Screening and Identication of Lactic Acid Bacteria with Antimicrobial Activity Against Food-Borne Pathogen

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Abstract

This study focused on screening and identification of lactic acid bacteria with antimicrobial activity against *Escherichia coli* from milk samples. There were 250 isolates, showing different antimicrobial activities. Five isolates with predominant anti-*E. coli* efficiency were chosen for the study of bacteria identification, bacterial growth as well as the stability in pH change, heating and proteolytic treatment. The sequences of 16s rDNA indicated that selected isolates were *Lactobacillus* sp.. The antimicrobial efficiency, described in the term of Antimicrobial Index (AI) ranged from 0.63 to 0.84. However, the anti-*E coli* activity did not change when performing the test under proteinase-treated condition and high temperature-treated condition. Conversely, the pH adjustment of cell-free bacterial culture to 7.0 demolished their antimicrobial activity. This data implied that their antimicrobial activities were results of acidic condition, produced by lactic acid bacteria. From this study, the selected lactic acid bacteria showed their potential as a starter culture for fermented foods.

Keywords: Lactic Acid Bacteria; Antimicrobial Activity; Food-borne Pathogen

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1. Introduction

Microbial contamination in food is a major concern worldwide. It not only causes economic losses in food industry, but also leads to the foodborne illness problems in public health. (Zhang *et al*., 2014) It has been estimated that about one-third of world's food production is lost from food spoilage each year (Castellano *et al*., 2008) Likewise, it has been reported that as many as 30% of the world population suffered from food-borne diseases annually (Sara, 2004) In this regard, more than 90% of the cases, suffering from food-borne diseases, are caused by common pathogenic bacteria, such as *Salmonella* spp., *Listeria monocytogenes*, *Staphylococcus aureus*, *Vibrio parahaemolyticus* and *Escherichia coli* (Sokovic *et al*., 2012) Despite there are a lot of attempts to develop synthetic antimicrobial agents to prevent the growth of microorganisms in foods; a lot of adverse effects on human health have been reported so far (Fleming-Jones and Smith, 2003)

However, one of the possible ways to control the growth of undesirable microorganisms in food preservation technique is the use of protective cultures for food fermentation. Lactic acid bacteria (LAB) are recognized as a group of bio-preservative bacteria. They have long been used in spontaneous food fermentations, such as dairy, fish, meat and vegetable for centuries (Chagnaud *et al*., 2001) They have helped to improve flavor, texture and prolong food-shelf life. Moreover, they own ability to produce antimicrobial substances, for example, organic acids, hydrogen peroxide, ethanol, diacetyl and bacteriocin (El-Shafei *et al*., 2000; Sung-Mee and Im, 2009) For these reasons, the harmless LAB has drawn a lot of attentions as bio preservative agents. Several studies have addressed the study on isolation of LAB from dairy, meat, fish, products and fermented foods, but studies on LAB isolated from raw milk sample are few.

Therefore, two main objectives of this study were i) to screen and identify lactic acid bacteria with inhibiting effect to the growth of food-borne pathogens; *Escherichia coli* and ii) to study the nature of inhibiting agent, produced from lactic acid bacteria. The knowledge gained from this study would beneficially provide the potential starter culture to be used in food fermentation and characteristic details on their inhibiting agents.

2. Materials and Methods

2.1 Screening for Lactic Acid Bacteria

Sixty raw milk samples were collected in Kalasin area. Serial dilutions of samples with MRS broth were carried out and spread-plated onto MRS agar, containing 1% $CaCO_3$.

The plates were incubated anaerobically at 37°C for 48 hours. Colonies forming clear zone in the MRS agar were selected to store at -20°C in MRS broth with 30% glycerol. Gram staining and catalase production test were performed to identify the nature of LAB. Selected lactic acid bacteria were stored at -20°C in MRS broth with 30% glycerol for further study.

