

Selection of Biopreservative-produced Lactic Acid Bacteria from Chilled Seafood Products

Nucharee Tuntisuwanno^{1*}, Wilawan Charernjitrakul¹,
Nuttapong Bovornruengroj² and Preeyanuch Borvornruengroj¹

¹Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

²Department of Technology and Industry, Faculty of Science and Technology, Prince of Songkla University, Pattani 94000, Thailand

*Corresponding author. E-mail: hyok_wc@hotmail.com, preeyanuch.b@psu.ac.th

ABSTRACT

*In order to reduce usage of chemical preservatives and to maintain nutritional quality of seafood products, this study screened biopreservative-producing, lactic acid bacteria (LAB) from chill-stored seafood products. The viable acid-producing bacteria from chill-stored seafood products, such as shrimp, oysters, crabs, squid and fish, cultured in Bacteriocin screening medium (BSM) were between 3.0×10^5 – 1.4×10^8 and 3.4×10^5 – 2.6×10^7 CFU/g, respectively. Preliminary morphological and biochemical characterization studies revealed that, of the 266 acid-producing bacteria isolated from chilled seafood, 159 isolates belonged to LAB. All LAB were screened for their inhibitory activity against *Staphylococcus aureus* ATCC 29213, *Listeria monocytogenes* ATCC 15313, *Escherichia coli* PSU 95 and *Vibrio parahaemolyticus* PSU 1681 by agar spot test. Among all selected LAB isolates, five isolates showed strong inhibitory activity. Further, in agar well diffusion assay, lyophilized cell free supernatant of four LAB (HYL-20104, POL-20108, FSK-L 5101 and L-SQ-L 25104) inhibited the growth of *Vibrio parahaemolyticus* PSU 1681 and *Listeria monocytogenes* ATCC 15313. Biochemical characterization studies envisaged that the LAB belonged to the genus *Lactobacillus* and *Carnobacterium*. Further, molecular characterization studies confirmed that the potential LAB FSK-L 5101, POL-20108 and HYL-20104 were identified as *Carnobacterium divergens* and L-SQ-L 25104 was identified as *Carnobacterium maltaromaticum*.*

Keywords: Lactic acid bacteria, Bacterial inhibition, Biopreservative, Chill-stored seafood

INTRODUCTION

International trade in fish and seafood products continues to grow, reflecting consumption not only in the European Union and the United States, but in all other regions, including Asia. Thailand has one of the world's largest fish and seafood industries, valued at USD 2.5 billion in 2008 (THAIFEX - World of Food Asia,

