Isolation and Screening of Lactic Acid Bacteria Capable of Inhibiting Food-Spoilage and Food Borne Pathogens from Fermented Food in Southern Thailand

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ABSTRACT

One hundred isolates of lactic acid bacteria were isolated from 40 samples of fermented foods including fermented durian flesh (Tempoyak), fermented rice, fermented fish (Pla Som and Budu), fermented tapioca (Tapai), sausage and fermented fruits using MRS medium. After cultivation for 24 hours, the packed cells were removed and the cell free supernatant (CFS) of each isolate was investigated its ability to inhibit growth of eight gastrointestinal tract pathogenic bacteria by an agar well diffusion method. It was found that CFS of six isolates namely ST48, ST57, ST62, ST68, ST70 and ST93 could inhibit growth of *Bacillus cereus*. Interestingly, the ST93 CFS could tolerate temperature as high as 60°C and could tolerate pH as low as 4.0. This isolate ST93, isolated from fermented crab from Naradhiwat province, was identified as *Lactobacillus plantarum*.

Keywords: Lactic acid bacteria, Antimicrobial substance, Fermented food

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Introduction

Lactic acid bacteria (LAB) can be defined as a group of Gram-positive, non-spore forming, rods or cocci, normally have anaerobic respiration and lack catalase (Woraprayote *et al*., 2016). Fermentation patterns of lactic acid bacteria are homofermentative and heterofermentative. During fermentation, these bacteria do not produce only lactic acid but they are also known to produce and excrete compounds with antimicrobial activity. Cell free supernatant (CFS) of lactic acid bacteria fermentation has been reported to be able to inhibit growth of some spoilage bacteria and food-borne pathogens (Hwanhlem *et al*., 2014; Prasirsak *et al*., 2013). Generally, lactic acid bacteria found in fermented foods come from the environment. It is rather hard to control the quality of fermented foods produced by villagers in terms of taste, texture, amount and type of microorganisms presented in the foods, both non-pathogenic and pathogenic microorganism (Swetwiwathana *et al*., 2015).

Many strains of lactic acid bacteria including *Lactobacillus* sp., *Leuconostoc* sp., *Pediococcus* sp. and *Enterococcus* sp. have been reported to be able to produce cell free supernatant to inhibit specific species of spoilage bacteria and food borne pathogens (Todorov *et al*., 2005; Adnan *et al*., 2007; Sabo *et al*., 2014; Swetwiwathana *et al*., 2015; Woraprayote *et al*., 2016). Therefore, screening of lactic acid bacteria from southern Thai fermented foods for production of cell free supernatant against gastrointestinal tract pathogenic bacteria that cause public health problems in Thailand could lead to modernize and improve the quality of fermented foods produced by local people.

Objective of the study

The aim of this study was to isolate and screen for antimicrobial substance producing lactic acid bacteria from fermented foods of southern Thailand.

Materials and Methods

1. **Isolation of lactic acid bacteria (LAB)**

Forty samples of fermented foods such as fermented durian flesh (Tempoyak), fermented rice, fermented fish (Pla Som and Budu), fermented tapioca (Tapai), sausage and fermented fruits were all bought from local markets in southern Thailand. Ten grams of each sample were suspended in 100 ml of NaCl solution, 0.85% (w/v). Subsequently, 1 gram or 1 milliliter of each sample was cultured in 9 ml of MRS broth containing bromocresol green, 0.02% (w/v), as a pH indicator. The cultures were incubated under anaerobic conditions in an anaerobic jar with Anaerocult® A (Merck KGaA, Germany) at 37 °C for 24 hours. Only the culture tube that was changed from green to yellow color was streaked on MRS agar containing bromocresol green (Hwanhlem *et al*., 2011; Todorov *et al*., 2010). The yellow colonies were re-streaked on MRS agar until pure cultures were obtained.