2.2 Antibacterial activity of Lactic acid bacteria against *E. coli*

The agar-disc diffusion technique was performed to test for the antibacterial activity against *E. coli*. Selected lactic acid bacteria were cultivated in MRS broth for 24 hours. Cell-free culture of bacterial suspension was obtained by centrifugation and filtration through 0.45 μm pore-size filter. The aliquot of 10 μl of cell-free culture was dropped on sterile blank discs with 6 mm diameter. The disc was individually placed on NA agar plates, covered with 100 μl of the indicator strain. The plates were incubated at 37°C for 24 hours. Cell-free cultures showing inhibition zone were chosen for the study of the nature of inhibiting agents. The determination of antimicrobial property was done in triplicate and evaluated by measuring the diameter of inhibition zone of each cell-free culture against the indicator strains in millimeter (mm). The efficiency of anti-*E. coli* activity was reported as a ratio of Antimicrobial index (AI), described in equation below. The "Da" and "Db" were the diameter of inhibition zone of cell-free culture and paper disc against the indicator strain, respectively.

$$\text{Antimicrobial index (AI)} = \frac{Da - Db}{Db}$$

2.3 Study of the nature of inhibiting agents

2.3.1 Effects of pH

Selected cell-free cultures were normalized to pH 7.0 by 1 M NaOH and kept at room temperature for 4 hours. The remaining inhibitory activity was assayed as mentioned above.

2.3.2 Effects of proteolytic enzyme treatment

Selected cell-free cultures were incubated with proteinase K at the final concentration of 1 mg/ml for 12 hours. The proteinase K-treated cultures were heated at 100°C for 3 minutes to inactivate the proteolytic enzyme. The remaining inhibitory activity was assayed as mentioned above.

2.3.3 Effects of high temperature treatment

Selected cell-free cultures were incubated at 50 and 100°C for 10 min. After cooling down, the high temperature-treated cultures were tested for the remaining inhibitory activity as mentioned above.

2.4 The growth curve of LAB isolates

An overnight culture of selected LAB was inoculated into 100 ml MRS broth and incubated at 37°C. Samples were taken every 12 hours to measure optical density (OD 600 nm), pH and antimicrobial activity.

2.5 Identication of lactic acid bacteria by 16S rDNA sequencing

Species of LAB with dominant antibacterial activity were identified by using the sequence of 16s rDNA. The 16s rDNA region was amplified by using two primers, 20F (5'-GAG TTT GAT CCT GGC TCA G-3') and 1500R (5'-GTT ACC TTG TTA CGA CTT-3'). The purified PCR products were checked by 0.8% (w/v) agarose gel electrophoresis and purified with a GenepHlowTM Kit (Geneaid Biotech Ltd., Taiwan). The purified PCR products were performed on an ABI Primers® 3730XL DNA Sequence by sequencing service provider. Sequence analysis was performed by BLASTN, available online at the National Center for Biotechnology Information, NCBI.

3. Results and Discussion

Sixty raw milk samples were collected from Kalasin University dairy farm and private dairy farms in Kalasin area. The samples were screened for lactic acid bacteria. Total bacterial count from samples ranges from 107-108 CFU/ml. After screening by MRS media selection, there were 250 bacterial isolates, showing clear zones on MRS agar, supplemented with 1% CaCO3. These isolates were subsequently chosen for anti-*E. coli* test by an agar disc diffusion method. Though, the amount of isolates found from raw cow milk samples differed from that found from raw goat and camel milk (59 and 352 isolates, respectively) (Saidi *et al*., 2011 and Jans *et al*., 2012). This difference might be due to the factor of environmental condition such as climate, season and temperature. Apart from the environmental factors, types and ages of samples also influenced for the amount and diversity of lactic acid bacteria (Kwantrairat *et al*., 2009)

Results of the screening for the inhibition activity of cell-free cultures showed that each cell-free culture possessed different degree of anti-*E. coli* efficiency. To obtain LAB with dominant antimicrobial activity, five cell-free cultures which showed leading inhibitory efficiency to indicator strain were chosen for gram staining and catalase producing tests. The result showed that they were all gram positive and catalase-negative bacteria. These five chosen isolates included A27, A29, A39, B29 and C35 (Table 1). The ratio of Antimicrobial index (AI) was used to compare the anti-*E. coli* efficiency of

each isolate. It was found that the AI ratio ranged from 0.63-0.84.

To identify the nature of antimicrobial activity, produced from each strain, cell-free cultures of the five isolates were tested for their anti-*E. coli* properties under different conditions. The results demonstrated that the AI ratios of cell-free cultures were in about the same range among normal condition (0.63-0.84), proteinase K-treated condition (0.62-0.68) and high temperature-treated condition (0.65-0.77 for 50°C-treated condition and 0.61-0.82 for 100°C-treated condition). This data indicated that proteolytic enzymes and heating had no impact on the anti-*E.coli* activity. In contrast, the ratio of AI could not be observed when performing the test under the condition of pH change. AI ratios of all selected strains were 0 when the cell-free cultures were neutralized to pH 7.0. The loss of antimicrobial activity after neutralization suggested that the anti-*E. coli* activity was a result of acidic condition, produced by lactic acid bacteria.

