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Original Article

Screening and identification of lactic acid bacteria from raw seafoods and Thai fermented seafood products for their potential use as starter cultures

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Abstract

The number of lactic acid bacteria (LAB) was analyzed from 52 samples of raw seafoods (shrimp and mussel), and Thai fermented seafood products including fermented shrimp (kung-jom), mussel (hoi-dong), and fish (pla-jom). The viable LAB were 3.0×10^3 to 3.4×10^8 CFU/g. LAB were isolated and screened for their inhibitory activities against eight indicator bacteria by agar spot test. Among all selected LAB isolates, 52 isolates showed strong inhibitory activity. They were further characterized for their ability to resist hydrochloric acid, lactic acid, bile salts, and sodium chloride, and their ability to produce bacteriocins and amino acid decarboxylase. The selected LAB isolates, 1IS11 and 4IS17, were bacteriocin-producing strains, and showed no amino acid decarboxylase activity, which was suitable property for starter cultures. The isolate 1IS11 could resist both hydrochloric and lactic acid at the lowest pH of 2.0, while the isolate 4IS17 was able to tolerate hydrochloric and lactic acid at the lowest pH of 1.5 and 2.0, respectively. Both isolates could grow in MRS broth containing a high concentration of sodium chloride (10 %) and bile salts (1.5%). They were identified by morphological characterization, biochemical test, and 16S rDNA sequence analysis. The isolate 1IS11 was found to be *Enterococcus faecium*, whereas the isolate 4IS17 was *Enterococcus faecalis*.

Keywords: lactic acid bacteria, seafood products, bacteriocins, biogenic amine, probiotic, starter culture

1. Introduction

Starter cultures are large amounts of microbial cells added into raw materials to produce fermented food (Leroy and De Vuyst, 2004). Using starter cultures in food fermentation resulted in an increased rate of fermentation and improved stability of the products (Visessanguan *et al*., 2006). Lactic acid bacteria (LAB) have been used as starter cultures in various fermented food such as fermented dairies and fermented meat products. LAB play an important role in the fermentation process by rapid acidification of raw materials through the production of organic acids (Leroy and De Vuyst, 2004). Suitable characteristics of LAB are (a) their ability to produce antimicrobial compounds (bacteriocins, organic acids) for growth inhibition of harmful bacteria, (b) their ability to resist high concentrations of salts (in food), acids, and bile salts (in gastrointestinal tract), which is beneficial to health, and (c) their inability to produce amino acid decarboxylase (no biogenic amine accumulation) (Goldin and Gorbach, 1992; Ammor and Mayo, 2007).

LAB are widespread in nature. They are commonly found in raw fish and prawn (Nair and Surendran, 2004), in gastrointestinal tracts of marine fish (Buntin *et al*., 2008), and in oyster (Shiflett *et al*., 1966). Mauguin and Novel (1994) reported that *Lactobacillus*, *Lactococcus* and *Carnobacterium* were isolated from seafood. Furthermore, LAB are also found in Thai fermented foods such as pla-jom (fermented fish), kung-jom (fermented shrimp) and hoi-dong (fermented mussel) (Tanasupawat *et al*., 2000). Tanasupawat *et al*. (1998) reported that forty-seven strains of LAB (*Lactobacillus* and *Leuconostoc*) were isolated from fermented fish products in Thailand. However, isolation and characterization of LAB

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from raw and fermented seafood are few. Thus, the objective of this study was to select new bacteriocin-producing LAB with probiotic and other suitable properties from raw and fermented seafood products to be used as potential starter cultures.

2. Materials and Methods

2.1 Samples

Twenty samples of raw seafood such as shrimp (*Litopenaeus vannamei*) and green mussel (*Perna viridis*) and 32 samples of Thai fermented seafood products such as kung-jom (fermented shrimp), pla-jom (fermented fish) and hoi-dong (lowly salted fermented mussel) were purchased at local markets in Bangkok and Prachinburi, Thailand.