2013). Seafood is considered a healthy food choice because it contains high-quality protein and is lower in calories and saturated fat when compared to other protein-rich animal foods. However, fish and seafood products are susceptible to spoilage due to biochemical and microbiological degradation (Dalgaard et al., 2006; Mejlholm et al., 2008). The diverse nutrient composition of seafood provides an ideal environment for growth and propagation of spoilage microorganisms and common food-borne pathogens (Emborg et al., 2002 and Dalgaard et al., 2006). Pathogenic bacteria found in seafood can be categorized into three general groups (Calo-Mata et al., 2008; Mejlholm et al., 2008): (1) Bacteria (indigenous bacteria) that belong to the natural microflora of fish, such as *Clostridium botulinum*, pathogenic *Vibrio* spp., *Aeromonas hydrophila*; (2) Enteric bacteria (non-indigenous bacteria) that are present due to faecal and/or environmental contamination, such as *Salmonella* spp., *Shigella* spp., pathogenic *Escherichia coli*, *Staphylococcus aureus*; and (3) bacterial contaminants introduced during processing, storage, or preparation for consumption, such as *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium perfringens*, *Cl. botulinum*, *Salmonella* spp. Accordingly, the development of effective processing treatments to extend the shelf life of fresh seafood products, avoiding microbial contamination, has become mandatory. In addition, consumers increasingly demand high-quality, but minimally-processed, seafood (Campos et al., 2012). The problem of combating bacteria in foods has been influenced in recent years by several trends in food consumption. These include an increased consumption of pre-prepared food at home; an increased consumption of chilled and frozen food; and a reduced use of salt, sugar and chemical preservatives. Coupled with this, food processing developments now allow for the marketing of minimally processed products (Ohlsson, 1994). These improve the convenience and shelf life of a commodity, without significantly altering its fresh sensory attributes and nutritional qualities. Refrigeration and modified atmosphere packaging are used to reduce microbial spoilage. Among alternative food preservation strategies, particular attention has been paid to biopreservation techniques, which extend the shelf life and enhance the hygienic quality, thereby minimizing the negative impact on the nutritional and sensory properties. Biological preservation usually refers to the use of a natural or controlled microflora and/or its antimicrobial metabolites (Nilsson et al., 2005 and Garcia et al., 2010). Lactic acid bacteria (LAB) are interesting candidates for this approach. In fact, they often are naturally present in food products and may act as powerful competitors to contaminating spoilage microorganisms by producing a wide range of antimicrobial metabolites, such as organic acids, diacetyl, acetoin, hydrogen peroxide, reuterin, reutericyclin, antifungal peptides and bacteriocins. In the last two decades, using LAB and their metabolites for natural food preservation has drawn increasing attention (Cleveland et al., 2001, Nilsson et al., 2005 and Gálvez et al., 2007, Nes, 2011). Hence, this study aimed to screen biopreservative-producing LAB from chill-stored seafood products for the presence of antimicrobial activity and identification of the selected strain for use as biopreservatives in minimally-processed seafood products.

MATERIALS AND METHODS

Samples

Chill-stored seafood product samples, such as shrimp, oyster, crab, squid and fish, were purchased at supermarkets in Hat Yai District, Thailand. All the samples were brought to the laboratory in ice and stored at 4°C. Samples were analyzed within 24 h (Fang et al., 2003).

Isolation of lactic acid bacteria (LAB)

Twenty-five grams of each raw seafood were homogenized for 2 min with 225 ml of 0.1% sterile tryptone broth 225 ml in a stomacher. Samples were serially diluted in sterile tryptone broth (Fang et al., 2003) and 1 ml of each diluted sample was pour plated on Bacteriocin screening medium (BSM) supplemented with 0.004% Bromcresol purple and incubated at 35 and 8°C for 24 hrs and 3-7 days, respectively. Two replicates of at least three appropriate dilutions were enumerated. All plates were examined visually for typical colony morphology (Koutsoumanis et al., 2002). Duplicate agar plates of between 30 and 300 colonies were counted and mean counts were calculated. Colonies with distinct morphological differences (such as color, shape and size) were selected randomly and purified by streaking using the same medium. Further, isolates were subjected to catalase reaction and examined microscopically before stock preparations. Gram-positive, catalase-negative bacterial isolates were purified and frozen stocks were made in 30% (w/v) glycerol and stored at -80°C.

Screening of LAB for antimicrobial activity

Bacterial pathogens. The bacterial pathogens used in this study (*E. coli* PSU 95, *S. aureus* ATCC 29213, *L. monocytogenes* ATCC15313, *V. parahaemolyticus* PSU 1681) were obtained from the Department of Microbiology Laboratories, Prince of Songkla University, Thailand.

Determination of antibacterial activity by agar spot assay. LAB was grown in BSM broth at 35 and 8°C for 24 hrs and 3-7 days, respectively. The agar spot test was a modification of that described by Fleming et al. (1985). Overnight cultures of LAB to be tested for antimicrobial activity were spot inoculated on the surface of BSM agar plates and incubated at 35°C and 8°C for 24 hrs and 3-7 days, respectively, to allow colonies to develop. Anaerobic conditions were maintained to minimize the formation of hydrogen peroxide and acetic acid (Schillinger and Lucke, 1989). Colonies were overlaid with 7 ml of TSA soft agar (0.7% agar) containing 10⁶ CFU/ml of indicator strains (*E. coli* PSU 95, *S. aureus* ATCC 29213 and *L. monocytogenes* ATCC15313), except *V. parahaemolyticus* PSU 1681 inoculated into 7 ml of TSA soft agar containing 1.5% NaCl. After aerobic incubation for 24 h at 35°C, the presence of a clear zone around the spot was measured.