Table 1 Antimicrobial index of cell-free cultures from selected lactic acid bacteria

Strain	Condition				
	Normal	pH adjustment to 7.0	Proteinase K treatment	High temperature treatment	
				(50°C)	(100°C)
A27	0.70	0	0.63	0.77	0.76
A29	0.84	0	0.62	0.72	0.72
A39	0.83	0	0.68	0.79	0.82
B29	0.80	0	0.67	0.73	0.70
C35	0.63	0	0.67	0.65	0.61

Monitoring the antimicrobial activity of these 5 isolates additionally indicated that the efficiency of anti-*E. coli* related to the growth-associated patterns. The growth curve of isolate A29 was given as an example and shown in Figure 1. The maximum optical density (OD 600 nm) was observed at the hour of 24 after inoculation and then remained constant. This pattern was similar to the pattern of inhibition zone diameter. Following pH changes of cell-free culture, it was found that the pH of cell-free culture dropped from 5.6 to 4.2 within 24 hours of inoculation, leading the inhibition zone to the highest. After 24 hours of inoculation, the pH of cell-free cultures stayed the same which also in agreement of the inhibition zone diameter. It was found that the pH of

cell-free cultures of another 4 isolates were in the same pattern as the isolate A29. Their pH values of cell-free culture decreased from about pH 5.6 to be in the range of 4.2-4.3 in 24 hours and remained the same since that. The decreasing in the pH value of cell-free cultures within 24 hours led their inhibition zones to the highest which were similar to that of A29. These results could be explained that the antimicrobial property might be from the acidic condition, produced by lactic acid bacteria or the inhibiting agents which were sensitive to pH change. The result implied that the anti-*E. coli* activity, found in this study might not come from bacteriocin because bacteriocin is protein which could normally be denatured by proteolytic treatment and heating. A similar result had been reported from lactic acid bacteria, isolated from gastrointestinal tract of Nile tilapia (*Oreochromis niloticus*) (Kwantrairat et al.,2009) The authors found that acidic condition was believed to play a vital role in inhibiting fish pathogen for lactic acid bacteria, isolated from Nile tilapia. Besides acidic condition produced by LAB, some isolated lactic acid bacteria with other antimicrobial agents, had been reported so far (Saidi et al., 2011; Petsuriyawong and Khunnajark, 2010; Khunnajark *et al*., 2008; Todorov *et al*., 2006)

The sequence of 16s rDNA of these 5 isolates indicated that 4 out of 5 isolates were identified as *Lactobacillus rhamnosus* and the other was *Lactobacillus paracasei* subsp. *tolerans*. To compare the results of antimicrobial activity in this study to others was difficult. It was because the difference in LAB found from samples, the type of indicator strain used and the resistance to antibiotics. These factors played important roles in antimicrobial activity. Although, these isolates did not produce bacteriocin which is one of the most powerful antimicrobial agents from nature, they still could be good candidates to be used as starter culture in food fermentations as a result of their antimicrobial properties.

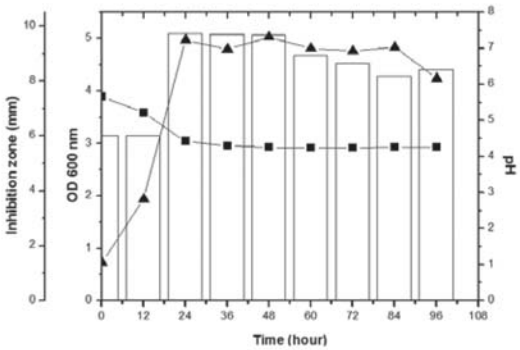

Figure 1 Cell growth (▲, OD 600 nm), pH (■) and inhibition zone (bar) of LAB isolated A29, incubated at 37°C

4. Conclusions

The lactic acid bacteria, isolated from raw milk samples, exhibited different anti-*E. coli* activities. The AI ratio against *E. coli* was in the range of 0.63-0.84. Their anti-*E.*

coli abilities might be from the acidic condition, produced by lactic acid bacteria. These lactic acid bacteria were able to be applied as starter cultures in food fermentations.

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