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Selection of High Acid Producing Lactic Acid Bacteria and Potential Application in Pineapple Juice Fermentation

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Abstract: Lactic acid bacteria are used popularly for fruit juice fermentation because they are good sources of probiotics for human. In this study, ten lactic acid bacteria were isolated from 7 samples of naturally fermented pineapple juice. All isolates could grow in the MRS medium pH 1.5 and reach high densities (6.80 – 6.95 log CFU/mL) after 2 h of incubation at 37°C. Moreover, *Lactobacillus* sp. Y1 produced highest lactic acid concentration (1.20% w/v) and identified as *L. acidophilus*. The different diluted ratios of pineapple juice (0, 10, 20 and 30% w/v) and different sucrose supplementations (0, 3, 6, and 9% w/v) were used for testing fermenting capacity of *L. acidophilus* Y1. The undiluted pineapple juice with 9% (w/v) of sucrose supplementation was found to be suitable for fermentation. Based on the results of sensory evaluation and bacterial density determination, the favorable conditions for pineapple fermentation were determined as follow: initial bacterial level at 5.0 log cells/mL, fermentation time for 36 h at 37°C. The results of storage testing showed that the suitable temperature for product storage was 4 – 6°C, bacterial density (8.06 log CFU/mL) of final product was maintained up to 3 weeks.

Keywords: Lactic Acid Bacteria, Lactic Acid Fermentation, *Lactobacillus acidophilus*, Pineapple, Probiotic

1. Introduction

Nowadays, scientists as well as nutritional experts always research to find bio-products which are good for health and nutrition. These products are called probiotic products. Probiotics are bacteria that help maintain the natural balance of organisms [1, 2]. A number of probiotic strains have been introduced in the market in dietary and pharmaceutical forms. Lactic acid bacteria (e.g. *Lactobacillus*) and *Bifidobacterium* constitute the main group of probiotics commercialized for human consumption. The mechanisms of action of probiotics against gastrointestinal pathogens addressed in diverse patent applications include: modification of the environmental conditions, competition for nutrients and adhesion sites, production of antimicrobial metabolites and modulation of the immune and non-immune defense mechanisms of the host [3, 4].

Lactic acid bacteria (LAB) are generally presented in the intestines, mouth, skin, urinary and genital organs of both humans and animals, and may have several beneficial effects on their health. They are used widely in probiotic products such as yogurt, fermented meat, fermented vegetables and fruit juices and so on [5-10]. These products are not only used as food but also used for treating several intestinal and stomach diseases because LAB can produce antibiotics to prevent and skill pathogenic bacteria [11].

Probiotic product is commonly used as fermented milk and yogurt. However, many consumers are intolerant of lactose as well as high cholesterol in the milk products. Thus, the fruit juice is recommended as a good medium to produce fermented probiotic products [12]. Fruit juices are good media for bacteria's growth and probiotic products. Fruits and vegetables are good for health as they contain antioxidants, vitamins, fibers and minerals. Pineapple is one of the most popular fruits in tropical countries especially in Vietnam. Pineapple is an important source of vitamins, enzyme bromelain and minerals. Pineapple provides several

health benefits such as potential anti-inflammatory, increasing digestive effect, antioxidant protection, immune support and protection against macular degeneration [13-15]. The aim of this study was to isolate high acid producing LAB and to study on conditions for pineapple juice fermentation.

2. Materials and Methods

2.1. Materials and Media

Pineapple was bought from local market in Can Tho City, Vietnam. MRS broth and MRS agar (De Man, Rogosa and Sharpe-Merck, Germany) were main media for growth and enumeration of LAB. Other chemicals such as NaOH, HCl, H_2O_2, $NaHSO_3$, crystal violet, iodine, acetone, ethanol and so on were bought as commercial products from Hi Media (India) and Merck (Germany).