2.2 Isolation of lactic acid bacteria

Total viable LAB counts in each sample were analyzed by spread plating the serially diluted samples onto MRS agar (deMan Rogosa Sharpe, Difco, pH 7.2±0.2) supplemented with 0.5% calcium carbonate. After incubation at 37°C for 48 hrs, colonies with clear zones were counted. Some of these colonies were selected and purified on MRS agar.

2.3 Screening of lactic acid bacteria for antimicrobial activity

Isolates of LAB were screened for their antimicrobial activity by the agar spot test (Schillinger and Lücke, 1989). Each isolate was grown in MRS broth at 37°C for 24 hrs. The cells were harvested by centrifugation, washed twice, and resuspended in 0.1% peptone solution. The cell suspension was adjusted to match the turbidity of 2 McFarland Standard. Then, 5 µl of cell suspension of each isolate was spotted onto the surface of agar plates (MRS with 0.2% glucose) and incubated under anaerobic condition at 37°C for 24 hrs to allow colonies to develop. Colonies were then overlayed with 10 ml of MRS soft agar containing 10^8 CFU/ml of LAB indicator strains (*Pediococcus acidilactici* TISTR 051 and *Lactobacillus bulgaricus* TISTR 541, obtained from the Microbiological Resources Centre for Southeast Asian Region, Bangkok MIRCEN) or TSYE soft agar (Tryptic soy broth supplemented with 0.6% yeast extract) containing other indicator bacteria (*Staphylococcus aureus* TISTR 118, obtained from Bangkok MIRCEN, *Vibrio parahaemolyticus* SH1, isolated from raw shrimp and *Escherichia coli* DMST 4212, *Listeria monocytogenes* DMST 11256, *Pseudomonas fluorescens* DMST 20076, *Salmonella* Typhimurium DMST 0562, obtained from the culture collection of the Department of Medical Sciences, Ministry of Public Health, Thailand). The plates were incubated overnight at 30°C for *P. fluorescens* or 37°C for other bacteria before recording the diameter of inhibition zones.

2.4 Selection of acid, bile salt and sodium chloride tolerant isolates

To study lactic acid or hydrochloric acid tolerance, 190 µl MRS broth acidified with 85% (w/v) lactic acid or 5 M hydrochloric acid adjusted to eight different pH values (pH 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, and 7.0) were added in each well of a microtiter plate. The inoculum suspension (10 µl) of each LAB strain was then added in each well, and subsequently incubated at 37°C for 24 hrs. The turbidity was measured at 620 nm using the microplate reader (Microplate reader/ Washer, Labsystems iEMS Reader MF). The lowest pH of lactic acid or hydrochloric acid that provided growth of each isolate was recorded.

To study bile salt or sodium chloride tolerance, the experiments were performed using similar procedure as described above, but MRS broth with porcine bile salts (0, 0.15, 0.3, 0.5, and 1.0 % (w/v)) adjusted to pH 8.0 was used for bile salt tolerance study, and MRS broth with sodium chloride of 1.0 to 11.0 % (w/v) was used for sodium chloride tolerance study. The highest concentrations of porcine bile salts and sodium chloride that the isolates were able to grow were recorded.

2.5 Screening of amino acid decarboxylase activity

Screening for amino acid decarboxylase activity was performed following the procedure as described by Joosten and Northold (1989), and Bover-Cid and Holzapfel (1999) with a slight modification. Briefly, the isolates of LAB were grown and subcultured for five times in MRS broth containing 0.1% of each precursor (L-tyrosine, L-histidine monohydrochloride, L-ornitine monohydrochloride, and L-lysine monohydrochloride) supplemented with 0.005% pyridoxal-5-phosphate. Then, this LAB culture was streaked onto a surface of decarboxylation medium plates (Bover-Cid and Holzapfel, 1999) with and without amino acids (as control) and incubated at 37°C for 4 days under anaerobic conditions. The occurrence of a purple halo or the disappearance of tyrosine precipitate around the colonies indicated a positive reaction of amino acid decarboxylase activity of the isolates being screened. The LAB strains which showed a negative result (no amino acid decarboxylase activity) were selected.

