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# Isolation and Identification High-Biological Activity Bacteria in Yogurt Quality Improvement

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***Abstract:*** *Six strains of lactic bacteria and seven strains of acetic bacteria which were isolated from fermented food. The screening secured one strain of lactic acid bacterium LB4 which carried strong probiotic activity and one strain of acetic acid bacterium AB7 which was capable to generate high glucuronic acid. Activities of probiotic and glucuronic acid were useful biological activities for health protection and enhancing. The results of DNA sequencing showed LB4 was similar to Lactobacillus acidophilus and AB7 was similar to Gluconacetobacter nataicola with 100% of similar proportion. Both of these bacteria were safe that could be used in food fermentation.*

***Keywords:*** *probiotic, glucuronic acid, Lactobacillus acidophilus, Gluconacetobacter nataicola.*

## I. Introduction

The biological activity of yogurt was considered about probiotic activity - the popular biological activity in fermented milk products by lactic acid bacteria. The effects of probiotic in fermented milk products have been known for a long time [1]. Some recent researches have showed that lactic acid bacteria could be combined simultaneously with acetic acid bacteria in micro-aerobic condition. This combination significantly increased the concentration of glucuronic acid which was biosynthesized by acetic acid bacteria [2]. Glucuronic acid contained high biological activity, useful effects on human health. It reduced cholesterol in blood, eliminated the oxidizing agents and excreted toxins in human body. Nowadays, glucuronic acid has also been studied in combination with fucose sulfate and manose sulfate in the structure of U-fucoidan that promoted the Apoptosis which controlled cancer cells self-destroying. The combination of lactic acid bacteria and acetic bacteria have been considered significantly in a number of recent studies [3,4].

With the good effects of probiotic bacteria and glucuronic acid for human health. In this study, many strains of lactic acid bacteria which has high active probiotic and many strains of acetic acid bacteria which has high active glucuronic were isolated for the purpose of contribution these strains in yogurt production that was added high activity of glucuronic acid activity besides traditional probiotic activity.

## II. Materials And Methods

### *2.1. Isolates and culture media*

Lactic acid bacteria isolated from kimchi, pickled cucumber, tofu water, pickled vegetable, “com me” - Vietnam traditional fermented cooked rice, and “nem” - traditional fermented meat. The culture medium to isolate and reserve lactic acid bacteria was Man Rogosa Sharpe agar (MRS).

Acetic acid bacteria isolated from fermented coconut juice, fermented grape juice, vinegar, tea Kombucha. The culture media to isolate and reserve acetic acid bacteria was Heschin-Schramm agar (HS) medium.

### *2.2. Antibacterial activities evaluation*

Probiotic activity is evaluated by antibacterial ability by agar spot test and well diffusion agar test.
Raw bacteriocin concentration of lactic acid bacteria (AU/mL) was determined by the formula:

$$AU/mL = D_{FI} \times 1 / V_{bacteriocin}$$ [5]

The ability to survive in harsh conditions of human gastro-intestinal system of lactic acid bacteria were evaluated in terms of pH 2 and 0.3% bile salts.

Evaluation of the intestinal harmful bacteria resistant by bacteriocin which produced by lactic acid bacteria.

### *2.3. Method of measuring concentration of glucuronic acid of acetic acid bacteria*

Glucuronic acid concentration was determined by K-Uronic acid kits and measured absorption at 340nm by UV-Vis spectro 6000 spectrophotometer [4].

***2.4. Method of identification of microorganisms***

Lactic acid bacteria and acetic bacteria that obtained high biological activity were identified by the method 16S rDNA sequencing procedure with the primers $^{NK}$16s-F and $^{NK}$16s-R which were supplied by Nam Khoa Biotek Company.

