Isolation, identification and evaluation of Lactic acid synthesis of bacteria in traditional fermented products in Vietnam

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Abstract: Nowadays, many food products are promoted as being particularly healthy due to the characteristics of certain strains of lactic acid bacteria. Lactic acid bacteria (LAB) play a vital role in different fields, including food, pharmacy due to their benefit for young children, pregnant women and the elderly. LAB is one of the most significant groups of microorganisms used in food fermentations. They improve the flavour and texture of fermented foods. Moreover, LAB kills food spoilage bacteria by developing growth-inhibiting compounds. In this study, seven strains of LAB were isolated from traditional fermented products in VietNam including fermented rice, kimchi, and yoghurt. Moreover, LAB isolated was identified by amplification and sequencing of 16s rDNA gene. The isolated strains were Lactobacillus plantarum (M2 and M7), Lactobacillus paracasei (M4), Lactobacillus sakei (M10), Leuconostoc mesenteroides (M15), Lactobacillus curvatus (M17), Lactobacillus delbruekii sub sp. (M19). Cultural characteristics of M1 and M7 surface colony are 2mm wide, raised, smooth and white on MRS agar. The morphology of the colonies M4 and M10 is rounded, smooth, light yellow. Nevertheless, M4 and M10 are quite different in colour, white and light yellow, respectively. Moreover, M15 colonies are convex, with flat edges, smooth, semitransparent. M17 and M19 are small, normally rough and non-pigment. Acid lactic content produced by the isolated strains determined by spectral absorbance at 390 nm with FeCl₃ (0.2%), was highest in M2 strain (37.814g/l), followed by M17 (32.357g/l), M19 (27.336g/l), M4 (20.853g/l), M10 (9.408g/l), M7 (8.646g/l), and M15 (6.645g/l). In this study, the highest lactic acid content produced was M2 stain (Lactobacillus plantarum) and the lowest was M15 (Leuconostoc mesenteroides).

1. Introduction

Lactic acid bacteria (LAB) are Gram-positive, oxidase test and catalase test negative, spherical or rod-shaped, and non-spor forming bacteria [1]. LAB mainly presents in fermented products such as pickled cabbage, yogurt, fermented rice, or “Nem chua” (Vietnamese fermented pork roll) [2]. These bacteria are able to synthesis bacteria-inhibiting toxins (bacteriocin) such as lactin, sakacin, plantacin or helveticin hence acting antipathogens and food preservatives. LAB is the most significant category of micro-organisms that are used commercially to manufacture probiotic food. LAB has been in use in food fermentation and storage methods for decades. Although several probiotic strains were isolated and characterized, the search for more effective strains remains. The criteria for selecting probiotic strains include resistance to the enzymes in the oral cavity and antibiotic susceptibility. Other important properties to be considered during probiotic strain selection are the production of antimicrobial compounds because they provide competitiveness against pathogens and prevent or reduce their existence. Therefore, LAB is widely used in healthcare and veterinary applications, for instance health recovery, cancer prevention, improvement of immunity, and reduction of cholesterol [3-5]. Some potential benefits may result from the bacteria’s growth and activity during the production of cultivated...
foods. Several may result from the development and activity in the intestinal tract of some species of the lactic acid bacteria following ingestion of the foods containing them. In selecting a culture to produce a specific benefit, it is necessary to consider not only the wide variation between species of the lactic acid bacteria but also that between strains within a given species. Fermented food production is based on the use of starter cultures. New starter cultures of lactic acid bacteria are being produced recently with industrially significant functionality. Moreover, LAB is also used for waste treatment to reduce undesirable smells generated during waste degradation.

