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Characterization of lactic acid bacteria isolated from pickled vegetables as potential starters for yogurt preparation

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ABSTRACT

The process of preliminary isolation and selection of lactic acid bacteria from Vietnamese traditional fermented vegetables is done based on (i) isolation and (ii) qualitative and quantitative analysis of lactic acid as the basis for the inspection and selection of lactic acid bacteria with strong antibacterial characteristics. The selected lactic acid bacteria were applied for yogurt fermentation with different bacterial densities (10^4 - 10^8 CFU/mL). The changes in pH value during fermentation time and sensory quality of product were evaluated. The research results showed that 48 strains were isolated from Vietnamese traditional fermented vegetables. Among them, 35 isolates (33 rod-shaped and 2 spherical-shaped) met the criteria of lactic acid bacteria. There were 15 isolates that were able to produce the highest amount of lactic acid, in which the rod-shaped bacteria showed better lactic acid producing capacity compared to the spherical-shaped ones. Four strains (S1.2, S5.5, S5.7, XK1.4) of these 15 strains that were tested for antimicrobial activity based on the agar spot test and agar well diffusion test, could produce bacteriocin against *Bacillus subtilis*. Based on the result of DNA sequencing, strains XK1.4 and S1.2 with strong antibacterial activity belonged to *Lactobacillus plantarum* (similarities 97% and 99%). The yogurt that was prepared by inoculating 10^8 CFU/mL of starter cultures (strains XK1.4 and S1.2) had the pH value of 4.5 after incubating for 7 hours, and achieved the good sensory quality.

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1 INTRODUCTION

Lactic acid bacteria (LAB), non-pathogen microorganisms have been commonly used for fermentation, food production and preservation. They are also considered as generally recognised as safe (GRAS) by United State Food and Drug Administration (FDA) (Carminati *et al.*, 2015). There is a group of related bacteria that produces lactic acid as a major metabolic product from carbohydrate metabolism (Holzapfel and Wood, 2012). Lactic

acid fermentation represents the easiest and the most suitable way for increasing the daily consumption of fresh-like vegetables and fruits (Di Cagno *et al.*, 2015). Lactic acid fermented vegetables present in many traditional products in many countries around the world (Di Cagno *et al.*, 2015), including Vietnam. The lactic acid bacteria convert sugar into lactic acid and create a special aroma/taste for pickled vegetable products (Kader, 2002). Lactic acid fermentation allows the preservation of fresh vegetables and improves the digest-

ibility and nutritional value of the food (Caplice and Fitzgerald, 1999; Parvez *et al.*, 2006). In addition, lactic acid bacteria have been reported to exhibit inhibitory activity towards spoiled microorganisms and support for digestion (Yusuf and Hamid, 2013). Applying of isolated LAB from fermented food for other purposes such as exopolysaccharides production (Adebayo-tayo and Onilude, 2008) or bacteriocin production (Mohammed and Ijah, 2013) were reported. Lactic acid fermented foods are essential in the diet for people in general and Vietnamese in particular. Therefore, this study which was aimed to isolate and identify of LAB from fermented pickled vegetables to apply for dairy fermentation (making yogurt), a very popular product in Vietnam, will contribute to the self-control of starter culture and quality assurance of the products.

2 MATERIALS AND METHODS

2.1 Sample preparation and isolation

Twelve pickled vegetables samples such as pickled cabbage, bath tubs, cucumber, bamboo shoots were collected from the Co.opmart supermarket (Xuan Khanh market, Can Tho city) and Nhon Phu market (Mang Thit district, Vinh Long province). The pickled vegetables (both liquid and solid portions) were blended lactic acid bacteria and proliferated by adding 10 mL of blended product in 90 mL of De Man-Rogosa-Sharpe (MRS) broth and then incubated at 37°C for 24 hours in a shaking incubator. This suspension was diluted with sterilized distilled water at the concentrations of 10^1 to 10^5 CFU/mL. Then 0.1 mL diluted suspension was spread on MRS agar plates and incubated at 37°C for 48 hours. The colonies were purified several times on the MRS agar plates until no difference in colony characteristics was observed on each isolate. Finally, purified strains were collected and selected based on their characteristics (Randolph *et al.*, 1998).