## III. Results And Discussion

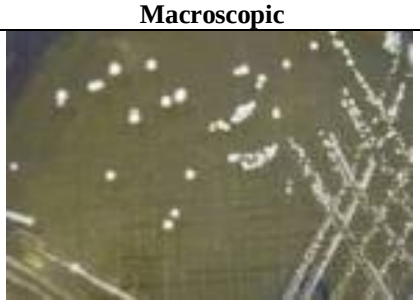
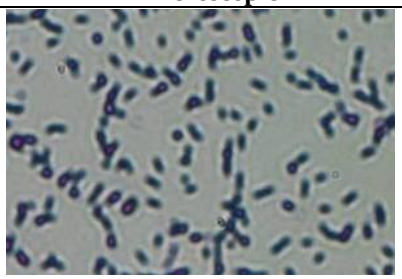
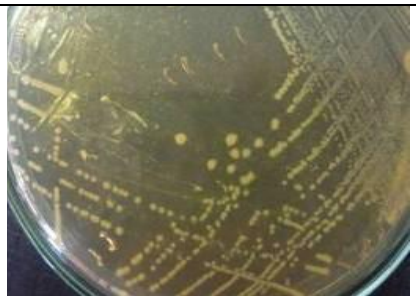
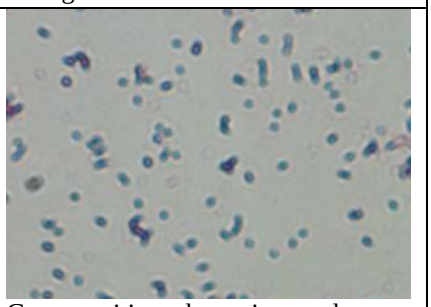
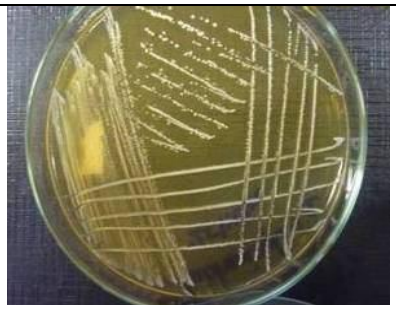
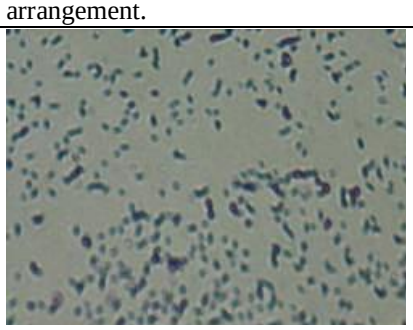
***3.1. Isolation and screening of lactic acid bacteria***

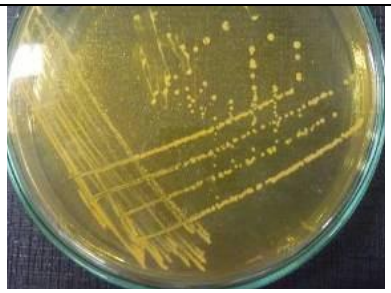
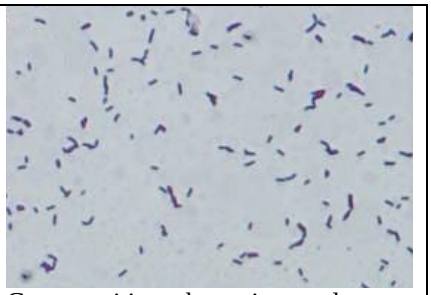
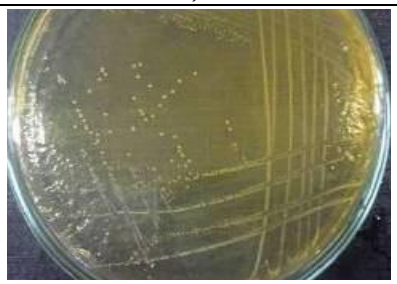
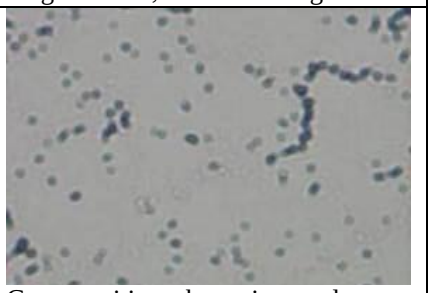
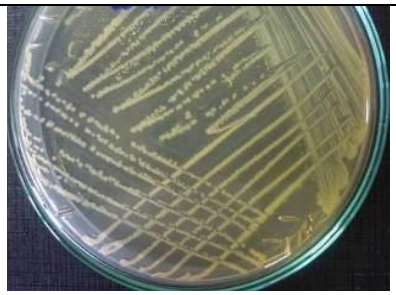
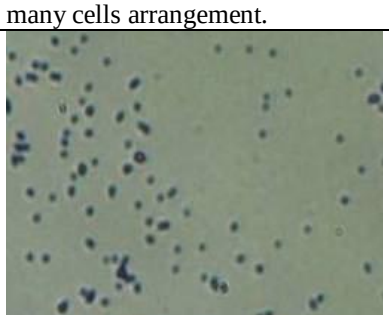
***3.1.1. Isolation of lactic acid bacteria***

