International Food Research Journal 20(1): 125-129 (2013)
Journal homepage: *http://www.ifrj.upm.edu.my*

Isolation of lactic acid bacteria strain *Staphylococcus piscifermentans* from Malaysian traditional fermented shrimp *cincaluk*

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Article history

Received: 11 November 2011
Received in revised form: 12 July 2012
Accepted:17 July 2012

Keywords

cincaluk
fermented shrimp
lactic acid bacteria
Staphylococcus piscifermentans

Abstract

Lactic acid bacteria is well known for it uses as starter culture in various fermented food, and it functions as a good natural antimicrobial agent. *Cincaluk*, a Malaysian fermented shrimp product commonly found in traditional dishes is commonly enriched with LAB. Out of 50 colonies from a local *cincaluk*, 7 strains were successfully isolated and shown to be positive in lactose utilization and catalase tests. The majority of the isolates from *cincaluk* showed Gram-positive cocci morphology and belonged to the group *Staphyloccoccus* spp. By using agar disc diffusion method, the anti-bacterial properties of these isolates (namely isolate 1, 2, 3, 4, 5, 6, and 7) moderately inhibited the growth of several pathogenic strains, i.e., *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium* and *Bacillus subtilis* which were used as indicator bacteria. Other than isolates 1, 2, 3 and 5; the 16S rRNA gene for isolate 6 and 7 were successfully amplified. The 16S rRNA gene fragment from isolate 7 was successfully cloned and sequenced. Based on rRNA sequences, both isolates 6 and 7 belonged to the group *Staphylococcus piscifermentans*, a rare strain previously reported to be specifically isolated exclusive from fish sources.

Introduction

Consumer interests in food that are natural, fresh and healthy are increasing in demand. There is a growing concern on the nutritional food losses upon processing and possible health risk of chemical preservatives. Despite of improved manufacturing standard and effective legislative control on processing procedures, the number of food borne illness still remains a public concern (Tharmaraj and Shah, 2009). The contamination and growth of psychrotropic and pathogenic spoilage microorganisms in refrigerated foods is a major risk in food industry. Consequently, the use of naturally produced anti-microbial agents without any adverse effect on human health to inhibit the proliferation of pathogenic microorganisms in food has been a prime concern.

Lactic acid bacteria or (LAB) is a group of related bacteria producing lactic acid as the result of carbohydrate fermentation (Ali *et al*., 2009). LAB is widely used as starter cultures for dairy, meat and vegetable fermentations (Jeppesen and Huss, 1993). In addition to flavour development and food preservation, they also produce variety of compounds with antimicrobial activity, including organic acids, hydrogen peroxide and bacteriocin (Choi *et al*., 1997; O'Sullivan *et al*., 2002). Bacteriocin produced by LAB could inhibit not only closely related species but also the growth of pathogenic bacteria (Jack *et al*., 1995; Klaenhammer, 1988; Tagg *et al*., 1976). Many LABs have important roles in the production of fermented foods, and some of the bacteria were capable of inhibiting the growth of a wide variety of food spoilage microorganisms (Lindgren and Dobrogosz, 2006). Thus, LABs are an attractive source of inhibitory compounds with promising natural food preservatives for improved food quality and safety. The purpose of the study is to isolate LAB from a Malaysian fermented shrimp product known as *cincaluk*. *Cincaluk* is a sauce in traditional Malay delicacies produced by fermenting small shrimp called *Acetes* sp., or locally known as *Udang geragau*, *Udang bubok* or *Udang gari* (Ali *et al*., 2009). The sauces, in various varieties, are also consumed in other Southeast Asian countries. Various strains from *Lactobacillus* sp. and *Lactococcus* sp. were

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the dominant LAB strains found in Thai fish product (Østergaard *et al*., 1998; Paludan-Muller *et al*., 1999). Similar works on various types of fermented shrimp products had previously isolated LAB from other genera such as *Leuconostoc mesenteroids*, *Streptococcus faecalis* and *Pediococcus halophilus* (Tanasupawat *et al*., 1998), *Tretragenococcus* sp. (Kobayashi *et al*., 2003) and *Leuconostoc durionis* sp. nov (Leisner *et al*., 2005). In an attempt to isolate γ-amino butyric acid (GABA) producing LAB, Ali *et al*. (2009) isolated *Leuconostoc* NC5 from *cincaluk*. In another work on Malaysian local fermented fish product or *budu*, the isolation of *Lactobacillus* spp. was reported (Liasi *et al*., 2009). In this work, we report on the isolation of LAB strain from a local *cincaluk*. However, further identification has led to the isolation of *Staphylococcus piscifermentans* a rare LAB strain which was never been reported from this source.