2.2. Isolation and Identification of LAB Isolates

Pineapple juice was contained in a sterilized glass and naturally incubated for fermentation at 37°C for 24 – 48 hours. Then, transferred 1 mL of fermentation juice into a tube containing MRS broth medium. After 24 h, transferred bacteria from MRS broth medium to MRS agar medium and re-streaked several times to obtain the purified bacteria. Genus identification by some typical tests such as Gram stain, catalase and oxidase reaction, and dissolution of $CaCO_3$.

2.3. Study on Acidity Tolerant Ability of LAB Isolates

Inoculated 1 mL of LAB inoculum (6.0 log cells/mL) into 9 mL MRS broth medium with different pH levels (1.5, 2.5, and 3.5). Then, bacterial density of each treatment was determined at initial inoculating time (at T_0) and 2 h after inoculating (at T_2) by plate counting method on MRS agar. All treatments were carried out in triplicate.

2.4. Study on Application in Producing of Fermented Pineapple Juice

Pineapple was pressed and put into the 50-mL tubes. Pasteurized by 140 mg/L of $NaHSO_3$ in 20 minutes, then inoculated the strains of LAB into tubes, each tube contained 36 mL of pineapple juice and 4 mL of LAB inoculum (initial density of bacteria was 5.0 log cells/mL). Incubated at 37°C for 48 h and then analyzed pH, soluble matter content (ºBrix), lactic acid content, density of LAB, and sensory evaluation.

The 16S rRNA gene of selected strain was isolated and amplified by polymerase chain reaction (PCR) in a thermal cycler. The primers 1492R (5'-TACGGTTACCTTGTTACG ACT-3') and 27F (5'-AGAGTTTGATCCTGGCTC-3') were used for PCR [16]. The purified PCR product was sent to 1st BASE Company (Singapore) for DNA sequencing. The 16S rRNA sequence was compared to the database of GenBank (NCBI) to determine the scientific name.

2.5. Study on Dilution Ratio of Pineapple Juice and Sucrose Supplementation

Experiments were conducted randomly with two factors, percentage of dilution ratio (0, 10, 20, 30% v/v) and percentage of sucrose supplementation (0, 3, 6, 9% w/v) in triplicate. Pineapple juice was diluted at 4 experimental levels and pasteurized by $NaHSO_3$ (140 mg/L) for 20 minutes. Then, distributed into 48 tubes, each tube contained 40 mL. Each dilution rate was mixed with 4 sucrose supplementation levels. Then they were measured pH and ºBrix. Inoculated 1% (v/v) bacteria with 7.0 log cells/mL into each tube. Then, incubated at 37°C for 48 h.

2.6. Study on Inoculum Density, Temperature and Fermentation Time

The experiment was conducted in three factors and duplication. The density of LAB (3.0, 5.0, and 7.0 log cells/mL), incubation temperature (30°C, 37°C, and 28 – 32°C (ambient temperature)), and fermentation time (24, 36, and 48 h). Experiment was performed the same procedure above but densities of LAB were diluted with saline for 5.0, 7.0, and 9.0 log cells/mL. Then inoculated into the tube 1% (v/v) of inoculum (initial concentration the bacteria corresponding 3.0, 5.0, and 7.0 log cells/mL) and incubated at different time and temperatures.

2.7. Study on Storage Temperature and Storage Time

Fermented pineapple juice samples were kept in cold condition (4 – 6°C), cool room (20 – 25°C) and ambient temperature (28 – 32°C). All samples were analyzed pH, ºBrix, lactic acid content, density of LAB, and sensory characteristics after 1, 2 and 3 weeks of storage.

2.8. Data and Statistical Analysis

The statistical analyses will be performed using the software Statgraphics Centurion XV (Statpoint Technologies, Inc., USA). Analysis of variance (ANOVA) was used to evaluate the differences among the treatments. The mean values were compared with least significant difference (LSD).

3. Results and Discussion

3.1. Results of Isolation and Identification of LAB Isolates

Totally, 10 strains of LAB were purified from 7 samples of naturally fermented pineapple juice. All colony morphologies were white color, circular shape, convex elevation, smooth texture. The bacterial cell shapes included cocci, short rod, short rod in pair, and long rod. Short rod was predominant type of cell.