Selection of psychrotrophic LAB. Psychrotrophic characteristic of LAB was a modification of that described by Matamoros et al. (2009). The selected isolates were cultivated at 4, 15, 30 and 35°C in BSM broth. Growth was monitored visually by checking turbidity after 24, 48 hrs and one week of culture.

Determination of antimicrobial activity by agar well diffusion method

Preparation of crude biopreservative. Each LAB isolate was grown in BSM broth at 30°C for 24 h. Cell-free supernatant was collected by centrifugation at 8,000×g at 4°C for 15 min, adjusted to pH 6.5 with 1 M NaOH, and subsequently filtered through 0.45 µm membrane filter before freeze-drying as described by Nanasombat et al. (2012).

Determination of antimicrobial activity of crude biopreservatives. Antimicrobial activity of crude biopreservatives against test indicator strains were tested using an agar well diffusion assay modified from Schillinger and Lucke (1989). Overlaid with 7 ml of TSA soft agar 0.7% agar containing 106 CFU/ml of indicator strains on BSM plate, except *V. parahaemolyticus* PSU 1681 inoculated into 7 ml of TSA soft agar containing 1.5% NaCl. Agar wells of 6 mm diameter were cut and the filter-sterilized freeze-drying supernatant (80 µl) was added into each well. The plates were incubated at 35°C for 24 hrs. The antimicrobial activity was determined by measuring the diameter of the inhibition zone around the wells (Narayanapillai et al., 2012).

Identification of LAB

Morphological and biochemical methods. The LAB exhibiting antimicrobial activity were subjected to morphological and biochemical tests, including Gram's staining, cell morphology, carbon dioxide production from glucose, growth at 10°C and 45°C, growth at pH 4.4 and 9.6, growth in 6.5% and 18% NaCl and catalase reaction by 3% hydrogen peroxide (Axelsson, 2004). Gas production from 1% glucose (w/v) containing Durham tube and carbohydrate fermentation of each LAB isolates was determined using cellobiose, galactose, lactose, maltose, mannitol, mannose, melibiose, raffinose, salicin, sucrose and trehalose, after incubation at 30°C for 18-24 hrs.

Molecular characterization of LAB. The genomic DNA of LAB isolates was extracted (Nanasombat et al., 2012) by using a Presto™ Mini gDNA Bacteria Kit. The 16S rDNA gene was amplified by polymerase chain reaction using primers 16S rDNA-27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 16S rDNA-1492R (5'-GGTACCTTGTTACGACTT-3'). PCR was carried out in a total volume of 20 µl containing 1X PCR buffer, 1 µl genomic DNA, 2.5 mM dNTPs 2 µl, 0.25 µl of Taq DNA polymerase, 10 µM 27F 0.5 µl, 10 µM 1492R 0.5 µl, Thermo buffer 2 µl and Dw 13.75 µl. The PCR conditions consisted of an initial denaturing step of 94°C for 3 min, followed by 35 cycles of denaturing for 0.45 min at 94°C, annealing for 0.45 min at 50°C and 2 min of primer extension at 72°C. A final extension at 72°C for 5 min was then performed. The 1,500 bp PCR products were purified with Gel/PCR DNA Fragments Extraction Kit. The PCR products were sequenced and sequence homologies were examined by comparing DNA databases.

RESULTS

Isolation of LAB

The viable acid producing bacteria from chill-stored seafood products (shrimp, oyster, crab, squid and fish) cultured in Bacteriocin screening medium (BSM) with 0.004% bromocresol purple incubated at 8 and 35°C were between 3.0×10^5 – 1.4×10^8 and 3.4×10^5 – 2.6×10^7 CFU/g, respectively (Table 1). Of the 266 acid producing isolates, 159 isolates were found to be LAB as preliminarily confirmed by examination for gram, staining and catalase test.