Lactic acid bacteria were isolated based on characteristics such as negative catalase, negative oxidase and the change of reagent U-ferment color would be selected for screening. The strains included rod-shaped, non-spore and Gram-positive will be selected for probiotic activity evaluation.

There were 6 strains that appropriated requirements could be identified as lactic acid bacteria. These strains were described in Table 1:

**Table 1:** Characteristics of isolated lactic acid bacteria strains

| No. | Strain | Morphological Characteristics | |
|---|---|---|---|
| | | **Macroscopic** | **Microscopic** |
| 1 | LB1 |  Milky color, circular, convex and entire of colonies, smooth surface. |  Gram-positive, sedentary and short rod cells, discrete or small group arrangement. |
| 2 | LB2 |  Milky color, circular, convex and entire of colonies, smooth surface. |  Gram-positive bacteria, sedentary, spherical cells, single or double arrangement. |
| 3 | LB3 |  Milky color, circular, convex and entire of colonies, smooth surface. |  Gram-positive bacteria, sedentary, spherical cells, discrete or group of many cells arrangement. |

| | | | |
|---|---|---|---|
| 4 | LB4 |  Milky color, circular, convex and entire of colonies, smooth surface. |  Gram-positive bacteria, sedentary, long rod cells, discrete arrangement. |
| 5 | LB5 |  Milky color, circular, convex and entire of colonies, smooth surface. |  Gram-positive bacteria, sedentary, spherical cells, discrete or group of many cells arrangement. |
| 6 | LB6 |  Milky color, circular, convex and irregular of colonies, smooth surface. |  Gram-positive bacteria, sedentary, single spherical cells. |

***Note:*** LB1 was isolated from kimchi, LB2 was isolated from sour water of tofu, LB3 from pickled vegetable, LB4 from pickled cucumber, LB5 from fermented cooked rice, LB4 from meat fermented.

Thus, there were 6 strains of bacteria had been isolated and identified as lactic acid bacteria by biochemical tests and morphological test. These bacterial strains were screened to select strain of high probiotic activity.

### *3.1.2. Screening of lactic acid bacteria which secured high probiotic activity*

Six strains of isolated lactic acid bacteria were capable to resistant the bacteria indicator *Bacillus subtilis* (Table 2). Both of strains LB1 and LB4 obtained strong of antibacterial ability; the strain LB2 had average antibacterial ability; LB3, LB5 and LB6 secured weak antibacterial ability.

