Techno-Science Research Journal 2(2014) 36-39

Content list available at ITC

Techno-Science Research Journal

Journal Homepage: www.

Techno-Science Research Journal

Isolation and Characterization of Lactic Acid Bacteria in Four Different Kinds of Fermented Vegetables Sold in the Markets

Reasmey Tan[1*], Soksreymeng Sdok[1], Len Sena[1], Sreynang Eang[1], Sivgech Chan[1], Sunly Heng[1], Kazuhiko Miyanaga[2], Yasunori Tanji[2]

[1] *Department of Chemical Engineering and Food Technology, Institute of Technology of Cambodia, Russian Ferderation Blvd., P.O. Box 86, Phnom Penh, Cambodia*

[2] *Department of Bioengineering, Tokyo Institute of Technology, 4259 J2-15 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan*

Abstract - *In Cambodia, the fermentation of different kinds of vegetables such as cucumber, Cambodian melon, green mustard and cabbage uses spontaneous fermentation process by which sugars are converted into lactic acid by lactic acid bacteria (LAB) naturally present on the skin of vegetables.Eventhough LAB were used, the type of LAB might be different for each vegetable. The types of LAB in these fermented vegetables have not yet been investigated so far. Therefore, the objective of this study was to isolate LAB in four different kinds of fermented vegetables sold in Cambodian markets and then to identify the isolated LAB by sequencing. Prior to sequencing, polymerase chain reaction and restriction fragment length polymorphism were first conducted to amplify the target DNA and grouping the bacteria into different groups, respectively. Among 16 groups of LAB, 15 were identified to be* Lactobacillus *and another one was* Pediococcus*. They were Gram-postive and catatase-negative. In addition, different kinds of LABwere found in each fermented vegetable.*

Keywords: Lactic acid fermentation; Lactic acid bacteria; MRS agar; Polymerase chain reaction; Sequencing.

1. INTRODUCTION

Vegetable fermentation is normally lactic acid fermentation processby which sugars are converted into lactic acid by lactic acid bacteria (LAB) in brine solution under anaerobic condition (Nguyen et *al.*, 2013, Xiong et *al.*, 2012).In Cambodia, different kinds of vegetables are being used for this spontaneous fermentation. It means that LAB used in this process are the bacteria that are naturally present on the skin of vegetable (Holzapfel, 2002). Although they use the same lactic acid fermentation process but the type of lactic acid bacteria might be different because the raw materials used are different. The types of LAB in diferent fermented vegetables sold in the Cambodian markets have not yet investigated. It is also interesting to learn how molecular biology works in order to identify the bacteria.

The objective of this research wastherefore to isolate LAB in four different kinds of fermented vegetablessold in the markets including fermented small cucumber, Cambodian melon, green mustard and cabbage, and then to identify the isolated LAB by sequencing.

2. METHODOLOGY

2.1 Isolation of lactic acid bacteria

For this study, four kinds of fermented vegetables were chosen as shown in Figure 1. To isolate LAB, the fermented liquid samples were first collected from the markets and then serially diluted. After spreading on

[*] Coresponding authors:
E-mail: rtan@itc.edu.kh

lactobacillu MRS agar(Nguyen et *al.*, 2013).the samples were incubated at 37°C for 48h. After 48h, more than 30 single colonies were picked up for each kind of vegetable and then purified.

Figure 1. Fermented vegetables:(a) small cucumber, (b) Cambodian melon, (c) green mustard and (d) cabbage.

The composition of MRS agar used per 1 litreis as follows:5g polypeptone, 5g meat extract, 2.5g yeast extract, 10g D(+)-glucose, 1g potassium hydrogen phosphate, 0.5g tween 80, 1g di-ammonium hydrogen citrate, 0.25g sodium acetate, 0.29g magnesium sulfateheptahydrate, 0.35g manganese sulfate pentahydrate, and 15g agar.

2.2 Gram-staining and catalase test

The purified colonies obtained from different kinds of fermented liquid samples were first tested for their Gram-staning and catalase. If they are LAB, they should be Gram-positive and catalase-negative. After these two tests, only 30 colonies for each vegetable (120 colonies in total) were selected for the next processes.

2.3 Polymerase chain reaction (PCR)

To identify the name of each bacteria, sequencing should be conducted. Before sequencing, the PCR should be done using the overnight pure culture. PCR is a biochemical technology in molecular biology used to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. In this study, the DNA amplification by PCR was conducted using primers 27F(5'−AGAGTTTGATCMTGGCTCAG−3') and 1492R (5'−CGGTTACCTTGTTACGACTT−3')with the expected DNA size of 1.5kbp(Yan et *al.*, 2008). PCR volumes of 25µl contained 2.5µl 10×KOD-plus neo buffer, 2.5µl dNTPs (2mM), 1.5µl $MgSO_4$ (25mM), 0.75µl forward primer 27F (10µM), 0.75µl reverse primer 1492R (10µM), 1µl DNA template (overnight culture),15.5µl free-bacterial water, and 0.5µl KOD-plus enzyme(1U/µl). A simplePCR was done using a TaKaRa thermocycler(Takara Bio Inc., Shiga, Japan). The initialization step was started at 94°C for 2 min, following by an initial denaturing step at94°C for 10sec. The annealing temperaturewas at 49°C for 30sec and an extension at 68°C for 30sec (25 cycles), followed by a final extension at 68°C for 10 min andcooling to 4°C. The size of target DNA was then checked by gel electrophoresis using 1% agarose gel.