Table 1. Total viable acid producing bacteria in raw seafood products and the number of lactic acid bacteria isolates on BSM medium incubated at 8 and 35°C for 24 hrs and 3-7 days, respectively.

Raw seafood products	No. of samples	Total viable acid producing bacteria (CFU/g) at incubation temperature (°C)		No. LAB (isolates) at temperature (°C)	
		8	35	8	35
		Shrimp	4	3.4×10^5 – 8.6×10^6	3.4×10^5 – 1.8×10^7
Oyster	6	3.0×10^5 – 1.3×10^7	5.5×10^5 – 1.6×10^7	23	20
Crab	2	2.6×10^6 – 1.4×10^8	3.6×10^6 – 2.6×10^7	6	14
Squid	5	8.1×10^6 – 3.5×10^5	3.4×10^6 – 4.4×10^5	8	18
Fish	8	7.3×10^6 – 9.3×10^6	3.4×10^5 – 4.3×10^6	11	35
Total	25	3.0×10^5 – 1.4×10^8	3.4×10^5 – 2.6×10^7	62	97

Screening of LAB for antimicrobial activity

The antibacterial activity of 159 LAB isolates against bacterial indicator strains were preliminarily determined by an agar spot test. Five isolates exhibited excellent growth inhibitory zone (Table 2). Antimicrobial activity observed for LAB indicated broad-spectrum activity against both gram-positive and gram-negative pathogenic and spoilage organisms. Greater inhibition was observed against *L. monocytogenes* ATCC 15313, *V. parahaemolyticus* PSU 1681 and *S. aureus* ATCC 29213. In contrast, *E. coli* PSU 95 was not inhibited by any LAB isolates.

Table 2. Antimicrobial activity of the LAB isolates against gram-positive and gram-negative pathogenic food and spoilage microorganisms as determined by agar spot test.

Raw seafood products	LAB Isolate	Incubation temperature (°C)	Indicator strains			
			<i>E. coli</i> PSU 95	<i>S. aureus</i> ATCC 29213	<i>L. monocytogenes</i> ATCC 15313	<i>V. parahaemolyticus</i> PSU1681
Fish	FSK-L 5101	35	-	-	7.10±0.60	6.80±0.90
Crab	POL-20108	35	-	12.20±1.40	6.75±0.30	6.90±0.10
Oyster	HYL-20104	35	-	12.75±0.25	7.50±0.25	6.65±1.35
Squid	L-SQ-L 25104	8	-	-	23.78±1.27	13.48±2.97
Shrimp	L-SH-L 25104	8	-	-	24.83±0.43	15.58±0.22

Note: Results are expressed as diameters of the inhibition zone in mm. (-) : no inhibition zone.

Selection of psychrotrophic LAB

Different strategies for the biopreservation of seafood products have already been conducted (Brillet et al., 2004; Altieri et al., 2005) but none of them focused on the cold adaptation of protective strains. Lactococcus or Leuconostoc species are not usually described as psychrotrophic LAB. For that reason, the growth rate as a function of temperature was tested for lactic acid isolates (Matamoros et al., 2009) according to OD measurements. All five selected LAB isolates were found to be psychrotrophic organisms, could grow at low temperature (4 and 15°C) with maximum growth temperature below 30°C (data not shown) (van de Guchte et al., 2002).

Determination of antimicrobial activity by agar well diffusion method

The antimicrobial activity of all five LAB were assessed against gram-positive and gram-negative pathogenic and spoiling strains relevant to seafood. The mean inhibitory activity of lyophilized cell-free supernatant of each isolate is presented in Table 3.

Table 3. Antimicrobial activity of crude biopreservative produced by the selected LAB isolates against *L. monocytogenes* ATCC 15313 and *V. parahaemolyticus* PSU 1681.