2.2 Assessment of lactic acid production, antimicrobial properties of isolated bacterial strains and applications for yogurt processing

The lactic bacteria strains (after preliminary identification, Gram staining, spore staining, oxidase, catalase and CaCO_3 resolution test) were proliferated in liquid MRS medium, after 24 - 48 hours, lactic acid production was characterized by the U-

ferment reagent and quantitative measurement by Therner acidity method. Selection of lactic acid bacteria was based on the highest of lactic acid production and the antimicrobial activity employed by agar spot test and well diffusion agar methods. DNA sequencing identification method was used to identify the selected bacteria strains based on 16S rRNA sequences and using primers 27F, then finding their sequences in the NCBI data bank using BLAST N software.

After testing the antibacterial activity, lactic acid bacteria were selected and applied for yogurt fermentation. The selected lactic acid bacteria were proliferated in liquid MRS medium for 24 hours. Then, these strains were proliferated in milk (about 12 hours) and then used for milk fermentation. Effect of bacterial densities (10^4 , 10^6 , 10^7 , 10^8 CFU/mL of milk) on pH value of yogurt was investigated during fermentation time. The sensory values of these final products were also evaluated by using quantitative descriptive analysis for the texture (homogeneity, smoothness, thickness), taste (sweetness, sourness) and odor parameters (creamy, acidic). The quantitative descriptive analysis was performed by a 20-member panel using a 5-score scale for description of the intensity/adequacy of selected parameters (1: untypically; 5: very typically expressed) (Karagül-Yüceer and Drake, 2013).

3 RESULTS AND DISCUSSIONS

3.1 Characteristics of bacteria

Forty-eight strains were isolated from samples collected (as mentioned above). Among them, 35 strains with colony characteristics were such as opaque white, smooth, convex, entire or lobed edge (Table 1). Colony diameter ranged from 2 to 4 mm after culturing on MRS agar at 37°C for 48 hours (Fig. 1). On MRS agar (supplemented with 0.05% bromocresol purple), the colonies of 35 out of 48 strains were opaque white or slightly yellow. There was a zone around the colony and color shifted from purple to yellow (Fig. 2) due to the change of pH from neutral to acid as a result of lactic acid production by colonies. Among 35 strains, there were 33 rod-shaped and 2 spherical-shaped strains (Fig. 3), Gram positive (Fig. 4), catalase negative (Fig. 5), oxidase negative (Fig. 6) and CaCO_3 resolved (Fig. 4).