To confirm these isolated strains belonged to LAB genera, some basically physical and biochemical properties were tested. They were all recognized as Gram positive, catalase negative, and oxidase negative. They could produce acid to dissolved $CaCO_3$ and formed clear

zones around colonies on the medium (MRS agar containing 1.5% $CaCO_3$). Due to the all above characteristics, these isolates were generally identified belong to the group of lactic acid bacteria.

3.2. Acidity Tolerant Ability of Lactic Acid Bacteria

After incubation at 37°C for 2 h with different pH levels, all strains increased densities to 6.80 – 6.95 log CFU/mL (Table 1). At pH 1.5 and 2.5, the bacterial densities reduced significant at initial time point (T_0) with values in range of 1.51 – 1.96 log CFU/mL, but they recovered and increased to 6.80 – 6.93 log CFU/ml after 2 h of incubation at 37°C. Each bacterial species was specialized to live in basic pH. If they moved stress pH conditions, metabolic imbalances of bacteria and environment occurred, that led to the death of bacteria. At pH 3.5, most of bacteria tolerated and reduced slightly at initial time point (T_0) with the densities of 4.23 – 4.28 log CFU/mL, as well as, increased and achieved values of 6.81 – 6.95 log CFU/mL after 2 h incubation at 37°C.

Table 1. *The evaluation criterion of LAB density.*

pH	Strains	LAB density at T_0 (log CFU/mL)	LAB density at T_2 (log CFU/mL)
1.5	A1	1.55^{ij}	6.85^{efg}
	P1	1.53^{jk}	6.87^{cde}
	Y1	1.56^{i}	6.82^{ghi}
	L1	1.52^{k}	6.81^{ij}
	B1	1.53^{jk}	6.93^{a}
	D1	1.52^{k}	6.86^{def}
	K1	1.55^{ij}	6.83^{hi}
	K2	1.56^{i}	6.81^{ij}
	K3	1.53^{jk}	6.86^{abcd}
	K4	1.51^{k}	6.80^{j}
2.5	A1	1.93^{gh}	6.89^{bc}
	P1	1.96^{e}	6.88^{bcd}
	Y1	1.91^{h}	6.89^{bc}
	L1	1.93^{fgh}	6.85^{efg}
	B1	1.91^{h}	6.86^{def}
	D1	1.96^{e}	6.80^{j}
	K1	1.92^{h}	6.83^{ghi}
	K2	1.95^{ef}	6.87^{cde}
	K3	1.92^{gh}	6.82^{hij}
	K4	1.94^{efg}	6.83^{hi}
3.5	A1	4.28^{a}	6.90^{b}
	P1	4.235^{d}	6.95^{a}
	Y1	4.24^{cd}	6.93^{a}
	L1	4.25^{bcd}	6.94^{a}
	B1	4.26^{abc}	6.84^{fgh}
	D1	4.28^{a}	6.81^{ij}
	K1	4.23^{d}	6.88^{bcd}
	K2	4.26^{abc}	6.87^{cde}
	K3	4.26^{abc}	6.83^{ghi}
	K4	4.27^{ab}	6.81^{ij}
CV%		7.38%	6.43%

*Note: Value in the table was average value of triplication; the average values with the same letter were not significantly different at the 95% confidence level.

3.3. Potential Application in Pineapple Juice Fermentation

In general, ºBrix and pH after fermentation reduced after inoculating for 48 h because of converting sucrose to lactic acid by LAB. *Lactobacillus* sp. Y1 had a preponderance of lactic acid content (1.20% w/v) while all other strains produced only 0.73 – 0.88% (w/v) (Table 2). The density of *Lactobacillus* sp. Y1 at initial time (T_0) was the highest density (4.35 log CFU/mL) and significant difference with the others. Yoon et al. (2004) reported that the lactic acid cultures reduced the pH of tomato juice to 4.1 and increased the acidity to 0.65%, and the viable cell counts reached 1.0 – 9.0 x 10^9 CFU/mL after 72 h fermentation.