Indicator strains	LAB isolates	Diameter of inhibition around the wells (mm)
<i>L. monocytogenes</i> ATCC 15313	POL-20108	17.52±0.22
	FSK-5101	18.60±0.10
<i>V. parahaemolyticus</i> PSU 1681	POL-20108	17.38±0.12
	FSK-L 5101	17.05±0.05
	HYL-20104	18.88±0.03
	L-SQ-L25104	19.35±0.57

Among the four isolates (crude biopreservations 15X concentration), POL-20108, FSK-L 5101, HYL-20104 and L-SQ-L 25104 showed a strong inhibitory activity against *V. parahaemolyticus* PSU 1681 and *L. monocytogenes* (Figure 1).



Figure 1. Antimicrobial activity of crude biopreservative produced by the selected LAB isolates against *L. monocytogenes* ATCC 15313 using supernatant of lactic acid bacteria isolates POL-20108 (1) and FSK-L 5101 (2).

Identification of LAB

All selected isolates were gram-positive cocci and catalase negative. They could grow at 6.5% NaCl, at 10°C and at pH 4.4. However, they did not produce CO₂ from glucose. Thus, all potential isolates were identified based on key characteristics and tests and found to belong to the genus *Carnobacterium* and *Lactobacillus* (data not shown). Further, isolates were characterized through molecular approach. It was confirmed that the isolates FSK-L 5101, POL-20108 and HYL-20104 were found to be *Carnobacterium divergens* (99% similar to *Carnobacterium divergens*), whereas the isolate L-SQ-L 25104 was *Carnobacterium maltaromaticum* (100% similar to *Carnobacterium maltaromaticum*).

DISCUSSION

Lactic acid bacteria (LAB) are usually normal microflora of aquatic animals (Nanasombat et al., 2012). These results are compatible with previous reports of Nanasombat et al. (2012), who observed the number of LAB in raw shrimp and mussel were in the range of 3.0×10^4 to 3.0×10^6 CFU/g. In addition, Shiflett et al. (1966) reported that *Lactobacillus* was the major microflora found in specific oysters and accounted for 75% of total microflora after storage at 7°C for 2 days. Several investigators concluded that microflora associated with aquatic animals reflect microbial population of surrounding waters.

In this study, the inhibitory effects of organic acids and hydrogen peroxide were eliminated by growing LAB in BSM (sugar 0.2%) and cultivating in anaerobic conditions. These isolates were selected for further studies. This is probably because LAB could produce various antimicrobial compounds, such as organic acids, diacetyl, hydrogen peroxide and bacteriocin or bactericidal proteins during lactic fermentations (Holzapfel et al., 1995). The bacteriocins from them are generally recognized as safe (GRAS) and LAB have attracted a great deal of attention as an alternative approach to control pathogens in food (Savadogo et al., 2004).

L. monocytogenes is commonly found in ready-to-eat foods and rarely in preserved seafood products (Ben Embarek, 1994). *Vibrio parahaemolyticus* is a human pathogen that is widely distributed in marine environments. This organism is frequently isolated from a variety of raw seafood, particularly shellfish. Consumption of raw or undercooked seafood contaminated with *V. parahaemolyticus* may lead to development of acute gastroenteritis characterized by diarrhea, headache, vomiting, nausea and abdominal cramps (Su and Liu, 2007). This study revealed the biopreservative potential of LAB against these particular pathogens.

Selecting biopreservative LAB isolates possessing this psychrotrophic characteristic is of interest because they exhibit a growth inhibitory effect, even at chilled-storage temperatures, making them more competitive against the spoiling flora that generally develops at this temperature (Matamoros et al., 2009).

Psychrotrophic LAB, *C. divergens* and *C. maltaromaticum*, from raw seafood products are able to grow in a wide variety of refrigerated raw and processed meat and seafood products stored aerobically, vacuum packaged or packaged under MAP conditions (Groth Laursen et al., 2005). They produce antimicrobial

metabolites against pathogenic and food spoiling bacteria, such as *L. monocytogenes* ATCC 15313 and *V. parahaemolyticus* PSU 1681. LAB with biopreservative potential might be helpful in maintaining the quality of seafood products, particularly in regards to extending the shelf life of seafood products. However, their antimicrobial metabolites need further characterization before applying in seafood products.

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