Materials and Methods

LAB samples and characterisation

A bottle of fermented shrimp product, *cincaluk* used as the sample source was purchased from domestic and home-made supplier at Melaka, Malaysia. Approximately, 25 ml of sauce sample was mixed with 225 ml of buffered peptone water to obtain a 1:10 dilution. Serial dilution of the samples was made using 0.1% peptone water. The diluted samples were spread on the De Man, Rogosa and Sharpe (MRS) agar plates and incubated, anaerobically, for 48 h at 37°C. Single colonies were picked out using sterile tooth pick and subjected to further studies. The morphological, physiological and biochemical characterization of the isolates were determined by the standard procedure of Gram staining, and tested for catalase and gas production. In lactose utilization test, the selected colonies were streaked onto nutrient agar (NA) with the addition of lactose containing 0.005 g/L of bromo-cresol purple, as pH indicator dye. The plates were incubated at 30°C for 24 h. Isolates utilizing lactose and producing acid were differentiated by the change in media color from violet to yellow. Catalase activity was tested by adding a drop of 30% hydrogen peroxide solution onto the culture smears. Positive reaction was seen as bubbles or froths generated from the colonies, indicating a rapid production of oxygen gas from hydrogen peroxide.

Antagonist test using disc diffusion method

The antagonistic activity of the isolated LAB against *Salmonella* typhimurium was determined by using agar disc diffusion method based on Kirby (Kirby *et al*., 1966). LAB isolate was propagated in MRS broth medium and incubated anaerobically at 30°C for 48 h. The propagated cell was pipetted onto filter paper disc (diameter of 5 mm, Whatman No.1) and dried for 10 min. The paper discs were put on the agar surface pre-spread with 500 µl of indicator organism. Four indicator strains; *Bacillus subtilis*, *E. coli* ATCC 35215, *Staphylococcus aureus* and *Salmonella* Typhimurium obtained from Institute of Medical Research, Malaysia (IMR) were used. The test was performed in triplicates. Then, the disc on the plates were incubated at 37°C for 24 h and the zone of inhibitions formed surrounding the disc were observed.

16S Ribosomal (rRNA) gene amplification, sequencing and analysis

The 16S rRNA gene fragment of ~1.5 kb was amplified by using a pair of universal primers pA forward(5'-AGAGTTTGATCCTGGCTCAG-3')and pE reverse (5'-CCGTCAATTCCTTTGAGTTT-3') (Edwards, 1989). The amplified product was purified with spin columns (Qiagen, USA). The amplifications were performed with initial denaturation at 94°C for 4 min, and with 29 cycles of denaturation at 94°C for 2 min; annealing at 55°C for 1 min and extension at 72°C. All DNA templates used were approximately at 5 ng DNA. The amplified fragments were then cloned by using blunt end of PCR product into pJET1.2/blunt cloning vector (Fermentas, Lithuania) and transformed into *Escherichia coli* competent cells (JM101). The DNA was analysed by using 1.0% (w/v) agarose gel electrophoresis in 1x TAE buffer at 90 V for 65 min; and was visualized by using gel documentation system (Alpha Imager). For successful transformant, the recombinant plasmid was extracted (Qiagen, USA). Then, the extracted plasmid was further verified by PCR using vector targeted primer. The insert was then sequenced with vector-targeted primers by using sequencing service available at 1st Base Laboratory Sdn. Bhd, Malaysia. The purified PCR product was also sequenced directly with 16S rRNA primers and further primer walked to get the complete rRNA sequence. The sequences of the whole gene fragment were used for the similarity search against NCBI GenBank database using the BLAST program, available at website *http://blast.ncbi.nlm.nih.gov/Blast.cgi*.