2. **Test bacteria**

The test microorganisms including *Escherichia coli*, *E. coli* O157:H7, *Salmonella* Typhi, *Shigella dysenteriae*, *Staphylococcus aureus*, Methicillin-resistant *S. aureus* (MRSA), *Bacillus cereus* and *Vibrio cholerae*

were obtained from the Microbiology section, Department of Biology, Faculty of Science, Chiang Mai University, Thailand.

3. Screening of antimicrobial substance producing LAB

3.1 LAB fermentation and preparation of cell free supernatant (CFS)

Each isolate of LAB obtained was grown in MRS broth under anaerobic condition at 37°C for 24 hours. Each cell free supernatant was obtained by centrifugation (8,000 g at 4°C for 10 minutes) and was adjusted to pH 7.0 by NaOH, 1M (Moraes *et al*., 2010).

3.2 Determination of antimicrobial activity

The antimicrobial activity of each CFS obtained was determined by an agar well diffusion method as described by Castro *et al*., 2011. Briefly, the Mueller Hinton agar (MHA) was thoroughly swabbed with 1×10^6 CFUs/ml of each test bacteria prior to addition of each CFS, 50 µl, into each well sizing 5 millimeters in diameter. MRS broth and 1 mg/ml of gentamicin were used as negative and positive controls, respectively. All test plates were incubated at 37°C for 24 hours. The inhibitory clear zones were observed and recorded (Hwanhlem *et al*., 2014; Woraprayote *et al*., 2016).

4. Investigation of high temperature tolerance and effect of initial media pH on CFS activity

Each CFS obtained above was placed at 50, 60, 70, 80, 90 or 100°C for 5 minutes in water baths prior to antimicrobial activity evaluation by an agar well diffusion method as described above. Meanwhile, each LAB isolated was grown in MRS broth with the initial media pH ranging between 4 and 10. The cultures were incubated under anaerobic condition at 37°C for 24 hours before centrifugation to obtain CFS. The CFS obtained was adjusted to pH 7 using NaOH, 1M, and sulfuric acid, 1M. Subsequently, each treated CFS was incubated at 30°C for 2 hours prior to determination of antimicrobial activity against test bacteria by the agar well diffusion method (Hwanhlem *et al*., 2011; Castro *et al*., 2011).

5. Identification of antimicrobial substance producing lactic acid bacteria

5.1 Phenotypic characterization

Morphological and biochemical characteristics of antimicrobial substance producing LAB were observed according to the Bergey's Manual of Determinative Bacteriology including Gram reaction, endospore formation, gas formation, catalase activity, motility test, growth at different temperatures (15-45°C), growth at pH 6.5 and 9.5, growth tolerance in NaCl, 6.5% (w/v), and sugar fermentation scheme (Todorov *et al*., 2005).

5.2 Genotypic characterization

Total DNA from LAB were extracted by protocol of Woraprayote *et al*., (2016) with some modification. The 16S rRNA gene was amplified with primers 27F (5´AGAGTTTGATCCTGGCTCA 3´) and 1429R (5´TACGGYTACCTTGTTACGACTT 3´). The PCR products were analyzed by First base laboratories, Malaysia. Sequence alignment was employed using the BLAST software from the Gen Bank. Multiple alignments of the sequence determined were performed with the CLUSTAL X program. Gaps and ambiguous bases were

eliminated prior to construction of a phylogenetic tree by the neighbor-joining method with the MEGA program (Prasirsak *et al*., 2013).

Results

1. Isolation and screening of lactic acid bacteria capable of producing antimicrobial substance

A total of 100 LAB isolates were isolated from 40 samples of fermented foods obtained from southern Thailand. Cell free supernatant of each isolate was investigated its antimicrobial substance against 8 food-spoilage and/or food borne pathogens (*Escherichia coli*, *E. coli* O157:H7, *Salmonella* Typhi, *Shigella dysenteriae*, *Staphylococcus aureus*, Methicillin-resistant *S. aureus* (MRSA), *Bacillus cereus* and *Vibrio cholerae*). It was found that the CFS of isolates ST48, ST57, ST62, ST68, ST70 and ST93 could only inhibit growth of *Bacillus cereus* with the inhibitory clear zone ranging between 8.6± 0.7 and 10.0 ± 0.3 millimeters (Table 1 and Figure 1) while the other test pathogenic bacteria could not be inhibited by any CFS. The isolate ST93 was selected for further studied due to its highest activity.