Table 2. *The evaluation criterion after fermentation of pineapple juice.*

LAB strains	ºBrix	pH	Lactic acid (% w/v)	LAB density at T_0 (log CFU/mL)	LAB density at T_{48} (log CFU/mL)
A1	8.0^{a}	3.42^{bc}	0.77^{cd}	4.25^{b}	7.21^{de}
P1	8.0^{a}	3.51^{a}	0.77^{cd}	4.22^{bc}	7.28^{bc}
Y1	8.0^{a}	3.39^{bc}	1.20^{a}	4.35^{a}	7.38^{a}
L1	8.0^{a}	3.41^{bc}	0.88^{b}	4.26^{b}	7.16^{e}
B1	8.0^{a}	3.38^{bc}	0.88^{b}	4.25^{b}	7.23^{cd}
D1	8.0^{a}	3.42^{bc}	0.73^{d}	4.25^{b}	7.31^{b}
K1	7.33^{c}	3.35^{c}	0.84^{bc}	4.25^{b}	7.26^{cd}
K2	7.83^{ab}	3.52^{a}	0.81^{bcd}	4.24^{bc}	7.21^{de}
K3	7.67^{b}	3.26^{d}	0.77^{cd}	4.07^{bc}	7.28^{bc}
K4	7.83^{ab}	3.44^{b}	0.81^{bcd}	4.18^{c}	7.25^{cd}
CV%	3.31	2.37%	16.03	12.27	8.85

*Note: Value in the table was average value of triplication; the average values with the same letter were not significantly different at the 95% confidence level.

After 48 h of incubation, LAB densities were increased above 6.0 log CFU/mL, varied from 7.16 to 7.38 log CFU/mL. The LAB densities were satisfied the requirement for probiotic products, therefore all of these LAB strains were able to apply in producing of fermented pineapple juice. In summary, lactic acid content and cell density of *Lactobacillus* sp. Y1 were the highest, 1.20% (w/v) and 7.38 log CFU/mL, respectively, so this strain was selected for pineapple juice fermentation.

The alignment result of the 16S rRNA sequence of selected *Lactobacillus* sp. Y1 with the database of GenBank (NCBI) indicated that this strain belonged to *Lactobacillus acidophilus* (100% of similarity to the strain *L. acidophilus* 1001H, accession no. JQ031741.1).

3.4. Dilution Ratio of Pineapple Juice and Sucrose Supplementation

At the same dilution of juice, the lowest bacterial densities were measured in the treatments of 0% of sugar supplementation (Figure 1). The reason is that nutrient-depleted environment after converting all sugar into lactic acid inhibited the bacterial growth and development. Besides, lactic acid itself could inhibit the development of bacteria [10]. In contrast, the treatment of 9% of sugar supplementation exhibited the highest bacterial densities, which means these were appropriate conditions for fermentation of pineapple juice. Moreover, bacterial densities at all dilution ratios (8.23 – 9.34 log CFU/mL) were above 6.0 log CFU/mL. This result was appropriate to the fermented tomato juice after 72 h fermentation (the viable cell counts of reached 1.0 – 9.0 x 10^9 CFU/mL) [17].