Results and Discussion

As shown in Table 1, out of 50 colonies, isolates 1, 2, 3, 4, 5, 6 and 7 appeared to be positive in lactose utilisation test. These isolates were able to ferment lactose to produce lactic acid that lowers the pH of the

Table 1. Morphological and biochemical tests for isolates 1 to 7

Characterization	LAB isolates						
	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5	Strain 6	Strain 7
Lactose utilization	+	+	+	+	+	+	+
Catalase activity	+	+	+	+	+	+	+
Gram staining	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Morphology	coccus	coccus	coccus	coccus	coccus	coccus	coccus
Agar Disc diffusion method (Indicator organism)							
B. subtilis	-	-	-	-	-	-	-
S. aureus	+	+	+		+	+	+
S. thyphimurium	-	-	-	-	-	+	+
E. coli	+	+	+	+	-	+	+

For agar diffusion method: degree of inhibition
– = no inhibition zone
+ = moderate inhibition (2-5 mm)

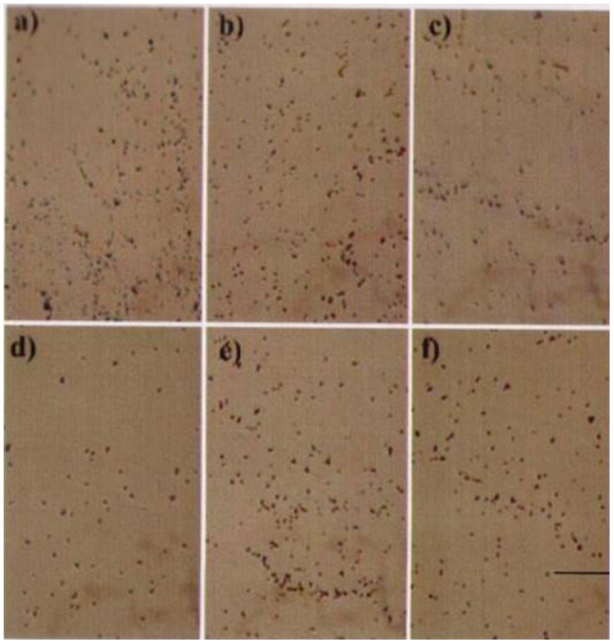

Figure 1. Gram staining test observed under light microscope (1000x) for isolate 1, 2, 3, 5, 6 and 7 (a to f, respectively). All isolates showed Gram positive coccoid morphology

MRS media that, in turn, changed the purple indicator dye to yellow indicative of fermentation activities. Gram reaction and morphology studies showed that all of these isolates from cincaluk as Gram-positive cocci (Figure 1). These are the common features of LAB whereby these organisms constitute a large group of non-sporulating Gram positive, catalase and oxidase negative rods or cocci that produce lactic acid as the major metabolite of the carbohydrate fermentation (Françoisea, 2010).

As indicated in Table 1, in the antimicrobial test, all 7 isolates produced zones of inhibition of sizes between 2 to 5 mm against pathogenic strain *S. aureus*. None of these isolates showed zones of inhibition against *B. subtilis*. Isolate 6 and 7 showed inhibitions of less than 5 mm in size against Gram negative indicator strain *S.* typhimurium. Meanwhile, only isolate 4 and 5 did not show any inhibition

Table 2. Top five similarity searches hits using BLAST for rRNA sequences for Isolate 6 and 7. The highest similarity was found to be *Staphyloccus piscifermentans*, followed by *S. carnosus* and *S. condimenti*

Sample	Match	Accession no.	Score (bits)	E value	Identity (%)
10 Isolate 6	*Staphylococcus piscifermentans* CIP103958	EU727184	2767	0.0	99
	Staphylococcus piscifermentans strain PU-87	Y15753	2760	0.0	99
	Staphylococcus carnosus subsp. *carnosus* TM300	AM295250	2756	0.0	99
	Staphylococcus condimenti CIP105760	EU727183	2750	0.0	99
	Staphylococcus sp. M5-7-5	FJ832082	2750	0.0	99
10.3 Isolate 7	*Staphylococcus piscifermentans* CIP103958	EU727184	2784	0.0	99
	Staphylococcus piscifermentans strain PU-87	Y15753	2776	0.0	99
	Staphylococcus carnosus subsp. *carnosus* TM300	AM295250	2772	0.0	99
	Staphylococcus condimenti CIP105760	EU727183	2767	0.0	99
	Staphylococcus sp. M5-7-5	FJ832082	2767	0.0	99

The table showed the BLAST result after the respective rRNA sequence from the Isolated 6 (denote as sample 10) and isolate 7 (sample 10.3), respectively, was searched through similarity searches using BLAST search engine tool at NCBI website using standard/default parameters using non-redundant nucleotide databes at Genbank (nr/nt). The match list (the first column) is the top 5 similarity hit, with the second column to indicate the respective Accession no for the gene in the hit list, followed by column for the Score obtained; the E-value and the Identity in the hit sequence with our sample in %