Table 1: Colonies and cells morphology of isolated bacteria

No.	Bacteria strains	Colonies morphology				Cell morphology
		Color	Form	Margin	Elevation	
1	S 1.1	Opaque	Circular	Entire	Raised	Long rod-shaped
2	S 1.2	Opaque	Circular	Entire	Raised	Curved, rod-shaped
3	S 1.3	Opaque	Circular	Entire	Raised	Long rod-shaped
4	S 1.4	Opaque	Circular	Entire	Raised	Rod-shaped
5	S 1.5	Opaque	Circular	Entire	Raised	Long rod-shaped
6	S 2.1	Translucent	Circular	Lobate	Flat	Spherical-shaped
7	S 2.2	Opaque	Circular	Entire	Raised	Rod-shaped
8	S 3.1	Opaque	Circular	Lobate	Raised	Spherical-shaped, in pair or chains
9	S 3.2	Opaque	Circular	Lobate	Raised	Rod-shaped, in chains
10	S 4.1	Opaque	Circular	Entire	Flat	Rod-shaped
11	S 5.3	Opaque	Circular	Entire	Raised	Rod-shaped
12	S 5.5	Opaque	Circular	Entire	Raised	Long rod-shaped, curved
13	S 5.6	Opaque	Circular	Entire	Raised	Rod-shaped
14	S 5.7	Opaque	Circular	Entire	Raised	Rod-shaped, in pair
15	XK1.1	Opaque	Circular	Entire	Raised	Rod-shaped, curved
16	XK1.2	Opaque	Circular	Entire	Raised	Rod-shaped
17	XK1.3	Opaque	Circular	Entire	Raised	Rod-shaped
18	XK1.4	Opaque	Circular	Entire	Raised	Rod-shaped
19	XK2.1	Opaque	Circular	Entire	Raised	Rod-shaped
20	XK2.2	Opaque	Circular	Lobate	Raised	Rod-shaped
21	XK2.3	Opaque	Circular	Entire	Raised	Rod-shaped
22	XK2.4	Opaque	Circular	Entire	Flat	Rod-shaped
23	XK3.1	Opaque	Circular	Entire	Raised	Rod-shaped, curved
24	XK3.2	Opaque	Circular	Entire	Raised	Rod-shaped
25	XK4.1	Opaque	Circular	Entire	Raised	Long rod-shaped
26	XK4.2	Opaque	Circular	Entire	Raised	Long rod-shaped
27	XK4.3	Opaque	Circular	Entire	Flat	Long rod-shaped
28	XK5.1	Opaque	Circular	Entire	Raised	Rod-shaped
29	XK5.2	Opaque	Circular	Entire	Raised	Rod-shaped
30	XK5.3	Opaque	Circular	Lobate	Raised	Long rod-shaped
31	XK6.1	Opaque	Circular	Entire	Raised	Rod-shaped
32	XK6.2	Opaque	Circular	Entire	Raised	Rod-shaped
33	XK6.3	Opaque	Circular	Entire	Raised	Rod-shaped
34	DC2	Opaque	Circular	Entire	Flat	Long rod-shaped
35	DC3	Opaque	Circular	Entire	Raised	Long rod-shaped



(a)

(b)

Fig. 1: Colony of S1.3 (a) and XK2.4 (b) on MRS agar for 48 h

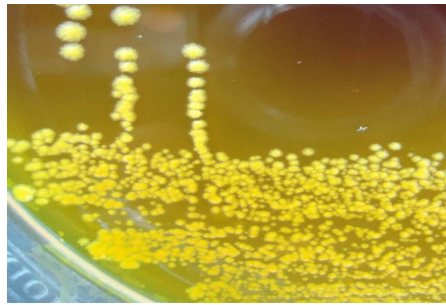
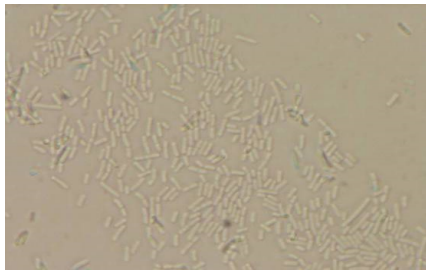
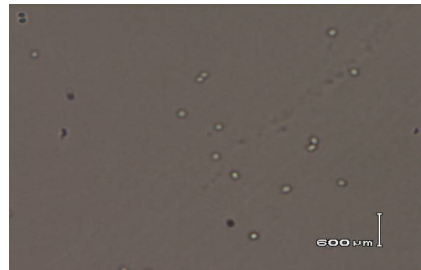


Fig. 2: Colony of XK1.4 on MRS agar supplemented with 0.05% bromocresol purple



(a)

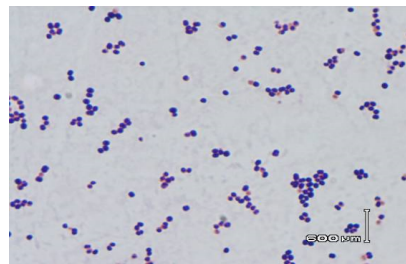


(b)

Fig. 3: Morphology of isolated bacterial strains in 1000 X zoom
(a) DC2 with long rod-shaped; (b) S2.1 with spherical-shaped



(a)



(b)