**Table 2:** The antibacterial property of strains lactic bacteria

| No. | Strain | Sterile loop diameter (mm) | Antibacterial activity | No. | Strain | Sterile loop diameter (mm) | Antibacterial activity |
|---|---|---|---|---|---|---|---|
| 1 | LB1 | 12 | +++ | 4 | **LB4** | 15 | +++ |
| 2 | LB2 | 7 | ++ | 5 | LB5 | 4 | + |
| 3 | LB3 | 4 | + | 6 | LB6 | 3 | + |

***Note:*** Sterile loop diameter > 10mm (+++): high antibacterial activity, 5-10mm (++): average antibacterial activity, <5 mm (+): weak antibacterial activity, 0mm (-): no antibacterial activity.

Thus, there were two strains which obtained high antibacterial ability were LB1 isolated from kimchi and LB4 was isolated from pickled cucumber. Two strains would be studied in bacteriocin production, ability to exist in low pH and high bile salts concentration.

The results about the ability to survive in harsh conditions of the digestive system and bacteriocin generation of LB1 and LB4 were illustrated in Table 3.

**Table 3:** The ability to exist in harsh conditions and bacteriocin production

| Bacteria strain | Bacteriocin concentration (AU/mL) | Proportion of survival (%) | |
|---|---|---|---|
| | | pH 2 | 0.3% bile salt |
| LB1 | 60 | 10.14 | 22.34 |
| **LB4** | **100** | **43.01** | **42.4** |

After incubating about 24 hours with these conditions at 37°C, LB4 synthesized highest bacteriocin which concentration bacteriocin was 100 AU/mL and the bacteriocin concentration of LB1 was 60 AU/mL. At the same time, the strain LB4 also showed the ability to survive in harsh conditions of the digestive system with a proportion was over 40%, higher than the result of LB1 in the same survey condition. Specifically, the ability to exist in pH 2 of LB4 was 43.01%, higher than the strain LB1 4 times (10.14%); survival ability in 0.3% bile salts was 42.4%, doubled the survival ability of LB1. Bacteriocin concentration was generated by LB4 strain equivalent the research of Ogunbanwo et al. (2003) and Sarika et al. (2010) [6,7]. The survival ability of this strain is also higher than the other lactic acid bacteria had been studied [2]. Therefore, LB4 could be confirmed as a powerful probiotic strain. However, the biological activity of this strain should be further confirmed by the ability to inhibit pathogenic bacteria commonly exist in the digestive system of the human.

Th results of inhibition of intestinal bacterial pathogenic by bacteriocin by lactic acid bacteria LB1 and LB4 discharged were presented in Table 4 with bacteriocin generation to resistant bacteria is indicated by diameter of inhibition loop.

**Table 4:** Results of inhibition of intestinal bacterial pathogenic

| Lactic acid bacteria strain | Diameter of inhibition loop (mm) | | | | | |
|---|---|---|---|---|---|---|
| | ***E.coli*** | ***Sal*** | ***Shi*** | ***Sta*** | ***Vib*** | ***Lis*** |
| LB1 | 9 | 4 | 3 | 0 | 0 | 0 |
| **LB4** | 14 | 9 | 10 | 2 | 3 | 7 |

***Note:*** *E.coli: Eschrichia coli, Sal: Salmonella,*
*Shi: Shigella, Sta: Staphylococcus aureus*
*Vib:Vibrio parahaemolyticus, Lis: Listeria monocytogenes*
Diameter of inhibition loop > 10mm: high of bacterial pathogenic ability,
Diameter of inhibition loop 5 - 10mm: average of bacterial pathogenic ability,
Diameter of inhibition loop <5 mm: low of bacterial pathogenic ability,
Diameter of inhibition loop 0mm: could not inhibit bacterial pathogenic ability.

The strain LB4 resisted more harmful bacteria than the strain LB1. The ability of harmful bacteria resistance of LB4 also showed that it was more sensitive with harmful bacteria than the results of Ouwehand et al. (2004) [40]. The lactic acid bacteria were isolated by Ouwehand et al. were almost only sensitive with *E. coli* and *L. monocytogenes* and some strains were sensitive with *S. aureus* and *Salmonella.*

Thus, the strain LB4 was highest about probiotic activity in six isolated strains, and this activity was also higher than the results of other studies as mentioned above. Therefore, the strain LB4 could be asserted as a strong probiotic activity strain.