The annual growth of lactic acid consumption was estimated at 12-15%, and half of the acid lactic products are used in beverages and food products [6]. Lactic acid is also used for biodegradable polymers with great potential in solving energy and environmental issues [7-8]. In Vietnam, fermented products are usually produced on a manual scale at natural conditions. However, product quality is not stable. The scale of industrial production has not been paid attention. Therefore, it is necessary to consider carefully the factors that can affect the quality of fermented foods. It can create a consistent quality product after the fermentation process and initially guide product preservation. Therefore, LAB and lactic acid's potential application is deemed worthy of a study to evaluate lactic acid bacteria isolated and identified from fermented products for the selection of highly productive strains.

In this paper, we identified lactic acid bacteria based on CaCO₃ resolution on MRS agar medium. After, we performed amplification and sequencing of 16S rDNA region with a size of about 1000bp. The isolates we identified were identified by comparisons on Genbank and phylogenetic tree construction. Moreover, Acid lactic content was determined by spectral absorbance at 390 nm with FeCl₃ (0.2%). Figure 1 illustrated the step approach to carrying out a simulation study.

2. Material and methods

2.1 Fermenting products
The researched traditional fermented products included kimchi, fermented rice and yoghurt made by households living in District 6, HCMC, Vietnam (Figure 1).

![Fermented rice](image1)
![Yogurt domestically](image2)
![Kimchi](image3)

Figure 1. Traditional fermented products in Vietnam.

2.2 Experiments
Isolation: Lactic acid bacteria were isolated in MRS agar medium [9] supplemented with CaCO₃ 0.5%, the content included beef extract 10 g/L, protease peptone 10 g/L, yeast extract 5 g/L, glucose 10 g/L, tween 80 1 g/L, diammonium citrate 2 g/L, magnesium sulfate 0.1 g/L, sodium acetate 5 g/L, manganese sulfate 0.05 g/L, and dipotassium phosphate 2 g/L. The bacteria were cultivated in AnaeroPack – Anaero (MGC, Japan) anaerobic cultivation bag [9].

Sequencing and identification: The bacterial strains were identified following the procedures of The ISOLATE II Genomic DNA Kit – BIOLINE. PCR was done using the bacterial universal primers for amplification of 16S rDNA bacterial sequences, the forward 8F primer and the reverse 1391R primer were listed in table 1 [10]:

| Primer’s name | Primer sequence                |
|---------------|--------------------------------|
| 8F            | (Forward) 5‘-AGA GTT TGA TCC TGG CTC AG-3’ |
| 1391R         | (Reversed) 5’-GAC GGG CGG TGG TCT CA-3’   |
The PCR mixture included BiH₂O, PCR buffer 5X, 200 mM dNTP, 50 mM MgCl₂, 1U Taq, 10 pmol primer 8F, 10 pmol primer 1891R, 100 ng DNA. The PCR reaction was performed in the thermal cycle of: 94 °C (5 minutes), 30 cycles of 94 °C (1 minute), 60 °C (1 minute 30 second), 72 °C (2 minutes), and 72 °C (5 minutes) [10]. PCR products were examined by electrophoresis on agarose 1% gel, and then were purified, and sequenced by Sanger method (from both directions) by 1st BASE company (Malaysia).

Sequencing results were analyzed by the SeaView software version 5.04 and were compared against the sequences in GenBank database.

Acid lactic productivity: Isolated strains were cultivated in liquid MRS medium anaerobically at 28 °C in 48 hours for determination of lactic acid content. The culture liquid was centrifuged to harvest the liquid phase, and the acid lactic content in there was determined by spectral absorbance at 390 nm at the sample volume of 100 µl supplemented with 2mM FeCl₃ (0.2%) [11].

3. Results and discussions

3.1. Lactic acid bacteria isolation.

The dissolution of CaCO₃ detected lactic acid bacteria by lactic acid products on the agar medium (Figure 3). A total of 20 strains were isolated from three types of traditional fermented foods, including kimchi, fermented rice and yogurt, the resulted dissolution of CaCO₃ and colony morphology were presented in Table 2. Upon preliminary diagnosis, 7 out of 20 isolates LAB were gram-positive and capable of fermented glucose and releasing acid. The M2 and M4 strains were isolated from fermented rice, M7, M10, and M15 strains from kimchi, and M17 and M19 strains from yogurt. Cultural characteristics of M2 and M7 surface colony are 2mm wide, raised, smooth, and white on MRS Agar.