Table 1 Antimicrobial activity of CFS produced by isolates ST48, ST57, ST62, ST68, ST70 and ST93 against *Bacillus cereus* using the agar well diffusion method.

Isolate	Inhibitory clear zone against *Bacillus cereus* (mm)
ST48	9.6 ± 0.7
ST57	9.6 ± 0.7
ST62	10.0
ST68	9.0
ST70	8.6 ± 0.7
ST93	10.0 ± 0.3

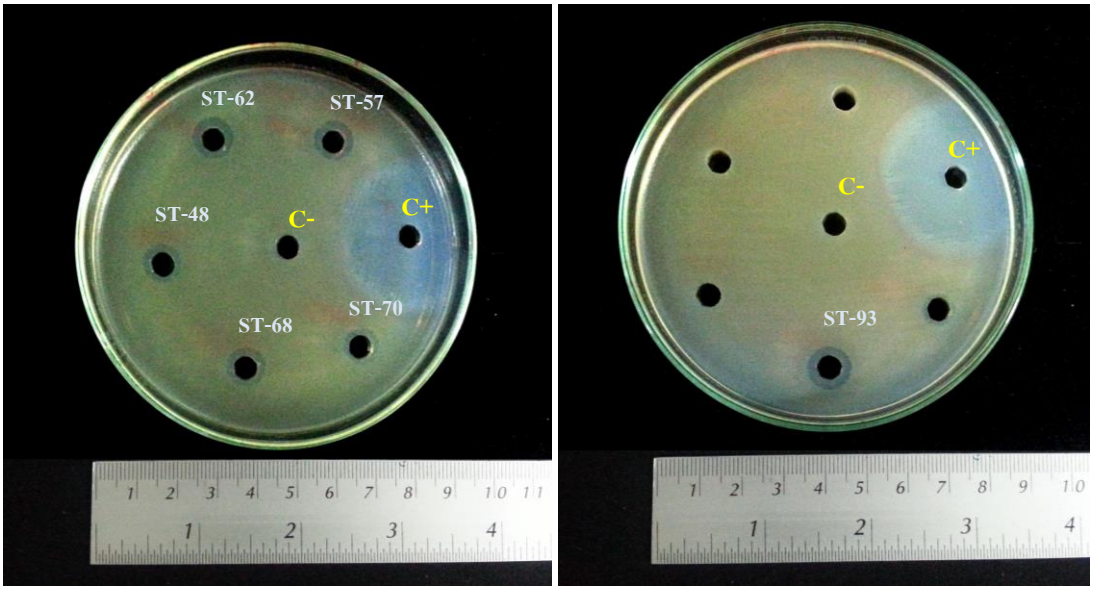


Figure1 Antimicrobial activity of cell free supernatant (CFS) produced by the isolates ST48, ST57, ST62, ST68, ST70 and ST93 against *Bacillus cereus* determined by the agar well diffusion assay on Mueller Hinton agar (MHA). The plates were incubated at 37°C for 24 hours. C+ and C- referred to positive and negative controls, respectively.

2. Investigation of high temperature tolerance and effect of initial media pH on CFS activity

The CFS of the isolate ST93 was further evaluated its properties to tolerate high temperatures including 50, 60, 70, 80, 90 and 100°C. The results showed that the inhibitory clear zone against *Bacillus cereus* of the ST93 CFS was decreased by 20% when temperature was increased up to 50 and 60°C, and was vanished when temperature was higher than 70°C (Figure 2).