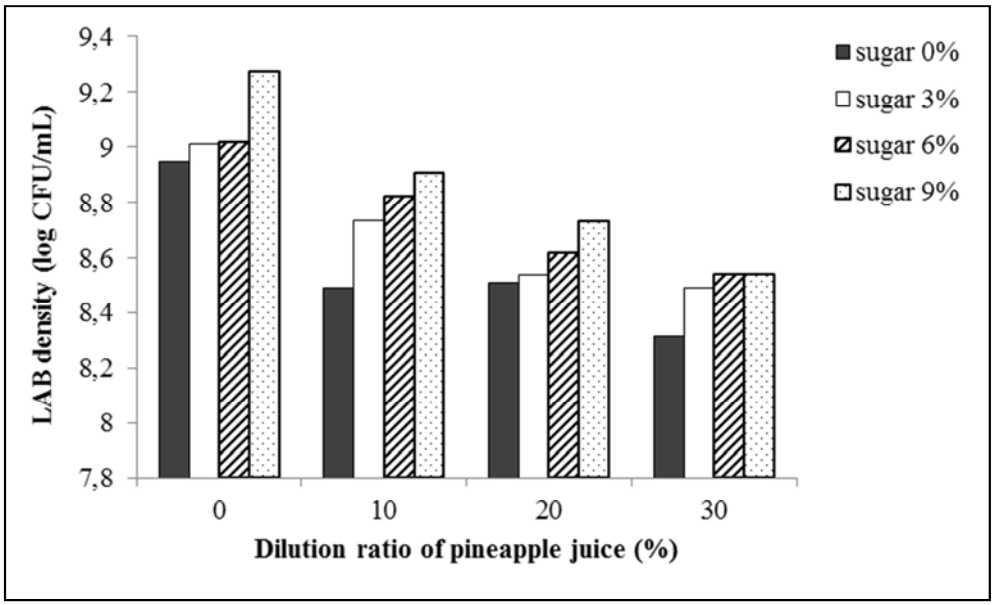

Figure 1. *Changes of LAB density by different dilution ratio and sugar content.*

Table 3. *The result of sensory evaluation of product.*

Dilution ratio (% w/v)	Sucrose ratio (% w/v)	Sensory score	CV %
	0	4.00^{g}	5.00%
	3	4.10^{e}	9.76%
0	6	4.40^{b}	2.27%
	9	4.65^{a}	1.08%
	0	3.70^{l}	5.41%
	3	3.95^{h}	1.27%
10	6	4.00^{g}	7.50%
	9	4.35^{c}	5.75%
	0	3.70^{l}	1.35%
	3	3.50^{o}	1.43%
20	6	4.15^{d}	3.61%
	9	4.15^{d}	1.20%
	0	4.05^{f}	1.23%
	3	3.80^{j}	2.63%
30	6	3.75^{k}	4.00%
	9	3.85^{i}	6.49%

*Note: Values in the table were average values of triplication; Sensory score was the average score of color, smell, taste, and appearance (maximum score is 5.0); the average values with the same letter were not significantly different at the 95% confidence level.

Suitable dilution ratio and sugar supplementation as well as appropriate sensory evaluation were very important to determine the fermented juice quality. The evaluation was carried out with 4 parameters (color, smell, taste, and appearance) and the sensory result is shown in Table 3. The fermented juice samples without dilutions were statistically significant different ($p<0.05$). The treatments of without sugar supplementation were given the lowest score because of bland taste. The undiluted treatments were more favorable than the others. Particularly, the treatment of undiluted juice and 9% (w/v) of sugar supplementation had the highest score because of gentle smell and comfortable sweet-sour taste.

In short, fermented juice samples from undiluted juice and 9% (w/v) of sugar supplementation treatments were better than those in other treatments.

3.5. Inoculum Density, Fermentation Temperature and Time

Results indicated that with the same inoculum density, the highest values of lactic acid were achieved at 36 h of fermentation time (Figure 2). In the treatments of 5.0 and 7.0 log cells/mL, the highest values of lactic acid were achieved at 37°C after 36 h of fermentation, 1.65% and 1.89% (w/v), respectively. These results were proved that 36-h fermentation was appropriate time for the development of bacteria in pineapple juice.

In addition, with the same inoculum and incubation time, lactic acid content at 37°C was higher than that at 30°C and 28 – 32°C, showing that 37°C was optimal temperature for the growth and development of bacteria. This could lead to high lactic acid was converted.