**Figure 2.** The sequential screening strategy flowchart developed in this study
M4 colony morphology is about 1 mm in diameter, rounded, smooth, and white. M10 colony morphology is the same as M4, but it’s light yellow. M15 colonies are convex, with flat edges, smooth, semitransparent. M17 and M19 are small, ordinarily rough, and non-pigment [13].

![Image of lactic acid bacteria isolated in agar plates.](image)

**Figure 3.** Lactic acid bacteria isolated in agar plates.

**Table 2.** The dissolution of \( \text{CaCO}_3 \) and Colony morphology of 20 sample. (Samples in bold format yielded positive result in dissolution of \( \text{CaCO}_3 \))

| Sample | The dissolution of \( \text{CaCO}_3 \) | Colony morphology |
|--------|----------------------------------|-------------------|
| M1     | -                                | Colony is round, usually golden yellow colonies. |
| M2     | +                                | Smooth colonies with relatively large size and milky color. |
| M3     | -                                | Round, color is glisten. |
| M4     | +                                | Uniformly circular, whiter, and small colonies. |
| M5     | -                                | Rough, opaque, fuzzy white with jagged edges. |
| M6     | -                                | Colonies are small, dry. |
| M7     | +                                | Smooth colonies with relatively large size and milky color |
| M8     | -                                | Transparent, small, flat and circular colonies |
| M9     | -                                | White round, opaque, flat, drying |
| M10    | +                                | Smooth, circular and golden yellow colonies |
| M11    | -                                | White round, flat, drying and smooth |
| M12    | -                                | Golden yellow round, flat and smooth |
| M13    | -                                | Rough, opaque, fuzzy slightly yellow with jagged edges |
| M14    | -                                | Large flat colonies wrinkled, sticky colonies |
| M15    | +                                | Colonies are convex, with flat edges, smooth, semitransparent. |
| M16    | -                                | Large, translucent, flat and filamentous colonies |
| M17    | +                                | Transparent, small, flat and circular colonies |
| M18    | -                                | Circular, flat, sticky, golden yellow colonies |
| M19    | +                                | Transparent, small, flat and circular colonies |
| M20    | -                                | Round and mucoid, color is glisten |
3.2. 16S rDNA sequence amplification, sequencing and identification.

![Gel electrophoresis of PCR amplification products from 7 samples isolated strains](image)

**Figure 4.** Gel electrophoresis of PCR amplification products from 7 samples isolated strains. The positive control was *Staphylococcus epidermidis*. The 16S rDNA amplification products had the size of 1000 bp.

**Figure 5.** The phylogenetic tree shows the seven isolates (M2-M19) genetic relationships from traditional fermented foods with the closest sequences identified by BLAST at GenBank. M2M19.

Neighbor-joining analyses were conducted using the MEGAX package [12]. The sequence of *Acetobacter cerevisiae* (AJ419843) served as the outgroup. Bootstrap values (expressed as percentages of 1,000 repetitions) > 90% are indicated at each node. Bar 0.020 substitutions per nucleotide position.
Figure 4 showed the 16S rDNA amplification products from 7 samples of isolated strains, which had a size of 1000 bp. Sequencing results were compared by BLAST N program against the sequences in the NCBI BLAST database. With the total 1000 sequenced nucleotides, seven isolated strain was identified as (with over 99% similarity) as Lactobacillus plantarum, (M2 and M7), Lactobacillus paracasei (M4), Lactobacillus sakei (M10), Leuconostoc mesenteroides (M15), Lactobacillus curvatus (M17), Lactobacillus delbrueckii subsp. Bulgaricus (M19) (Figure 5).