The isolate ST93 was grown in MRS medium with various initial media pH and its CFS was further adjusted to pH 7 prior to antimicrobial activity determination. It was found that only the pH adjusted CFS of the initial media pH 4 exhibited the ability to inhibit growth of *Bacillus cereus* with the inhibitory clear zone of 8.6 ± 0.15 millimeters (Figure 3-4). Interestingly, the isolate ST93 grew welll in all initial media pH MRS broth but only the initial media pH 4 CFS had the inhibitory activity. Therefore, the mechanism of inhibition should be further elucidated in the future.

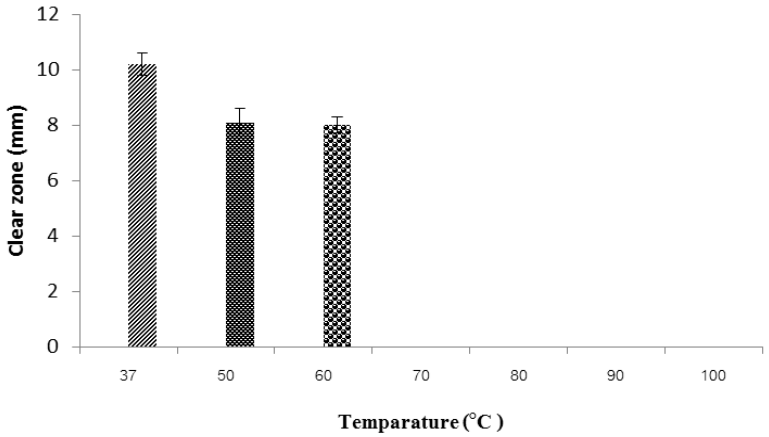


Figure 2 Effect of temperature on antimicrobial activity of ST93 CFS against *Bacillus cereus*. Each CFS was incubated at 37, 50, 60, 70, 80, 90 and 100°C for 5 minutes using water bath.

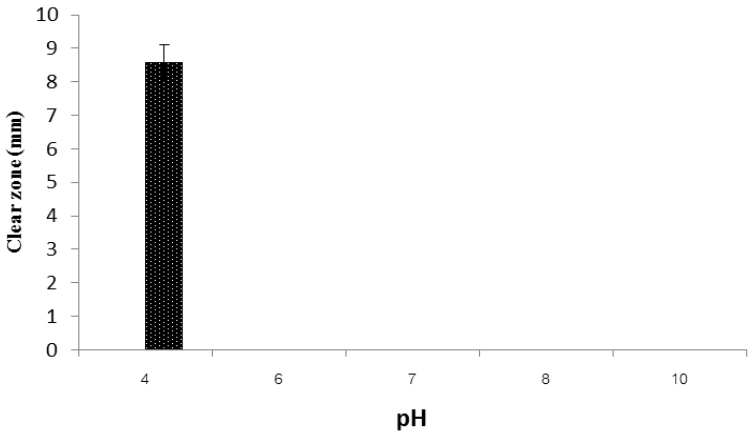


Figure 3 Effect of initial media pH on antimicrobial activity of the isolate ST93 CFS. The isolate ST93 was grown in MRS broth with various initial media pH at 37°C for 24 hours. Each CFS obtained was adjusted to pH 7 prior to antimicrobial activity evaluation against *Bacillus cereus*.