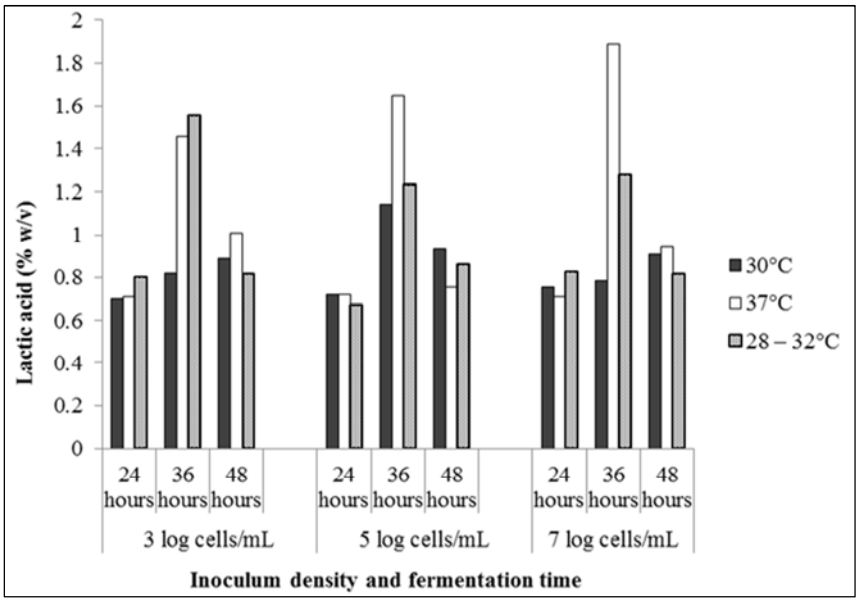

Figure 2. *Changes of lactic acid content by different inoculum density and fermentation time.*

At the same temperature and fermentation time, inoculum density of 7.0 log cells/mL gave higher lactic acid content than inocula of 3.0 and 5.0 log cells/mL. This might result from the fact that the higher the inoculum density, the higher lactic acid content was produced. Similarly, bacterial density was highest in sample of 36-h incubation, 7.0 log cells/mL and 37°C (8.43 log CFU/mL), and sample of 48-h incubation, 7.0 log cells/mL and 37°C (8.38 log CFU/mL) (Figure 3). Besides, bacterial density incubated at 37°C was higher than that at other temperatures. Therefore, 37°C was more appropriate for the growth and development of bacteria.

Bacterial density increased in proportion with the rise of incubation temperature, except for sample with 7.0 log cells/mL (Figure 3). In fact, bacterial density was highest after 48 h of incubation. Because of the longer incubation time, the better bacterial was grown and developed. Nevertheless, in the sample with 7.0 log cells/mL, the environment could not provide enough nutrients for bacteria to grow. Thus, death phase took place and led to decrease in bacterial density. Also, inhibitory substances released during fermentation could prevent bacteria from developing. Bacterial density required in probiotic products to be equal or more than 6.0 log cells/mL. Therefore, the samples with 3.0 or 5.0 log cells/mL after 24 h of incubation were not acceptable. Dissimilarly, the samples with more than 6.0 log cells/mL after 36 and 48 h of incubation were considered potential probiotic products. Shortly, quality probiotic products could be qualified if fermentation using minimal inoculum density of 3.0 log cells/mL at 30 – 37°C, and incubation time of 36-48 h.