2.6 Identification of the LAB isolates

2.6.1 Morphological and biochemical identification

LAB isolates were tested for characteristics of Gram staining, cell morphology, colony morphology, motility, carbon dioxide production from glucose, growth at 10°C and 45°C, growth at pH of 4.4 and 9.6, growth in 6.5% and 18% NaCl, and catalase reaction by 3% hydrogen peroxide (Axelsson, 2004). Then, carbohydrate fermentation patterns of each LAB isolates were determined using API 50 CH (bioMérieux, France).

2.6.2 Molecular identification by cloning and sequencing of 16S rRNA gene of LAB isolates

The genomic DNA of LAB isolates was extracted by using MasturePure™ Gram Positive DNA Purification Kit (EPICENTRE®, U.S.A.). The 16S rDNA gene was amplified by polymerase chain reaction using primers F16SrDNA-27bac (5′-AGAGTTTGATCCTGGCTCAG-3′) and R16SRDNA-1492bac (5′-GGTTACCTTGTT ACGACTT-3′). PCR was carried out in a total volume of 50 ml containing 1X PCR buffer, 100 ng genomic DNA, 1.5 mM of $MgCl_2$, 0.2 mM dNTPs, 0.25 mM of each primer and 2.5 U of *Taq* DNA polymerase (Promega, U.S.A.). The PCR conditions consisted of an initial denaturing step of 94°C for 10 min, followed by 35 cycles of denaturing for 30 sec at 94°C, annealing for 30 sec at 55°C and a 90 sec of primer extension at 72°C. A final extension at 72°C for 10 min was then performed. The 1,500 bp PCR products were purified with QIAquick PCR purification ki t (Qiagen, Germany) and ligated to TA-cloning vector (QIAGEN PCR Cloning kit, Germany). The ligation reaction was transformed into competent cells of *E. coli* DH5a, and the transformants were selected on LB agar containing 50 mg/ml of kanamycin. Plasmid DNA of the transformants was isolated using QIAprep® Spin Minoprep kit (Qiagen, Germany). It was then sequenced by Big-dye terminator reaction method using ABI PRISM® 3700 DNA analyzer (First Base Laboratories Sdn Bhd, Malaysia). Nucleotide sequences were analyzed using the BLAST program (Altschul *et al*., 1990).

2.7 Detection of inhibitory activity of selected LAB isolates

Selected LAB isolates were grown in MRS broth at 37°C for 24 hrs. Cell free supernatant of each isolate was obtained by centrifugation at 3,000×g at 4°C for 20 min. The supernatant was adjusted to pH 6.5 with 1M NaOH and subsequently filter sterilized through a 0.2 µm membrane filter (Whatman, Germany). Inhibitory activity was determined using agar well diffusion assay (modified from Schillinger and Lücke, 1989). Inhibitory effect of the hydrogen peroxide in the supernatant was eliminated by reacting with 5 mg/ml catalase added. Suitable agar medium containing 1% agar (45°C) was inoculated with each of the eight indicator strains. Agar wells of 5 mm diameter were cut and the filter-sterilized supernatant (20 µl) was added into each well. The plates were incubated at 37°C for 24 hrs, except for *P. fluorescens* DMST 20076, which was incubated at 30°C for 24 hrs. The inhibition zones around the wells were measured.

2.8 Characterization of bacteriocins produced by the selected LAB isolates

2.8.1 Preparation of crude bacteriocin

Each LAB isolate was grown in 10 ml of MRS broth at 37°C for 24 hrs. Cell-free supernatant was collected by centrifugation at 3,000×g at 4°C for 20 min, adjusted to pH 6.5 with 1 M NaOH, and subsequently filtered through 0.2 mm membrane filter before freeze-drying.

2.8.2 Effect of temperature, enzyme and pH on bacteriocin activity

To study the effect of temperature on stability of bacteriocin, crude bacteriocin was heated at three different temperatures and time combinations (100°C for 15, and 30 min, and 121°C for 15 min) before inhibitory activity was determined by agar well diffusion assay as described above.