3.3. Acid lactic productivity.

Figure 6 illustrated the lactic acid production by seven LAB grown at 28°C in 48 hours. As can be seen from the Figure 6, the M2 strain yielded was the highest acid content (37.814 g/l), followed by M17 (32.357 g/l) and M19 (27.336 g/l), corresponding with the species of L. plantarum, L. curvatus, và L. delbrueckii subsp. Bulgaricus, which are amongst the most popular species in researches and applications [14-16]. The M15 strain yield achieved the lowest acid content (6.645 g/l), followed by M10 (9.408 g/l) and M7 (8.864 g/l) corresponding with the species of Leuconostoc mesenteroides, L. plantarum, and L. sakei.

![Figure 6. Acid lactic production by selected LAB grown at 28°C in 48 hours](image_url)

Reuben et al. (2019) isolated and selected six strains that belonged to the species of Lactobacillus reuteri, Pediococcus acidilactici, Pediococcus pentosaceus, and Enterococcus faecium, which were regarded as optimal for probiotics [17]. Hariri et al. (2016) isolated six strains of L. curvatus, Streptococcus diacetylactis, Streptococcus fecalis, Streptococcus mitis, and Streptococcus thermophilus from carrot syrup. The acid lactic productivity of L. curvatus cultivated in carrot syrup supplemented with 1% Tween 80 achieved 29.95g/L [18]. This suggests that the ability to synthesize lactic acid of the L. curvatus strain we isolated is higher. In the study of Major 1985 [19], Lactobacillus delbrueckii strain was able to give lactic acid content of about 8.93 g/l when cultivated continuously at 42 °C. This study showed that our Lactobacillus delbrueckii strain showed a higher ability to synthesize lactic acid at 27.336 g/l. Our L. plantarum isolates showed higher lactic acid synthesis than Yoshida’s experiment (74.2 g/l).

Yoon et al. study (2006) showed that the efficiency of lactic acid synthesis of Lactobacillus plantarum C3, Lactobacillus casei A4, and Lactobacillus delbrueckii D7 was about 22.99 g/l; 18.15 g/l; 22.385 g/l [20-21]. However, Lactobacillus delbrueckii in this paper was lower than Yoon. Xu research about acid lactic productivity of Lactobacillus paracasei was 31.5 g/l higher than our results (M4: 20.853 g/l) [22]. Lactobacillus paracasei subsp. Paracasei CHB2121 was about 192 g/l acid lactic [23], the content of acid lactic of M4 sample was lower than Moon’s published. In this experiment, we haven’t investigated and optimized the acid lactic productivity to secrete bacteria isolated. Leuconostoc mesenteroides was determined to create a bacteriocin, referred to as mesenterocin, which is active against Listeria monocytogenes strains [24]. Lactobacillus curvatus created a heat-stable bacteriocin, which was inhibited a broad spectrum of inhibitory activity [25]. Moreover, Lactobacillus species produce a variety of antimicrobial agents which vary in their inhibitory spectra, mode of action, and biochemical characteristics and the strain with the highest lactic acid synthesis that we recorded was...
Lactobacillus lactis BME5-18M (210g/l) [26] and the next one was Enterococcus munditii QU 25 (119g/l) [26].

4. Conclusion
This study managed to isolate six LAB strains from the species of Lactobacillus plantarum, Lactobacillus paracasei, Lactobacillus sakei, Leuconostoc mesenteroides, Lactobacillus curvatus, Lactobacillus delbrueckii sub sp và Staphylococcus epidermidis from fermented products. Highest lactic acid yield (37.814 g/l) was achieved for strain L. plantarum M2, followed by Lactobacillus curvatus M17 (32.357g/l), Lactobacillus delbrueckii sub M19 (27.336g/l), Lactobacillus paracasei M4 (20.853g/l), Lactobacillus sakei M10 (9.408g/l), Lactobacillus plantarum M7 (8.864g/l), and Leuconostoc mesenteroides M15 (6.645g/l). Further researches are advisable for lactic acid productivity assessment of isolated strain and optimization of lactic acid production from the potential candidates.

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