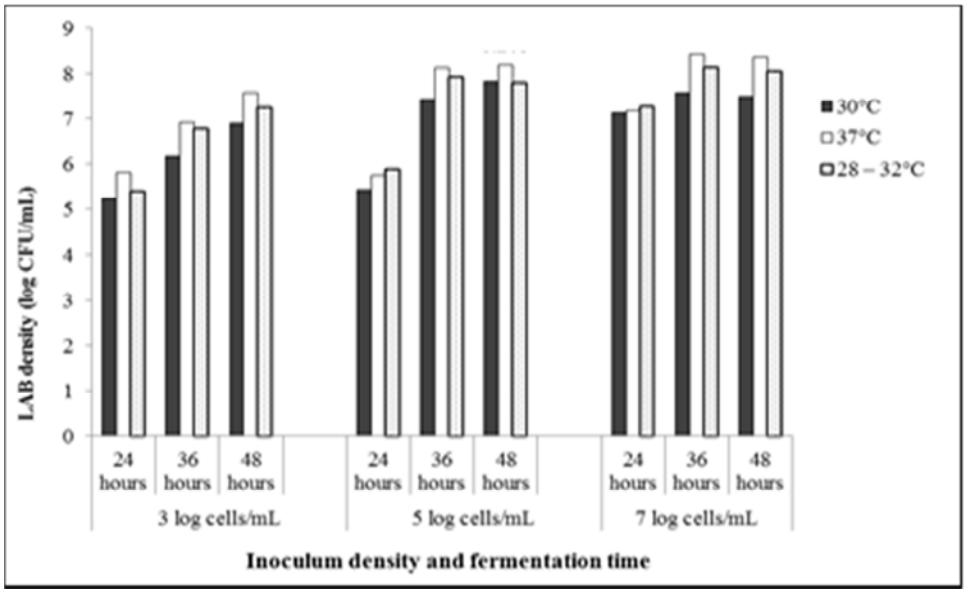

Figure 3. *Changes of LAB density by different inoculum densities, fermentation time and temperatures.*

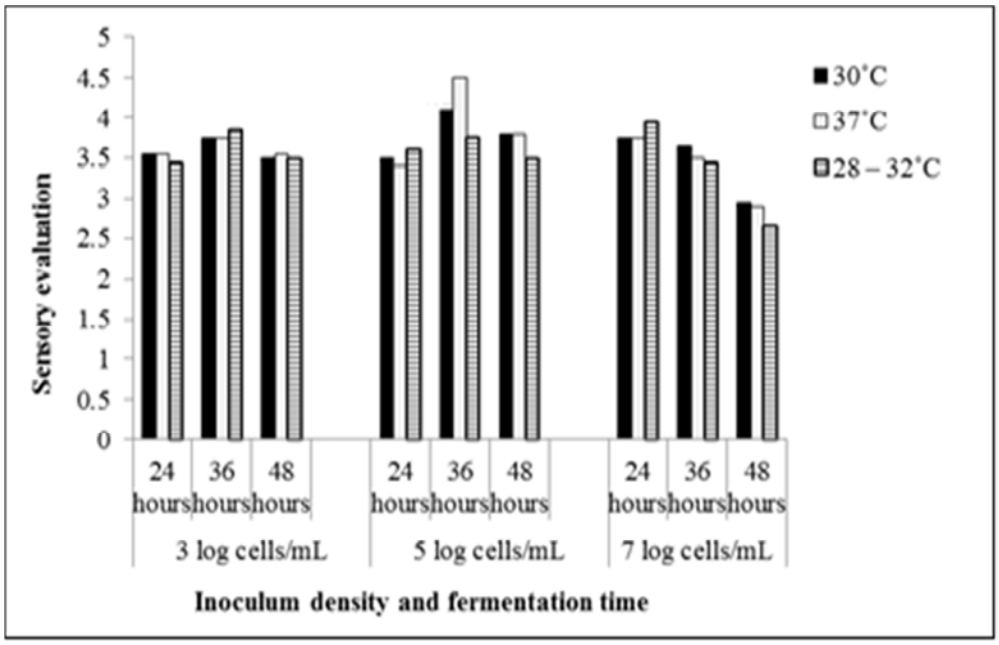

Figure 4. *Sensory evaluation of different treatment of fermented pineapple juice.*

Sensory evaluation results indicated that the sample had the highest score (4.5/5.0) with initial inoculum density of 5.0 log cells/mL, incubation at 37°C in 36 hours (Figure 4). The smell was gentle and the taste was favorable sweet-sour. In contrast, the sample had the lowest score with initial inoculum density of 7.0 log cells/mL, incubation at 28 – 32°C in 48 hours.

All in all, appropriate conditions for fermentation of pineapple juice were initial LAB density of 5.0 log cells/mL and 36 h of incubation at 37°C.