Fig. 4: Gram staining
(a) DC2 long rod-shaped; (b) S2.1 spherical-shaped

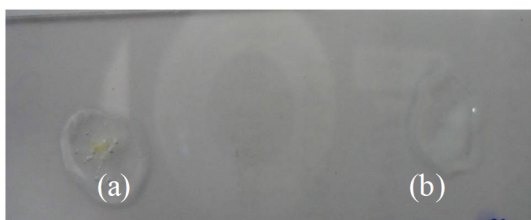


Fig. 5: Catalase test (a) S4.9: positive, (b) S5.3: negative

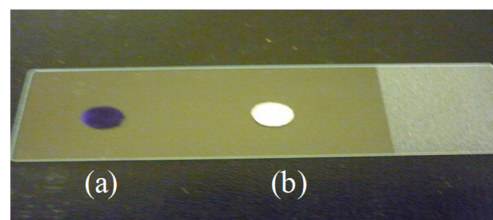


Fig. 6: Oxidase test (a) oxidase positive, (b) oxidase negative



Fig. 7: CaCO₃ resolution ability of S2.1

3.2 Lactic acid production ability of isolated bacterial strains

Bacteria were proliferated in MRS liquid for 3 days, then characterized by dropping 1mL of U-

ferment reagent. The results showed that 35 strains exhibited the lactic acid production ability causing the color change of U-ferment reagent from blue-violet to yellow (Fig. 8).



Fig. 8: Changes in the color of U-ferment reagent by *Lactobacillus* strains

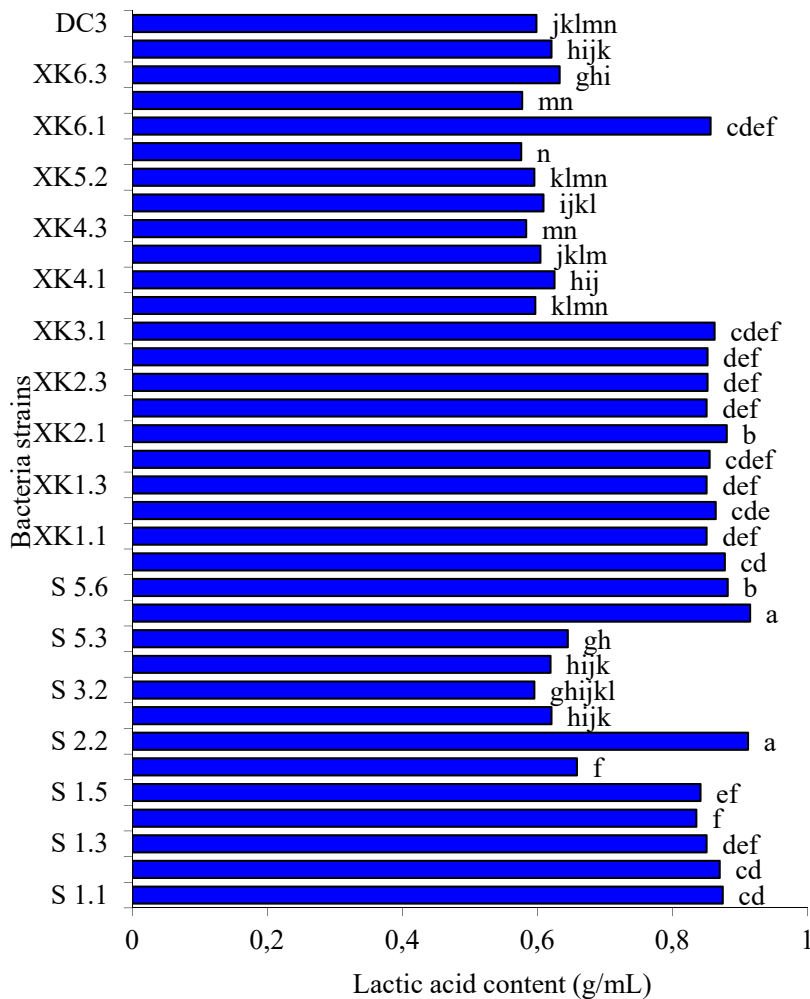


Fig. 9: The acid content produced by the bacterial strains

The result of quantitative acidity of isolated bacterial strains was done by Therner method (Emanuel *et al.*, 2005) and presented in Fig. 9. There were 15 lactic acid producing strains that were selected (from high to low), including S2.2, S5.5, S5.6, XK2.1, S5.7, S1.1, S1.2, XK1.2, XK3.1, XK6.1, XK1.4, XK2.3, XK2.4, XK1.1, XK1.3. They should be tested for the bacteriocin producing ability.