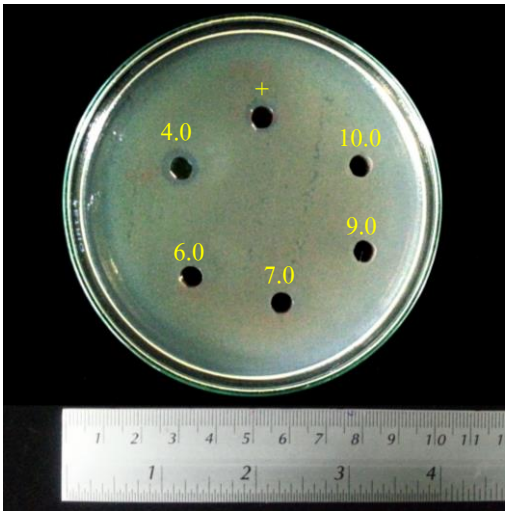
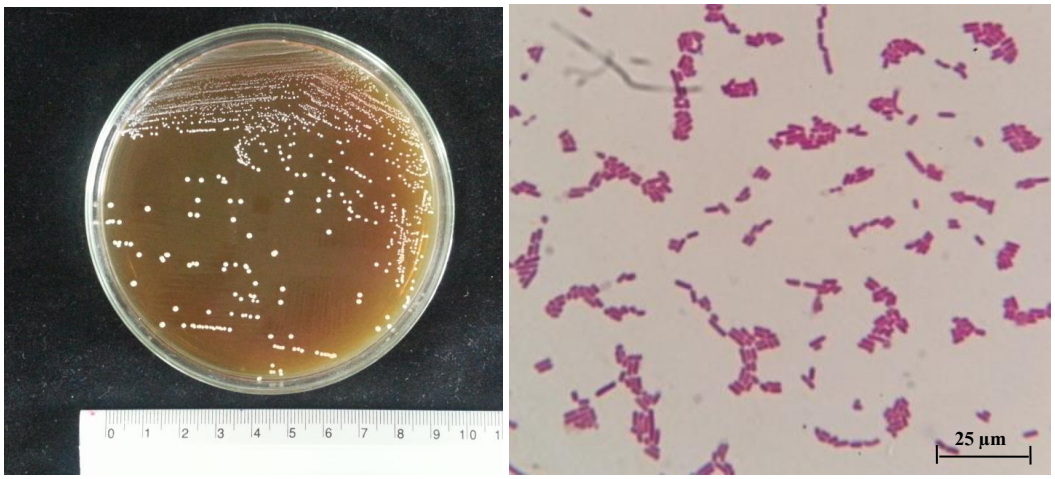


Figure 4 Effect of initial media pH on antimicrobial activity of the isolate ST93 CFS against *Bacillus cereus* by the agar well diffusion assay**.**

3. Identification of the isolate ST93

The isolate ST93 which its CFS could inhibit growth of *Bacillus cereus* was further studied. This isolate was Gram positive, rod shape, unable to produce catalase, non-motile and appeared circular and white colonies on MRS agar (Figure 5). The isolate ST93 grew well at 15-30°C but not at 45°C, could tolerate NaCl, 6.5% (w/v)**,** and was heterofermentative (Table 2). Furthermore, from the 16s rRNA sequence analysis, the ST93 was similar to *Lactobacillus plantarum* by 99% (Figure 6).



(A) **(B)**

Figure 5 Characteristic of the isolate ST93. Colonies on MRS agar (A) and cell morphology under a compound microscope (B).

Table 2 Morphological and biochemical characteristics of the isolate ST93

Characteristics	Isolate ST93
Colony shape	Circular, Convex, Entire
Cell shape	Rod
Gram stain	+
Catalase test	-
Oxidase test	+
Gas production	+
Endospore formation	-

Motility test	-
Growth at temperature (°C)	
15	+
30	+
45	-
Growth in NaCl, 65% (w/v)	+
Growth at pH	
6.5	+
9.5	+
Sugar fermentation	
D-glucose	+
D-raffinose	+
D-sorbitol	+
D-mannitol	+
D-xylose	+
D-mannose	+
D-cellubiose	+
D-lactose	+
D- sucrose	+
D-fructose	+
D- galactose	+
D-rhamnose	-
D-ribose	+