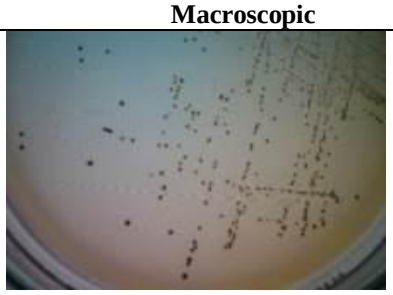
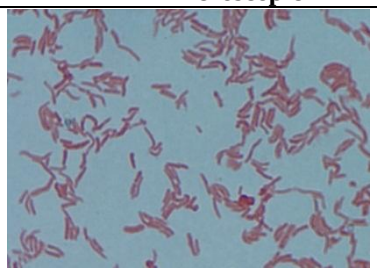
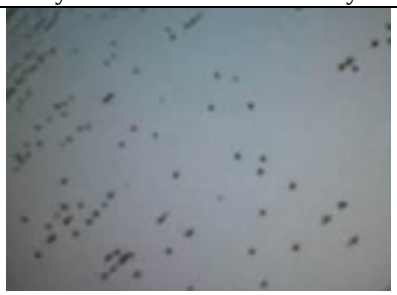
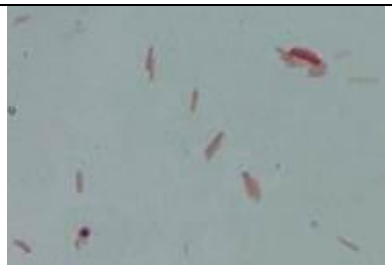
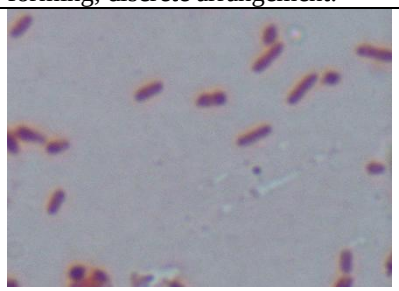
### *3.2. Isolation and screening of acetic acid bacteria*

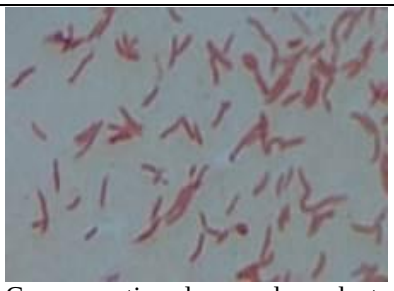
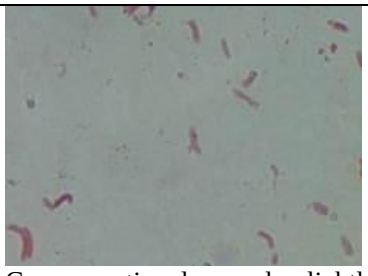
#### *3.2.1. Isolation of acetic acid bacteria*

Acetic acid bacteria were isolated from HS medium which was added 1% CaCO3 and 4% ethanol, the positive catalase test and halo-degradation ring would be selected for microscopic inspection. The rod-shaped bacteria, non spore-forming and Gram-negative staining will be selected to prepare test for ability to discharge glucuronic acid.

There are 7 isolated strains which are satisfied the conditions of test were confirmed as acetic bacteria. These strains are described in Table 5:

**Table 5:** Chracteristics of isolated acetic acid bacteria strains

| No. | Strain | Morphological Charateristics | |
|---|---|---|---|
| | | **Macroscopic** | **Microscopic** |
| 1 | AB1 |  Opalescent, punctiform, raised and mucus of colonies, 0.3-0.5mm of diameter, the old was yellow and darker than the young. |  Gram-negative, long rods, sedentary, no spore-forming, arranged in clusters 2-4 concentrate bacteria. |
| 2 | AB2 |  Circular and raised colonies, smooth surface, 0.5-1.5 mm of diameter, young colonies was limpid like the aerosol, the old became darker. |  Gram-negative, short rods, sedentary, no spore-forming, discrete arrangement. |
| 3 | AB3 |  White and flat colonies, around 0.1 mm of diameter. |  Gram-negative, long rods, no spore-forming, discrete arrangement. |
| 4 | AB4 |  White and flat colonies, smooth surface, the old colonies was slightly mucilaginous, the young was limpid like the aerosol, small of diameter, about 0.1mm. |  Gram-negative short rods, no spore-forming, usually discrete arrangement. |

| | | | |
|---|---|---|---|
| 5 | AB5 |  White and flat colonies, smooth surface, very small size of diameter 0.1-0.2mm. |  Gram-negative, long rods, sedentary, no spore-forming, and arranged in clusters or 2 discrete cells put together. |
| 6 | AB6 |  Yellowish, entire and raised colonies, smooth surface, diameter 1-2mm, the center of old colonies was dark. |  Gram-negative, long rods, sedentary, no spore-forming, discrete arrangement. |
| 7 | AB7 |  White, entire and flat colonies, smooth surface, small size of diameter 0.3-0.5mm. |  Gram-negative, long rods, slightly curved rod cells, no spore-forming, discrete or clustered arrangement in 2 cells. |

***Note:*** AB1, AB2 and AB3 old isolated from fermented coconut juice, AB4 isolated from fermented grape juice, AB5 from vinegar, AB6 and AB7 from tea Kombucha.

So, there were 7 strains of acetic acid bacteria had been isolated and identified based on biochemical and microscopic characteristics. These strains were screened by glucuronic acid generation.

### *3.2.2. Screening acetic bacteria capable of high glucuronic acid*

Seven strains of isolated acetic bacteria were cultured at 30°C in 36 hours in fresh milk for screening strains that produced high glucuronic acid concentration to increase biological activity of yogurt (Table 6) .

**Table 6:** Result of screening acetic bacteria

| No. | Strain bacteria | Concentration of glucuronic acid (mg/L) |
|---|---|---|
| 1 | AB1 | $22,19^{c}$ |
| 2 | AB2 | $9,13^{f}$ |
| 3 | AB3 | $0^{g}$ |
| 4 | AB4 | $12,04^{d}$ |
| 5 | AB5 | $11,52^{e}$ |
| 6 | AB6 | $25,37^{b}$ |
| 7 | **AB7** | **$28,04^{a}$** |

***Note:*** The letters from a to g showed the differences at 95% of reliability

In 7 isolated acetic acid bacteria strains, only 6 strains was capable in fermenting to generate glucuronic acid were AB1, AB2, AB4, AB5, AB6 and AB7. The strain AB3 was incapable of glucuronic acid production in milk. In 6 strains of bacteria that produced glucuronic acid, concentration of glucuronic acid was

generated by AB7 that was isolated from tea Kombucha was the highest and difference significantly to the others.

Thus, AB7 was the the highest glucuronic acid concentration strain in isolated acetic acid bacteria.

### *3.3. Identification of high biological activity bacteria strains*

#### *3.3.1. The results of 16S rDNA gene sequencing of LB4 strain*

The isolated strain LB4 were identified by 16S rDNA sequencing. The sequence of 16S rDNA gene of LB4:

AGCTAGTTGGTAGGGTAACGGCCTACCAAGGCAATGATGCATAGCCGAGTTGAGAGAC
TGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTT
CCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAG
CTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCAGAAA
GTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATT
GGGCGTAAAGCGAGCGCAGGCGGAAGAATAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGA
ACTGCATCGGAAACTGTTTTTCTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGG
AATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGCTGA
GGCTCGAAAGC

| Description | Max score | Total score | Query cover | E value | Ident | Accession |
|---|---|---|---|---|---|---|
| Lactobacillus acidophilus gene for 16S rRNA, partial sequence, strain: JCM 7711 | 952 | 952 | 100% | 0.0 | 99% | AB911464.1 |