3.3 Antibacterial ability of the isolated bacterial strains

The results showed that 7 out of 15 strains had antibacterial activity by applying the agar spot test method (Table 2). The diameters of inhibition zone by lactic acid bacteria isolated were various. Among them, two strains of S 1.2 and XK 1.4 showed the zone of inhibition diameters larger than 10 mm, presenting a strong antibacterial activity; 5 strains XK1.2, S5.5, S5.6, S5.7, and XK3.1 showed an average antibacterial activity (9-10 mm) (Fig. 10).

In particular, the S1.2 strain illustrated the strongest antibacterial activity (20 mm) (Fig. 11). According to Ouwehand and Vesterlund (2004), lactic acid bacteria widely produce organic acid, an important antimicrobial substance, and other antimicrobial substance including hydrogen peroxide, carbon dioxide and diacetyl. A few strains produce specific antimicrobial substances such as reuterin, pyroglutamic acid. Thus, the strains that could produce high amount of antibacterial substances on MRS agar medium had high antimicrobial activity.

The results showed that most of isolated bacteria belonging to the genus *Lactobacillus* had antibacterial activity which was in agreement with reports of several authors. Some previous studies have also demonstrated that bacteria of the genus *Lactobacillus*

are antagonistic to harmful bacteria. It was reported that *Lactobacillus* that isolated from buffalo milk showed activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Shigella dysenteriae* and *Bacillus subtilis* (Barua *et al.*, 2014). The study indicated that all strains of *Lactobacillus* that could generate zone of inhibition with the diameter ranging from 15 to 28 mm.

Well diffusion agar test has been used to test the antibacterial activity of bacteriocin that is produced from bacteria of the genus *Lactobacillus*. The size of the sterile zone around the wells presented the strength or weakness of various antibacterial solutions. Among 15 tested strains, 6 strains showed the bright sterile zones with various sizes from large to small including: XK1.4, S5.7, XK3.1, XK6.1, S5.5, where strain XK1.4 had the strongest antibacterial activity with the sterile zone diameter of about 23 mm (Fig. 12).

Table 2: The diameters of inhibition zone of isolated strains against *Bacillus subtilis* using the agar spot test

No.	<i>Lactobacillus</i> strains	Diameters of inhibition zone*	Antibacterial activity**
1	S 1.2	20.00 ^a	++
2	S 5.5	10.33 ^c	++
3	S 5.6	10.00 ^{cd}	++
4	S 5.7	10.00 ^{cd}	++
5	XK 1.2	8.67 ^{cd}	++
6	XK 1.4	12.67 ^b	++
7	XK 3.1	10.00 ^{cd}	++
CV (%)			4,02%

Note: * Data of diameters of inhibition zone has been rounded; where ** (-) $x < 1$ mm, (+) $1 \leq x \leq 5$ mm, (++) $6 \leq x \leq 20$ mm. Data is mean value of 3 repetitions. Means followed by the same letter are not significantly different at the 5% level

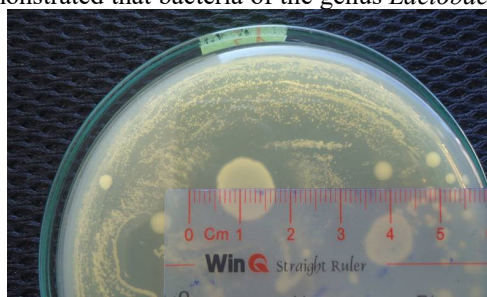


Fig. 10: S5.7 has an average antibacterial activity (10 mm)