3.6. Storage Temperature and Storage Time

Results in Table 4 shows that bacterial densities in all samples was over 6.0 log CFU/mL. Besides, bacterial densities in all treatments increased after one week of storage because there were enough nutrients for the growth and development of bacteria; however, the densities increased slowly. LAB densities decreased from the second week. Additionally, 20 – 25°C and 28 – 32°C are suitable ranges for bacteria to grow and develop. After using all soluble sugar and nutrients as well as increasing lactic acid content, the bacteria started to be inhibited and died. However, bacterial densities were still higher than 6.0 log cells/mL in the second and third weeks.

Table 4. *The evaluation criterion after storage.*

Storage time (week)	Storage temperature	pH	Lactic acid (% w/v)	LAB density (log CFU/mL)	Sensory score
0		3.51^{a}	0.91^{f}	8.50^{b}	4.55^{a}
1	4-6˚C	3.47^{b}	0.95^{e}	8.72^{a}	4.55^{a}
	20-25˚C	3.41^{cd}	1.01^{c}	8.61^{ab}	4.40^{bc}
	28-32˚C	3.40^{d}	1.06^{b}	8.21^{c}	4.10^{d}
2	4-6˚C	3.45^{b}	0.97^{de}	8.23^{c}	4.50^{ab}
	20-25˚C	3.36^{e}	1.05^{b}	8.33^{c}	3.95^{e}
	28-32˚C	3.11^{h}	1.12^{a}	7.52^{e}	3.55^{f}
3	4-6˚C	3.42^{c}	0.99^{cd}	8.06^{d}	4.30^{c}
	20-25˚C	3.27^{f}	1.09^{a}	7.13^{f}	2.70^{g}
	28-32˚C	3.13^{g}	1.12^{a}	6.88^{g}	2.40^{h}
CV%		3.97%	6.99%	7.64%	19.37%

*Note: Value in the table was average value of triplication; the average values with the same letter were not significantly different at the 95% confidence level.

After 3 weeks, lactic acid content of samples that were stored at 4 – 6°C increased slightly from 0.91 to 0.99% (w/v). Because LAB were still alive but they could not reduce lactic acid at low temperature. Moreover, stored at 20 – 25°C and 28 – 32°C, these samples included high lactic acid content, about 1.01 to 1.12% (w/v). At 28 – 32°C, LAB could grow well and continue to produce lactic acid. Therefore, lactic acid contents in these samples were high (1.06 – 1.12% w/v). In addition, storage of samples at 4 – 6°C gave high sensory score because they still had the same favorable smell and taste, while others had negative sour taste. Thus, suitable storage temperature in the three weeks was 4 – 6°C. The LAB densities were almost not change during 3 weeks of storage, and the LAB density was 8.06 log CFU/mL at the 3rd week. Yoon et al. [17] reported that the viable cell counts of LAB in the fermented tomato juice

varied from 10^6 to 10^8 CFU/mL after 4 weeks of storage at 4°C. While AdebayoTayo and Akpeji [19] demonstrated that the fermented pineapple juice samples with the initial LAB density of 1.05 – 1.10 × 10^9 CFU/mL were decreased to about 1.30 × 10^7 CFU/mL after 4 weeks of storage at 4°C. In the same condition, after 4 weeks at 4°C, the viable cell counts of *L. plantarum* and *L. delbrueckii* were still 4.1 x 10^7 and 4.5 × 10^5 CFU/mL, respectively, while *L. casei* did not survive in fermented cabbage juice and lost cell viability completely in the second week of storage [20].

4. Conclusions

Lactobacillus acidophilus Y1 could produce the highest lactic acid (1.20% w/v) and reach high cell density (7.38 log CFU/mL) from pineapple juice after 48 h of incubation. Fermentation of pineapple juice by *L. acidophilus* Y1 was successfully produced when using undiluted pineapple juice and 9% (w/v) of sucrose, incubating at 37°C for 36 h, and initial bacterial cell density of 5.0 log cells/mL. The final product was stable at 4 – 6°C up to 3 weeks of storage.

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