**Figure 1:** Comparison of 16S rDNA gene of LB4 strain with *Lactobacillus acidophilus* (accession number AB911464.1)

The sequence included 525bps and this gene was compared with sequences in the bacterial database by using NCBI BLAST Search tool. The results showed that 16S rDNA gene of strain LB4 was similar up to 100% in comparison with the DNA sequence of *Lactobacillus acidophilus* that had accession number AB911464.1 in International Gene bank. This probiotic bacteria were applied widely in fermented foods such as yogurt, kimchi ... and used in medical products such as digestive enzymes. So, this was useful microorganism and safe for health.

#### *3.3.2. The results of 16S rDNA gene sequencing of AB7 strain*

The isolated strain AB7 were identified by 16S rDNA sequencing. The sequence of 16S rDNA gene of AB7:

TGGCTCAGAGCGAACGCTGGCGGCATGCTTAACACATGCAAGTCGCACGAACCTTTCGGG
GTTAGTGGCGGACGGGTGAGTAACGCGTAGGGATCTGTCCACGGGTGGGGGATAACTTTGGGAAA
CTGAAGCTAATACCGCATGACACCTGAGGGTCAAAGGCGCAAGTCGCCTGTGGAGGAACCTGCGT
TCGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGATGATCGATAGCTGGTCTGAGAGGAT
GATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG
GACAATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGTTTTCGGATTGTAAAGC
ACTTTCAGCGGGGACGATGATGACGGTACCCGCAGAAGAAGCCCCGGCTAACTTCGTGC

| Description | Max score | Total score | Query cover | E value | Ident | Accession |
|---|---|---|---|---|---|---|
| Gluconacetobacter nataicola strain LMG 1536 16S ribosomal RNA gene, partial sequence | 821 | 821 | 100% | 0.0 | 100% | NR_041012.1 |

**Figure 2:** Comparison of 16S rDNA gene of AB7 and *Gluconacetobacter nataicola* (accession number NR_041012.1)

The sequence included 444bps and this gene was compared with sequences in the bacterial database by using NCBI BLAST Search tool. The results showed that 16S rDNA gene of strain AB7 similar to the DNA sequence of *Gluconacetobacter nataicola* that had accession number NR_041012.1 on the International Gene bank with the similar proportion up to 100%. This was the traditional microorganism that was used in many fermented foods such as vinegar, tea Kombucha... So, this identified strain was safe for human health.

## IV. Conclusion

In this research, there were 6 lactic acid bacteria strains and 7 acetic acid bacteria strains from traditional fermented foods were isolated for biological activity improvement of yogurt. The strain LB4 showed the strongest of probiotic activity, the sequence of LB4 was identified as *Lactobacillus acidophilus* in comparison with data on Gene Bank. AB7 was capable of production of the highest glucuronic acid in all

isolated strains. The sequence of this strain was similar to *Gluconacetobacter nataicola*. Both strains were safe microorganisms for human health and could be used in food fermentation.

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## BIOGRAPHY

Huong H.L. Ly is currently MSc Researcher in Department of Biotechnology, Ho Chi Minh City University of Technology, Vietnam National University of Ho Chi Minh City. She received Bachelor degree in 2011 in Advanced Biotechnology program that was corporation between Can Tho University - Vietnam and Michigan State University – USA. In 2014, she graduated MSc degree in Biotechnology at Ho Chi Minh City University of Technology, Vietnam National University of Ho Chi Minh City, Vietnam. Her main research includes food and environmental biotechnology, microbiology and bio-medicine.

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Huong T. Nguyen is presently working as Associate Professor in Biotechnology in Ho Chi Minh City University of Technology, Vietnam. She is Head of Department of Biotechnology, Ho Chi Minh City University of Technology, Vietnam National University of Ho Chi Minh City. Her main research includes microbiological engineering and genetics engineering.