To determine the effect of enzyme on bacteriocin activity, crude bacteriocin was treated with six different enzymes including α-chymotrypsin (from bovine pancreas, Merck), trypsin (from porcine pancreas, Fluka), protease (from *Streptomyces griseus*, Sigma), α-amylase (from *Aspergillus oryzae*, Fluka), lipase (from porcine pancreas, Fluka) and pepsin (from porcine gastric mucosa, Fluka). The first five enzymes were dissolved in 0.05 M phosphate buffer (pH 7.0) to a final concentration of 1 mg/ml. Pepsin was dissolved in 0.002 N HCl. All samples were incubated at 37°C for 1 hr and tested for inhibitory activity.

To study the effect of pH on bacteriocin activity, pH of crude bacteriocin was adjusted to pH 3-10 using 6 M HCl or 6 M NaOH and incubated at 30°C for 2 hrs, after which all samples were re-adjusted to pH 7. Then, inhibitory activity of the treated crude bacteriocin was determined by agar well diffusion assay.

3. Results and Discussion

3.1 Isolation of lactic acid bacteria

The number of LAB in raw shrimp and mussel was in the range of 3.0×10^4 to 3.0×10^6 CFU/g (Table 1). In some fermented seafood samples, such as kung-jom and pla-jom, the total viable LAB were found in higher numbers (upto 10^7 to 10^8 CFU/g). A large number of LAB was found in raw shrimps and mussels. These results are compatible with previous reports. Buntin *et al*. (2008) found that gastrointestinal (GI) tracts of shrimp and shellfish contained 4.5×10^4 cells of viable LAB/g wet weight. 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 bivalve molluscan shellfish such as mussels, which are filter feeders, bacteria from water are concentrated during feeding. Bivalve shellfish at harvest usually have LAB counts of 10^3-10^5 CFU/g shellfish, which are 1-2 log units higher than the total counts in surrounding water (Cook, 1991). In this study, higher numbers of LAB were found in fermented seafood when compared to raw seafood. This may be because LAB are usually normal microflora of aquatic animals, a raw material for production of fermented seafood. Moreover, LAB starter

Table 1. Total lactic acid bacteria in raw seafoods and fermented seafood products and the number of selected isolates with antimicrobial activity

Type of samples	Number of samples	pH of samples	Total LAB counts (CFU/g)	Selected LAB (isolates)	LAB with antimicrobial activity[a] (isolates)
raw shrimp	10	6.08 – 6.63	3.8×10^4 - 9.1×10^5	44	11 (4.9 %)
raw mussel	10	6.08 – 6.19	3.0×10^4 - 3.0×10^6	47	10 (4.5 %)
Hoi-dong (fermented mussel)	12	4.53 – 4.58	3.2×10^3 - 2.0×10^5	30	10 (4.5 %)
Kung-jom(fermented shrimp)	10	4.09 – 4.28	1.2×10^5 - 9.3×10^7	65	19 (8.6 %)
Pla-jom(fermented fish)	10	4.30 – 5.56	3.0×10^3 - 3.4×10^8	36	2 (0.9 %)
Total	52	-	-	222	52

[a]The LAB isolates inhibited the indicator bacteria of at least one species by agar spot test.

cultures are also used in some commercial fermented seafood products. Tanasupawat *et al*. (1998) also reported that Thai fermented fish, shrimp and mussels contained high numbers of LAB (10^7 to 10^{11} CFU/g).

3.2 Screening of lactic acid bacteria for antimicrobial activity

In this study, some isolates of LAB were able to inhibit growth of some indicator microorganisms by agar spot test. Fifty-two (24.3 %) of 222 LAB isolates were able to inhibit growth of at least one of the eight indicator strains (Table 1). These strains were selected for further studies. This is probably because LAB could produce various antimicrobial compounds. Holzapfel *et al*. (1995) stated that LAB produce various metabolic products with antimicrobial properties such as organic acids (lactic and acetic acid), hydrogen peroxide, enzymes (lactoperoxidase system with hydrogen peroxide and lysozyme), low-molecular metabolites (reuterin, diacetyl and fatty acids) and bacteriocins (nisin and others). De Carvalho *et al*. (2006) investigated that LAB isolated from Italian salami could inhibit growth of *Listeria monocytogenes*. Similarly, Olasupo *et al*. (1994) reported that four out of 200 LAB isolates from 'wara' (fermented skimmed cow milk product from West Africa) could inhibit growth of *Lactobacillus sake* DSM 20071.