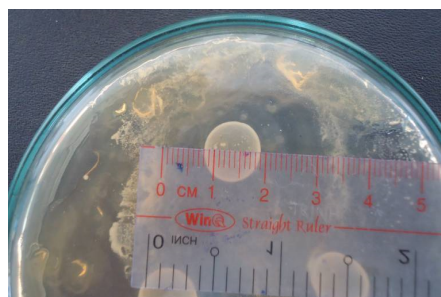


Fig. 11: S1.2 has a high antibacterial activity (20 mm)

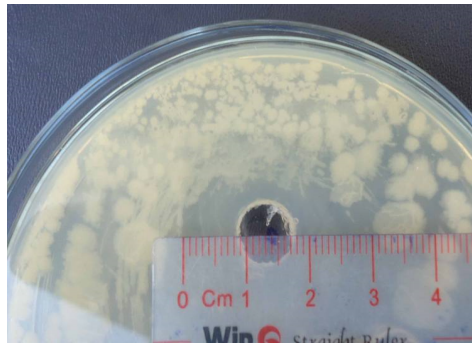


Fig. 12: Sterile zone from XK1.4 strain

The results of the agar spot test and well diffusion agar test indicated that 6 isolated *Lactobacillus spp.* strains expressed antibacterial activity against *Bacillus subtilis* by lactic acid, H₂O₂ and other bacteriocins.

3.4 Sequencing Results

The XK1.4 and S1.2 strains that produced bacteri

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5' ATGCGGCAGCAGCCAGATCGAAATGCGAGTTCGAACGAACTCTGGTATTGATTGGTGCTTGCA
TCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCAGAA-
GCGGGGATAACACCTGAAACAGATGCTAATACCGCATAACAACCTGGACCG-
CATGGTCCGAGTTTCAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGC-
TAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGG-
TAATCGGCCACATTGGGACTGAGACACGGCCAACTCCTACGGGAGGCAGCAG-
TAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAA-
GAAGGGTTTCGGCTCGTAAACTCTGTTGTTAAAGAAGAACATATCTGAGAG-
TAACTGTTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAG-
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCG-
CAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCG-
GAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTG-
TAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTG-
TAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAG-
TCCATACCGTAAACGATGAATGCTAAGTGGTTGGAGGGTTTCCGCCCTTCAGTGCTG-
CAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGCCG-
CAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAAGCGGTGGAG-
CATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTT-
GACATACTATGCAAATCTAAGAGATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTT-
GCATGGTTGTCGTCAGCTCGTGTGCGTGGAGATGTTGGGTAAAGTCCCGCAACGAGCG-
CAACCCTTATTATCAGTTGCCAGCATTAAAGTTGGGCACTCTGGGTGA-
GACTGCCGGTGACAAACCGGGAGGAAAGGTGGGGGATGACGTG-
CAAATCATCATCGCCCCTTAAGGACCTGGGCTACCACCGTGGCTAG-
GATGGAATCGGTCCACCCAGTTGCGGAATTCCGCAGAAGGAAGGCTAATCTCTTAAAA-
GCATTTCCAGTTTCCGATTTTATCGCTCGACTCCGCCCATGGGAGTCGGGGATCCCTTAA-
TATACTGGGAAATGTAGGCCGCGAGAAACACTCTCCCGCGCGCTTTGGGCCCTTGTACAG-
CACTTCCAGGAGGTTTTTCTCAACAACCAGGTGGGGGGAAAATATTATTAAGAAAAAC-
CAAACCTTGGAGTGGGGGGTTCATAAAGGGCAACGTCCCGCCCGGCCGAC-
CGGGCCCCCTGTGAACGGGAAACGGGAAAAATAGCAAAAAAAAAAAGTAAAC-
GACAAAAATAAACTAACAGAAAGAAAAAAAAAAGGGGGGGGGGG-
GAAAGGGGGGGGGGGGGGAAAAAAGGGGGGGGGGGGGGGGGGGGAAAAAAGGGGGGAAAA-
GAAAGGGGGGGGAAAAAAGGGGGAAAAAAGGGGGGGGGAAAAAAGGGGGGGGGGG-
GAAAAAATGGGGGAAAGGGGGAAAAAAGGGGGAAAAAAGGGCCCCAAAAAGGG-
GAAAAAATTTGCCA -3'
    
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ocin were selected for identification by DNA sequencing the 16S rRNA gene and using BLAST N software to find and compare the similarities with the sequences of the standard strains on NCBI gene bank. Result of DNA sequencing was as follows:

The sequence of the 16S rRNA gene of XK1.4 strain.