+ = positive or growth

- = negative or no growth

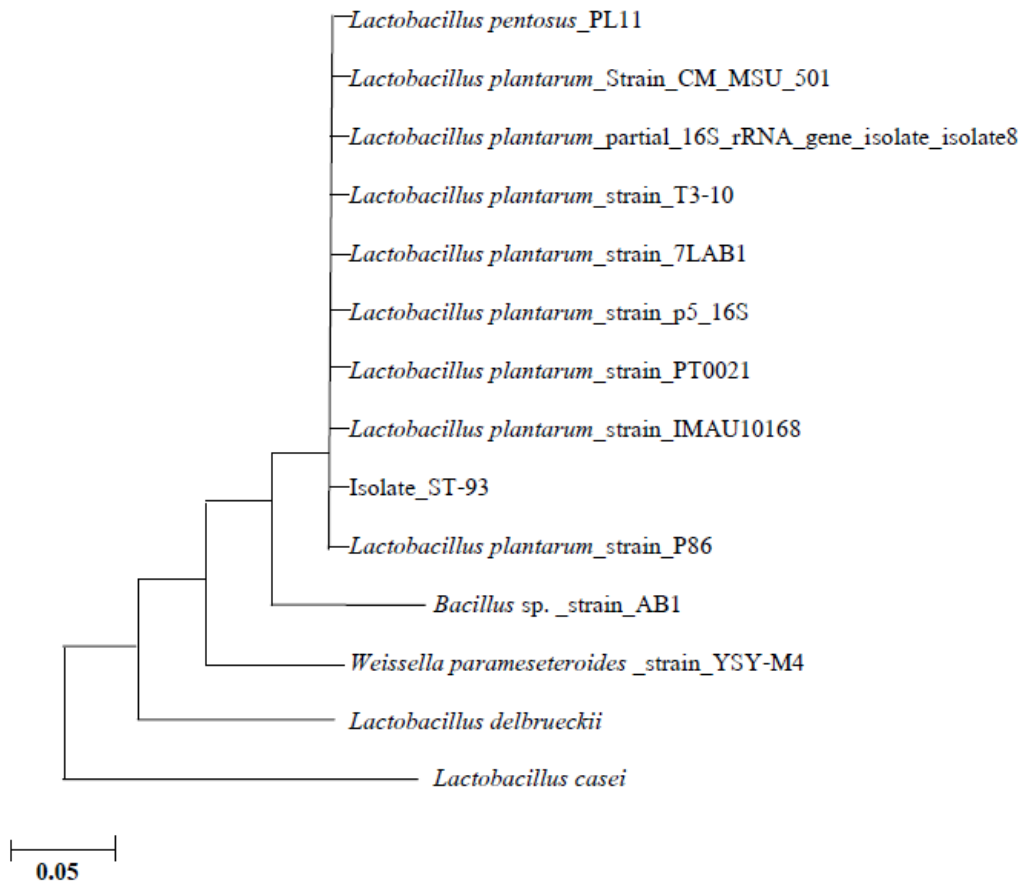


Figure 6 Phylogenetic tree showing the strain ST93 and other related strains based on 16S rRNA sequences

Discussion

Antimicrobial substance could be produced by lactic acid bacteria isolated from southern Thai fermented foods. Among 40 samples of fermented foods, 100 LAB isolates were discovered, however, only six isolates namely ST48, ST57, ST62, ST68, ST70 and ST93 were able to produce cell free supernatants that capable of inhibiting growth of *Bacillus cereus*, a food borne pathogen. Among six LAB isolates, only the ST93 CFS could inhibit growth of *B. cereus* after heating up to 60°C for 5 minutes indicating the heat resistance of the substance residing in the ST93 CFS. Furthermore, the ST93 CFS obtained from the culture broth of the ST93 grown in the MRS broth with initial media pH 4 was the only initial media pH that its CFS was able to inhibit growth of *B. cereus*. The result indicated that upon growing in low pH medium, the ST93 might be provoked its stress response resulting in production of some antimicrobial substance that effect of hydrogen ion was excluded. Therefore, this antimicrobial substance should be further studied in details in the future.

Conclusions

The cell free supernatant capable of inhibiting growth of *Bacillus cereus* could be found in *Lactobacillus plantarum* ST93 isolated from southern fermented crab. The *Lactobacillus plantarum* ST93 CFS could tolerate temperature as high as 60°C while its antibacterial substance could be efficiently produced when grown in MRS broth with initial media pH 4. The ST93 CFS had a potential to be further applied as a bio-control agent in food products.

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