3.3 Selection of acid, bile salt and sodium chloride tolerant isolates

From the study of hydrochloric acid and lactic acid tolerance, it was shown that LAB isolates (46.2%) were capable of growing at the lowest pH (1.5) in MRS broth adjusted pH with hydrochloric acid. However, only 11.5% of LAB isolates could grow at lowest pH (1.5) in MRS broth adjusted pH with lactic acid (Table 2). Considering the minimum pH for growth, most LAB isolates (24 isolates) could tolerate hydrochloric acid at lower pH values than lactic acid. The most acid-resistant LAB to both hydrochloric acid and lactic acid at pH 1.5 were the isolates 1IS3, 4IS19, and 4IS23. For sodium chloride and bile salt tolerance, 33 isolates (63.5%) were able to grow at the highest concentration (10% w/v) of sodium chloride, whereas 51 isolates (98.1%) were resistant to the highest concentration of bile salts (1.5%).

LAB isolates with probiotic properties may be used as starter culture in fermented food. Consumption of food with live probiotic bacteria is beneficial to human health. Several researchers suggest that probiotic bacteria may mediate various health effects such as the decrease of cancer risk, improvement of the clinical outcome in many intestinal disease targets, and improvement of immune and mucosal barrier function (Salminen, 1999). In order to gain health benefit, probiotic bacteria need to survive passage through the gastrointestinal tract. This means that they must tolerate hydrochloric acid in the stomach and bile salts in the small intestine. LAB isolates were able to tolerate hydrochloric acid (strong acid) more than lactic acid (weak acid). At low pH, undissociated lipophilic acid molecules of weak acids inhibit microorganisms by entering the cells and dissociating to hydrogen ion within the cells. This causes a decrease of the internal pH (Adam and Moss, 1995; Girgis *et al.*, 2003). The ability of LAB isolates to grow at high acid conditions may be due to their acid tolerant response (ATR). The ATR had been observed in *Leuconostoc mesenteroides* (McDonald *et al*., 1990), *Lactobacillus plantarum* (McDonald *et al*., 1990), *Enterococcus hirae* (Belli and Marquis, 1991) and *Lactoccoccus lactis* (Hartke *et al*., 1996). In addition, resistance of LAB isolates may be due to the function of F_0F_1 ATPase to maintain intracellular pH and protect the cells during exposure to acids by transferring protons out of the cell membrane (Girgis *et al*., 2003). Moreover, high tolerance of LAB isolates to bile salts was one of suitable properties to be starter cultures as they may tolerate to bile condition in duodenum. Normally, the pH of duodenum is 8.0 (Enger and Ross, 2000). This is the reason why MRS broth with bile salts used for bile salt tolerance study was adjusted to pH 8.0. Moser and Savage (2001) revealed that bile salt hydrolase was an important enzyme of intestinal bacteria. Bile salt hydrolase activity may relate to ability of bacteria to survive and multiply in gastrointestinal tracts.

Table 2. Hydrochloric acid, lactic acid, sodium chloride and bile salt tolerance of lactic acid bacteria isolated from raw seafoods and fermented seafood products.

