria ivanovii* DMST 4553, *Lactobacillus plantarum* ATCC 8014 and *Pediococcus pentosaceus* ATCC 33316. The isolate KSS-1 had the ability to inhibit *V. parahaemolyticus* DMST 5665 and *Listeria ivanovii* DMST 4553. By agar spot test method, the nine isolates namely, KPL-4, KPL-3, PLD-4, KPL-2, KPL-6, KLC, KPL-1 and KPL-5, had the ability to inhibit *Listeria ivanovii* DMST 4553, *Lactobacillus plantarum* ATCC 8014 and *Pediococcus pentosaceus* ATCC 33316. The

Table 1 Inhibitation results of lactic acid bacteria by spot test agar method.

Isolates	*Lb. plantarum* ATCC 8014	*E. coli* ATCC 25922	*S. aureus* ATCC 25923	*L. ivanovii* DMST 4553	*Sal.* Enteritidis ATCC 14028	*V. parahaemolyticus* DMST 5665	*P. pentosaceus* ATCC 33316
KPL-4	+++	-	-	+++	-	-	+++
KPL-3	+++	-	-	+++	-	-	+++
PLD-4	+	-	-	+	-	-	+
KPL-2	+++	-	-	+++	-	-	+++
KPL-6	+++	-	-	+++	-	-	+++
KLC	++	-	-	+	-	-	++
KPL-1	+++	-	-	+++	-	-	+++
PP2	-	-	-	+	-	-	-
KPL-5	+++	-	-	+++	-	-	+++

Notes +++ clear zone >0.5 cms.width
++ clear zone >0.3-0.5 cms.width
+ clear zone 0.1-0.3 cms.width
- without clear zone
Lb. = *Lactobacillus* *Sal.* = *Salmonella* *E.* = *Escherichia* *V.* = *Vibrio*
S. = *Staphylococcus* *P.* = *Pediococcus* *L.* = *Listeria*

Table 2 Efficiency of inhibition to test organisms by lactic acid bacteria.

Lactic Acid Bacteria	Efficiency of Inhibitation (Unit per ml) Test Organisms (10^6 CFU/ml)		
	Lb. plantarum ATCC 8014	*L. ivanovii* DMST 4553	*P. pentosaceus* ATCC 33316
KPL-1	400	200	400
KPL-2	400	200	400
KPL-3	800	200	800
KPL-4	1600	400	1600
KPL-5	800	400	800
KPL-6	800	200	800

isolate PP-2 could also inhibit *L. ivanovii* DMST 4553.

Only six isolates from the agar well diffusion method namely, KPL-1, KPL-2, KPL-3, KPL-4, KPL-5 and KPL-6 had the ability to inhibit *L. ivanovii* DMST 4553, *L. plantarum* ATCC 8014 and *Pediococcus pentosaceus* ATCC 33316. After classification found that six isolates were belong to *Enterococcus faecalis*.

Barefoot and Klaenhammer (1983) found that 63% of *Lactobacillus acidophilus* could inhibit some species of lactic acid bacteria. As well as Geis *et al*. (1983) found that 9% of *Lactococcus lactis* spp. and 7.5% *Lc. lactis* spp. *cremoris* could

Table 3 Suitability of pH and temperature for enzymes.

Enzymes	pH	Temperature (°C)
Trypsin	7.5	25
Pepsin	2.0	37
Lysozyme	6.5	25
a-Amylase	6.0	20

Table 4 Results of antibacterial substances with chilled crab meat.

Time (days)	Total Viable Count (CFU/g)	
	Control	Soaked in 10% antibacterial substances
0	1.4×10^{7a}	9.8×10^{6b}
1	1.7×10^{7a}	1.2×10^{7b}
2	1.8×10^{7a}	1.4×10^{7b}
3	1.9×10^{7a}	1.5×10^{7b}
4	2.1×10^{7a}	1.7×10^{7b}
5	2.3×10^{7a}	1.9×10^{7b}
6	2.9×10^{7a}	2.0×10^{7b}
7	3.4×10^{7a}	2.9×10^{7b}
8	4.2×10^{7a}	3.0×10^{7b}
10	1.0×10^{8a}	8.7×10^{7b}

Means in horizontal line followed by different letter are significantly different ($P<0.005$)

produce bacteriocin. Hoover and Steenson (1993) found that bacteriocin could inhibit the growth of many Gram positive bacteria including food pathogenic bacteria such as *Listeria monocytogenes*, so bacteriocin should be selected for a preservative.

CONCLUSIONS

KPL-4 was the best antibacterial substances, which showed the ability to inhibit *Lactobacillus plantarum* ATCC 8014 and *Pediococcus pentosaceus* ATCC 33316 at the maximum concentration of 1,600 unit per ml. The ability of antibacterial substances were reduced by enzyme trypsin and pepsin but stable to heat at 60°C for 15 minutes and 100°C for 30 minutes and the ability was lost at 121°C for 15 minutes.

The concentration of antibacterial substances at 10% could inhibit microorganisms and was chosen in chilled crab meat for 10 days. The results showed that the microorganisms decreased significantly in the present of crude extract compared with the ones without antibacterial substances.

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