The sequence of the 16S rRNA gene of S1.2 strain

5'GAAGGCGGCGAACGCACGAAATAGCAGTCGAACGAACTCTGGTATTGATTGGTGCTTGCATC
 ATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCAGAA-
 GCGGGGATAACACCTGGAACAGATGCTAATACCGCATAACAACCTGGACCG-
 CATGGTCCGAGTTTAAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGC-
 TAGATGGTGGGGTAAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGG-
 TAATCGGCCACATTGGGACTGAGACACGGCCAAACTCCTACGGGAGGCAGCAG-
 TAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAA-
 GAAGGGTTTTCGGCTCGTAAAACCTCTGTTGTTAAAGAAGAACATATCTGAGAG-
 TAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAG-
 CAGCCCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCG-
 CAGGGCGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCG-
 GAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACCTCCATGTG-
 TAGCGGTGAAATGCGTAGATATATGGAAGAACCAGTGGCGAAGGCGGCTGTCTGGTCTG-
 TAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAG-
 TCCATACCGTAAACGATGAATGCTAAGTGTGGAGGGTTTTCCGCCCTTCAGTGCTG-
 CAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGGCCG-
 CAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAG-
 CATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACAAGGTCTTGAC-
 GTACTGTGCTAATCTAAGAGATCAGACGTTCCCCTCGGGGACTTGGATA-
 CAAGTGGTGCCTGTTTGACGACAGGTTTTTCGACGAGCCAAGCTCGGTTAACTCCCGCGAC-
 GAGGCTCACCACATAAATACTTTCGTCAAGATGAAAATACACCCACTACGGTGAGCCCCAC-
 GAAAGAATAATTGGGCCGGGACCTTCGGGGTTGAGGGGGGG -3'

From the obtained results, two strains of XK1.4 and S1.2 had the similarities with *Lactobacillus plantarum* NCBI 97% and 99%, respectively.

3.5 Application XK1.4 and S1.2 in milk fermentation

The two strains of XK1.4 and S1.2 were selected and applied for yogurt fermentation. Results showed that the rate of pH changing was influ-

enced by the density of starter cultures. The low density of starter cultures (10^4 CFU/mL) resulted in slowly decreasing of pH value (pH 5 for 7 hrs of incubation), whereas the fermented milk, prepared with 10^8 CFU/mL of starter cultures, was effective in dramatically decreasing in pH value and reaching the standardized yogurt pH (pH 4.2 to 4.6) within 7 hours of incubation (Fig. 13).

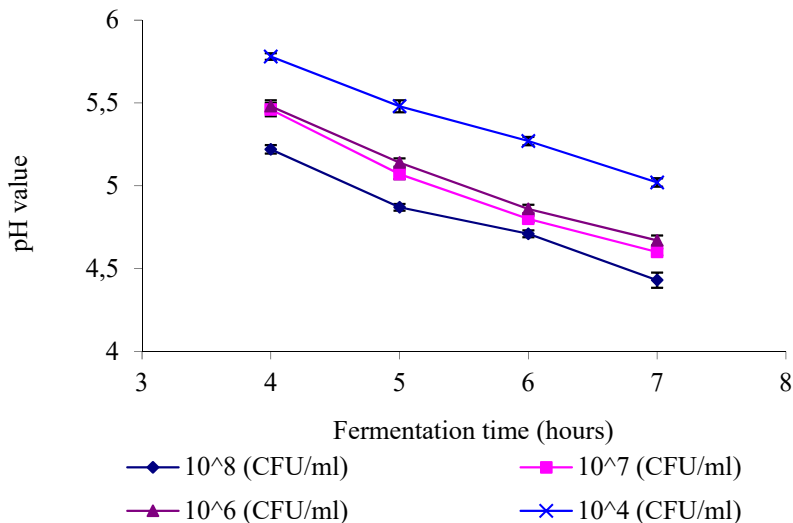


Fig. 13: Changes in pH of yogurts during fermentation depending on bacterial densities