Source of isolates	LAB isolates	pH[a] of HCl	pH[a] of lactic acid	NaCl (%w/v)[b]	Bile salts (%w/v)[b]
Raw mussel	1IS2	2.0	2.5	10.0	1.5
	1IS3	1.5	1.5	10.0	1.5
	1IS11	2.0	2.0	10.0	1.5
	39IS5	3.0	5.0	5.0	1.5
	41IS5, 45IS3, 47IS1	3.0	5.0	7.0	1.5
	42IS3, 46IS2	3.0	4.0	7.0	1.5
	45IS4	3.0	5.0	6.0	0.5
Raw shrimp	3IS1, 3IS17	2.0	1.5	10.0	1.5
	35IS3, 50IS3	3.0	5.0	5.0	1.5
	37IS1	3.0	5.0	9.0	1.5
	49IS1,49IS2,49IS3, 49IS5,	3.0	5.0	7.0	1.5
	52IS2, 52IS3	3.0	5.0	7.0	1.5
Hoi-dong	6IS5,6IS20	2.0	2.0	10.0	1.5
	6IS6, 6IS12, 6IS14,6IS15,	1.5	2.0	10.0	1.5
	6IS16, 6IS17, 6IS18	1.5	2.0	10.0	1.5
	6IS19	1.5	2.5	10.0	1.5
Pla- jom	33IS3	3.0	4.0	7.0	1.5
	33IS5	3.0	5.0	7.0	1.5
Kung- jom	4IS1, 4IS4, 4IS5	1.5	2.0	10.0	1.5
	4IS2	2.0	1.5	10.0	1.5
	4IS6	2.0	2.0	10.0	1.5
	4IS9, 4IS10, 4IS11, 4IS12, 4IS13,	1.5	2.0	10.0	1.5
	4IS14, 4IS16, 4IS18, 4IS20, 4IS21	1.5	2.0	10.0	1.5
	4IS17	1.5	2.0	10.0	1.5
	4IS19	1.5	1.5	10.0	1.5
	4IS22	2.0	2.5	10.0	1.5
	4IS23	1.5	1.5	9.0	1.5

[a] The lowest pH of acid at which the isolates were able to grow.
[b] The highest concentration of sodium chloride and bile salts at which the isolates were able to grow.

3.4 Amino acid decarboxylase activity of selected lactic acid bacteria

In this study, LAB isolates with no amino acid decarboxylase activity (negative test) were selected. Three isolates of 52 isolates (5.8%), 1IS11, 4IS16, and 4IS17, were amino acid decarboxylase negative. LAB isolates without amino acid decarboxylase may result in no accumulation of biogenic amine (BA) in fermented seafood products. Fermented foods with high levels of BA are health problems as BA could induce various diseases such as migraines, headaches, gastric and intestinal ulcers, and allergenic responses (Pereira *et al*., 2001). The factors affecting BA accumulation are the availability of free amino acids, the presence of microorganisms capable of decarboxylating amino acids and suitable conditions for growth of decarboxylase-producing microorganisms (Pereira *et al*., 2001). Shalaby (1996) reported that amino acid decarboxylase-producing bacteria were found in Enterobacteriaceae and in *Clostridium*, *Lactobacillus*, *Streptococcus*, *Micrococcus* and *Pseudomonas*.

3.5 Inhibitory activity of selected lactic acid bacteria

Agar well diffusion assays of 52 LAB isolates showed that culture supernatant of two LAB isolates, 1IS11 and 4IS17, could inhibit growth of *L. bulgaricus* with 8.7 mm and 10.4 mm inhibition zone diameter. However, only culture supernatant of the isolate 4IS17 was able to inhibit growth of *S. aureus* (17 mm). One of the inhibitory substances produced by these two LAB isolates could be bacteriocins because the inhibitory effects of organic acids and hydrogen peroxide were eliminated by adjusting the pH to neutral pH and adding catalase. Bacteriocins produced by LAB can inhibit growth of Gram-positive bacteria more than Gram-negative bacteria because the outer membranes of Gram-negative bacteria are barrier for bacteriocins to penetrate into their cell membranes

(Stevens *et al*., 1991; Savadogo *et al*., 2004). Buntin *et al*. (2008) reported that three strains of LAB, *P. pentosaceus* APa4, *P. pentosaceus* AIa1, and *Enterococcus faecium* ARa1, were able to inhibit growth of *S. aureus*, *Salmonella* sp., *E. coli* and *L. monocytogenes* by agar well diffusion method.