2.4 Restriction fragment length polymorphism (RFLP) and sequencing

In molecular biology, RFLP is a technique that exploits variations in homologous DNA sequences. It refers to a difference between samples of homologousDNA molecules that come from differing locations of restriction enzyme sites. In RFLP analysis, the DNA sample is broken into pieces (digested) by restriction enzymes and the resulting restriction fragments are separated according to their lengths by gel electrophoresis.The DNA of each sample after PCR was then cut by 2 different enzymes (HhaI and MspI) in order to group the 120 bacteria into different groups (Yan et *al.*, 2008). After grouping, the PCR products of each bacteria were purified by phenol/chloroform method, diluted, and the primers (27F and 1492R) were added separately in each PCR tube and then sent for sequencing at Biocenter of Tokyo Institute of Technology.

3. RESULTS AND DISCUSSION

The image of gel electrophoresis of PCR products of different bacterial samples is shown in Figure 2. By using the primer sets 27F-1492R, the DNA size to be amplified should be 1.5kbp by comparing with DNA λ marker with known DNA size and only one single band should be obtained.

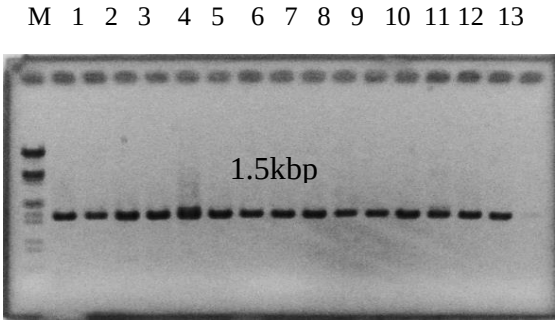

Figure 2. 1% gel electrophoresis of PCR products (M: DNA λ marker with known DNA size).

The PCR products that gave only single band were cut by 2 different enzymes (HhaI and MspI) in order to classify which bacteria are the same or different. When the band patterns of DNA were the same, they were classified into only one group.

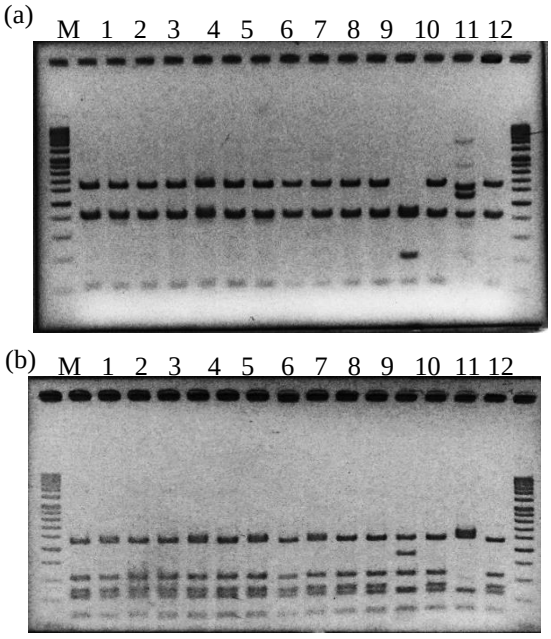

Figure 3. 2% gel electrophoresis after RFLP by two enzymes: (a) HhaI and (b) MspI (M: DNA ladder marker with known DNA size).

From RFLP results, the bacteria were then classified into 16 groups. Among these 16 bacteria, 5 different bacteria were identify in fermented small cucumber (sample A). And 4, 3 and 4 different bacteria were identify in fermented Cambodian melon (sample B), green mustard (sample C) and cabbage (sample D), respectively.In addition, all of them are Gram-positive and catalase-negative as indicated in Table 1.

Table 1. Gram-staining and catalase test.

Sample	Gram-staining	Catalase-test
A1	+	–
A2	+	–
A3	+	–
A4	+	–
A5	+	–
B1	+	–
B2	+	–
B3	+	–
B4	+	–
C1	+	–
C2	+	–
C3	+	–
D1	+	–
D2	+	–
D3	+	–
D4	+	–

Accordning to sequencing, these 16 bacteria were identified. Among them, 15 bacteria were Lactobacillus and another bacteria (A1) was Pediococuss. Eventhough these 15 bacteria were Lactobacillus, some of them have specific name such as *Lactobacillus fermentum*, *Lactobacillus planetarium*, *Lactobacillus delbrueckii* subsp. *Bulgaricus, Lactobacillus salivarius, Lactobacillus sp., Lactobacillus reuteri*.Similar results were obtained by Nguyen et *al.*, (2013). They found that the predominant LAB associated with mustard and beet fermentationand eggplant fermentation in Vietnam were identified as *Lactobacillus fermentum* (56.6%), *Lactobacillus pentosus* (24.4%) and *Lactobacillusplantarum* (17.1%). Less abundant species were *Pediococcus pentosaceus* (1.0%) and *Lactobacillus brevis*(0.5%).

4. CONCLUSIONS

In conclusion, among 120 colonies that were isolated from 4 different kinds of vegetables, only 16 bacteria were grouped according the RFLP results and these 16 bacteria were all Lactobacillus, except sample A1 which was Pediococcus accoding to sequencing results. Different kinds of vegetable used for fermentation, different kinds of LAB were found in be present.

ACKNOWLEDGMENTS

We are thankful to the ASEAN University Network-Southeast Asia Engineering Education Development Network (AUN/SEED-Net) for their financial support.

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