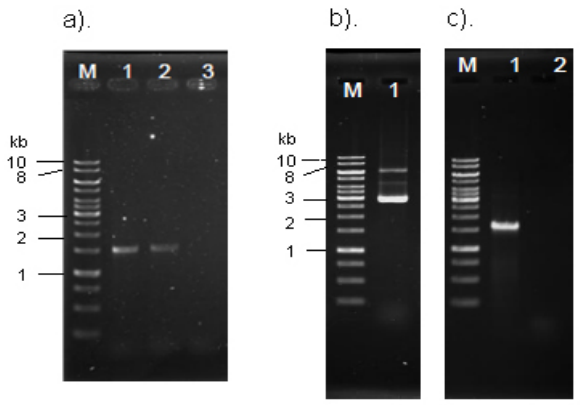

Figure 2a. PCR amplified 16S rRNA gene fragment of ~1.5 kb size of DNA samples from isolate 6 (Lane 1) and isolate 7 (Lane 2). Lane M is 1 kb DNA Ladder (Fermentas); Lane 3 is the negative control. Figure 2b. Recombinant plasmids containing the ~1.5 kb 16S rRNA gene fragment(Lane 1). Lane M is 1 kb DNA Ladder (Fermentas). Figure 2c. PCR product of ~1.6 kb in size was amplified from the recombinant plasmids, indicating the presence expected size (1.5 kb) insert with including the vector priming site. Lane M is 1 kb DNA Ladder (Fermentas); Lane 2 is the negative control.

against *E. coli*. Antagonistic activity of LAB towards pathogenic strain could be due to several factors. This included the production of organic acid or bactericidal substances such as bacteriocins (Karimi *et al*., 2008).

Despite of the amplification of 16S rRNA gene for isolates 1, 2, 3, 4 and 5 were not successful, the gene for isolate 6 and 7 were successfully amplified (Figure 2a). However, only 16S rRNA gene fragment from isolate 6 was successfully cloned. The transformant for isolate 6 (labelled 10) was tested by PCR, and the recombinant plasmid was extracted from the positive clone (Figure 2b). Then, the presence of 1.6 kb amplicon verified the presence of 1.5 kb insert in the plasmid plus the vector priming site (Figure 2c). For isolate 7 (labelled 10.3), the purified PCR product was sequenced directly with 16S rRNA primers and further primer walked to get the complete sequence.

The top five hits for each 16S rRNA sequence of

isolate 6 and 7 is shown in Table 2. The results showed that both sequences have high similarity (99%) with *Staphylococcus* species, and the highest score were with that of *Staphylococcus piscifermentans*. Other than common group lactobacilli and pediococci, *Staphylococcus piscifermentans* sp. nov was also originally reported to be found in fermented fish product and soy sauce in Thailand (Tanasupawat *et al*., 1992). However, the presence of non-motile, non-spore forming *Staphylococcus piscifermentans* is rather unique as this strain is newly recognized species. This coagulase negative species is phylogenetically and biochemically most closely related to *Staphylococcus carnosus* subsp. *carnosus*, *Staphylococcus carnosus* subsp. *utilis* and *Staphylococcus condiment* (Schleifer and Fischer 1982; Probst *et al*., 1998; Pantucek *et al*., 1999). This strain was also found in healthy dog feces, which was believed to have originated from fermented food residue presented in dog feed (Štetina *et al*., 2005). Nevertheless, there has been very little report on this species in food for the past decade (Probst *et al*., 1998). The strain alone, or in combination with other strains can be used as bacterial starter culture in food fermentation.

Conclusions

Several strains of lactic acid bacteria were successfully isolated from traditional Malaysian fermented shrimp samples. Being Gram positive cocci, they are positive on catalase and lactose utilization tests. Meanwhile, two of these isolates (6 and 7) were confirmed belonged to the rare group *Staphylococcus piscifermentans* of which this strain was originally shown to be found in fermented fish and soy sauce products in Thailand. In this work, *Staphylococcus piscifermentans* strain was isolated from cincaluk, a traditional fermented shrimp used in Malay dishes. These strain is however different from the previously isolated strains found in related works on fermented shrimp product in Malaysia or elsewhere. Nevertheless, these strains have huge potential for the use in food processing and preservation.

Acknowledgement

The authors wish to thank International Islamic University Malaysia (IIUM) for financial assistance in this work (IIUM Endowment Fund EDW BO905-282).

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