3.6 Effect of enzyme, heat treatment and pH on bacteriocins produced by the selected isolates

Bacteriocin activity of the selected isolates, 1IS11 isolated from raw mussel and 4IS17 isolated from kung-jom, were inactivated by α-amylase and some proteolytic enzymes, but they had inhibitory activity against *L. bulgaricus* after adding with lipase (Table 3). This indicated that these bacteriocins may contain heterogeneous groups of compounds including carbohydrate and protein molecules in their structures, but not lipid molecules. Since bacteriocins are proteinaceous substances released extracellular, they are inactivated by proteolytic enzymes such as trypsin, proteinase K, α-chymotrypsin, and pepsin (De Vuyst and Vandamme, 1994). The inhibitory activity of bacteriocins produced by the isolates 1IS11 and 4IS17 was completely eliminated at 100°C for 15 min or higher degrees of heat treatment (Table 3). However, bacteriocin activity of the isolate 1IS11 was stable to acid conditions at pH 3.0-4.0, but not for the isolate 4IS17.

3.7 Morphological, biochemical and molecular identification of selected lactic acid bacteria

The isolates 1IS11 and 4IS17 were Gram-positive cocci. Both isolates had similar characteristics with catalase negative. They could grow at 6.5% NaCl, at 10°C and 45°C and at pH 4.4 and 9.6. However, they did not produce CO_2 from glucose and did not grow at 18% NaCl. Thus, the isolates 1IS11 and 4IS17 were identified belonging to genus *Enterococcus* based on key characteristics and tests (Axelsson, 2004). Carbohydrate fermentation tests showed that the isolate 1IS11 could ferment melibiose, lactose, maltose, cellobiose, salicin, esculin, mannose, trehalose, sucrose, galactose, and ribose, but not melezitose, raffinose, sorbitol, xylose, rhamnose, and arabinose. This fermentation pattern was similar to that of *Enterococcus faecium.* Thapa *et al*. (2004) isolated and identified *Enterococcus faecium* H2:B3 from 'hentak' (traditional fermented fish products of North-East India) on the basis of sugar fermentation using the API system and found that the results of sugar fermentation were similar to the results of the isolate 1IS11. Similarly, the isolate

Table 3. Effect of enzyme, heat treatment and pH on stability of bacteriocins produced by the selected isolates 1IS11 and 4IS17 against indicator bacteria

Treatments	Isolate 1IS11		Isolate 4IS17	
	Inhibition zone diameter (mm)			
	Lactobacillus bulgaricus	*Staphylococcus aureus*	*Lactobacillus bulgaricus*	*Staphylococcus aureus*
Enzyme				
α-amylase	-[a]	-	-	-
α-chymotrypsin	-	-	-	-
Lipase	6.8		6.6	-
Pepsin	-	-	-	-
Protease	-	-	-	-
Trypsin	-	-	-	-
Heat				
15 min at 100 °C	-	-	-	-
30 min at 100 °C	-	-	-	-
15 min at 121 °C	-	-	-	-
pH				
3.0	-	11.5	-	-
4.0	-	9.9	-	-
5.0	-	-	-	-
6.0	-	-	-	-
7.0	-	-	-	8.5
8.0	-	-	-	-
9.0	-	-	-	-
10.0	-	-	-	10.3

[a]loss of bacteriocin activity

4IS17 was able to ferment melezitose, lactose, maltose, cellobiose, salicin, sorbitol, mannose, trehalose, sucrose, galactose, esculin, and ribose, but not arabinose, xylose, rhammose, melibiose, and raffinose. This fermentation pattern was similar to that of *Enterococcus faecalis*. These sugar fermentation results were compatible with the results reported by Thapa *et al*. (2006). *Enterococcus faecalis* CG1:B2 from sidra (sun-dried fish product of Eastern Nepal) was characterized by sugar fermentation tests (Wood and Holzapfel, 1995). The isolate 1IS11 was confirmed by molecular identification by 16S rDNA sequence analysis and it was shown to be *Enterococcus faecium* 1IS11 (99% similar to *Enterococcus faecium*). The isolate 4IS17 could be identified as *Enterococcus faecalis* 4IS17 (99% similar to *Enterococcus faecalis*).

4. Conclusions

The results of the present study clearly suggest that two bacteriocin-producing LAB strains, *E. faecium* 1IS11 and *E. faecalis* 4IS17 with probiotic properties could potentially be used as fermented seafood starter cultures. These LAB strains could provide significant health benefits and enhance safety of the products. However, they need further characterization regarding isolation, purification, and identification of their bacteriocins and fermentation ability in fermented seafood products.

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