The two strains could be used in lactic acid fermentation which results in good homogeneity and smoothness of yogurt. Amount of starter culture had significant influence on sensory quality of yogurt including the texture, taste and odor parameters (Fig. 14). The higher sourness, acidic odor and

thickness were found in the yogurts with higher starter's addition. The density of 10^8 CFU/mL and incubated time of 7 hours were appropriate for making yogurt with high sensory quality (Figure 15).

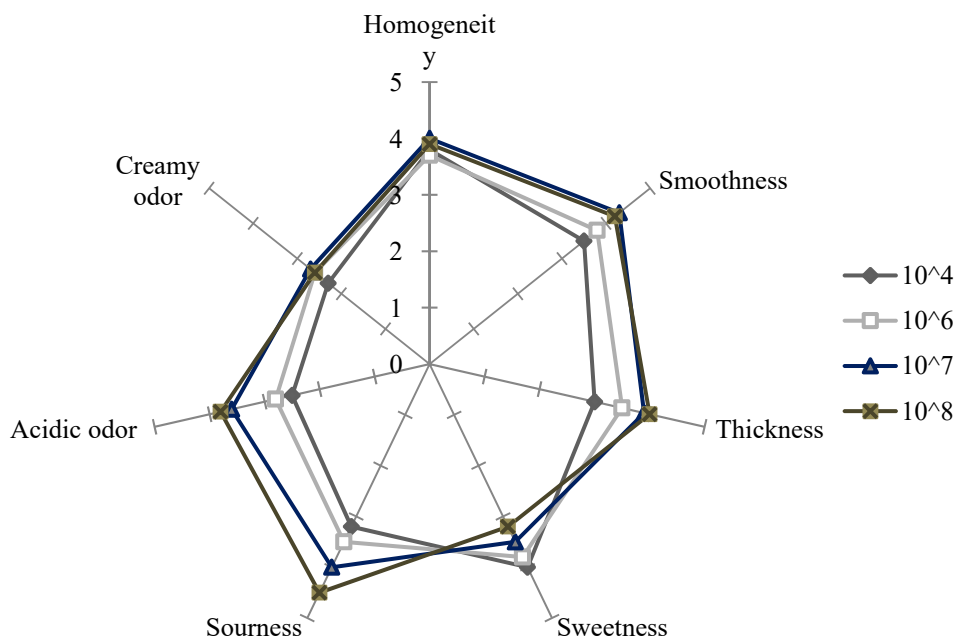


Fig. 14: Intensities of sensory attributes in yogurt fermented by combination of XK 1.4 and S 1.2 strains

(Note: 0: not present, 1: threshold, 2: slight, 3: moderate, 4: strong, 5: extremely strong)



Fig. 15: Yogurt fermented by combination of XK 1.4 and S 1.2 strains (bacteria density of 10⁸ CFU/mL and incubation for 7 hours)

4 CONCLUSIONS

Forty-eight bacterial strains were isolated from pickled vegetables. Among them, 15 isolates could produce high amount of lactic acid, in which strain XK1.4 was isolated from pickled cucumber reform (follow the traditional recipe) and strain S1.2 was isolated from pickled melon (Can Tho Co.opmart supermarket) showed the strongest antibacterial activity. Results of DNA sequencing showed that sequences of two strains of XK1.4 and S1.2 were similar to *Lactobacillus plantarum* (similarities 97% and 99%). The isolated lactic acid bacteria can be implicated in the yogurt preparation as starter culture. High sensory quality of yogurt can

be achieved by inoculating 10⁸CFU/mL of starter cultures and incubating for 7 hours.

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