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SCREENING YEAST FROM NATURAL HONEY, AND BEESWAX IN SON LA PROVINCE FOR LOW-ALCOHOL MEAD PRODUCTION

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Abstract. Son La is a northern mountainous province of Vietnam, where the climate and soil are suitable for various flowering plants and for many honey bee species to grow and produce high-quality honey. Yeasts in honey are expected to have valuable characteristics that can be applied in many fields. In this paper, seventy-two yeast strains isolated from natural honey and beeswax in Son La were screened for the ability of alcoholic fermentation to produce mead. Screening yeast strains for low-alcohol mead production were carried out based on the fermentation ability and the sensory of the final mead product. Yeast train NT95 was selected and characterized as *Zygosaccharomyces* sp. NT95. A suitable condition for the growth of *Zygosaccharomyces* sp. NT95 was Hansen broth containing a sugar concentration of 60 - 90 g/L at pH 4.0 - 4.5, and a temperature of 30 °C. A suitable condition for the alcoholic fermentation of *Zygosaccharomyces* sp. NT95 in the honey medium was at 30 °C with the inoculum of 5 x 10^7 cells/mL for 18 days. Under this condition, the mead produced by *Zygosaccharomyces* sp. NT95 contained approximately 4.5% alcohol (v/v) and the sensory score reached 14.51 ± 0.15 points. Further studies need to be done to improve the fermentation process and the completion of the product.

Keywords: alcoholic fermentation, honey, mead, yeast, *Zygosaccharomyces* sp. NT95.

1. Introduction

Yeast, especially some species of *Saccharomyces* genus, plays an important role in alcoholic fermentation. Yeast has been isolated from a variety of natural sources such as fruits, leaves, flowers, and honey. Honey is a natural product with primary components fructose (38%), glucose (31%), water (17%), maltose (7%), and sucrose (1%) [1]. Most microorganisms are not able to survive in an environment of high osmotic pressure like honey. Yeasts found in honey have been known as harmful factors that reduce the quality of honey [2] but are also expected to have some valuable characteristics which can be applied in different fields [3]. Some studies on yeast in honey were published in the world [2, 3], however, there is no publication on honey yeast and very few relevant publications on mead production in Vietnam [4].

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Son La is a mountainous province located in the northwestern region of Vietnam, where the climate and soil are suitable for many types of plants. The enormous natural forest area with many types of flowers creates a favorable condition for many honeybee species to grow and produce high-quality honey.

Mead, also known as honey wine, is a traditional beverage and typically contains between 8 to 18% (v/v) ethanol. Mead is normally produced from the alcoholic fermentation of diluted honey by yeasts [5]. Mead seems to be an alternative to increase the income of beekeepers. In recent years, mead production is becoming a significant economic activity in the world, especially in Europe and North America. The global mead market size was valued at USD 408.45 million in 2018 [6]. The market was estimated to expand further at a CAGR (compounded annual growth rate) of 10.41% from 2019 to 2025 and it was expected to reach USD 817.03 million by 2025, according to a report by Grand View Research, Inc [7]. To the best of our knowledge, two types of mead are now available on the Vietnamese market and contain about 12.5% (v/v) ethanol. No official statistic about mead consumption in Vietnam has been done so far, but it is believed that mead will become more popular in this market, due to its pharmaceutical values [8, 9]. Besides, it has been recommended that a low-alcohol drink would be a healthier option for people, particularly women and the elderly [10].

The process of making mead is a time-consuming fermentation and regularly takes several weeks or months to accomplish. The fermentation rate and sensory properties of mead depend on numerous factors, such as the yeast strain used, temperature, and pH value [11-13]. This study aims to screen potential yeast strains from honey and beeswax in Son La province for low-alcohol (3.5 - 7% v/v) mead production.

2. Content

2.1. Materials and methods

2.1.1. Materials

Natural honey and beeswax were obtained at different locations in Son La province for isolation. Beekeeping honey was purchased in the Moc Chau district for preparing fermentation medium.

All chemicals used were at analytical grade from Sigma-Aldrich, Merck, and Scharlau.

2.1.2. Methods

** Isolation of yeast from natural honey and beeswax*

Beeswax was ground in sterilized porcelain pestle and then 1 g of finely ground sample was added to 9 mL of sterilized saline solution (0.9% NaCl) to prepare 10^{-1} sample dilution. One mL of honey was added to 9 mL of sterilized saline solution to prepare 10^{-1} sample dilution. The 10^{-2} and 10^{-3} dilutions were prepared for each sample by serial dilution. Each dilution (100 µL) was spread on Hansen agar plates containing 150 g/L glucose. The plates were then incubated at 32 °C for 48 hours. Individual colonies were selected based on morphology and streaked on new Hansen agar slides for further studies.

** Observation of yeast colonial and cellular morphology*

Yeast colonies were observed and described based on several characteristics, such as color, elevation, form, and flavor. Yeast cells were stained with 1% fuchsin and observed under a light microscope (x1,000) for measuring and describing the size and shape of the cells.

** Mead fermentation and monitoring*

The fermentation medium was prepared by using diluted beekeeping honey in hot water at 80 °C and mixed to homogeneity. The pH was adjusted by citric acid and $NaHCO_3$. The ^{O}Brix value of the medium was determined before pasteurization. This fermentation medium was pasteurized at 65 °C for 10 minutes and then cooled down to room temperature before use [14].

All fermentation experiments were carried out in triplicate using 300 mL-glass bottles filled up to 2/3 of their volume by fermentation medium which had an initial ^{O}Brix of 22° ± 1°, pH 4.3. The bottles were maintained in a dry incubator at a specific temperature during alcoholic fermentation. Fermentations were daily monitored by weight and weight loss was used to estimate the amount of CO_2 emitted. CO_2 was released through a special pipe plugged into the lid of each bottle with a water lock. At the end of the fermentation, samples were taken from all bottles for distillation and determination of total alcohol by volume (ABV), fermentation efficiency (FE), and °Brix. Sensory evaluation of fermentation products was also carried, based on the method of TCVN 3217-79 [15].

Yeast inoculum was added to the fermentation medium to reach 10^7; 2.5 x 10^7; 5 x 10^7, and 10^8 yeast cells/mL, and then fermentation was carried out at 28 °C for 25 days to determine a suitable inoculum for mead fermentation.

An inoculum of 5 x 10^7 yeast cells/mL was used and the fermentation was carried out at 20 - 24 - 28 - 32 °C for 25 days to determine a suitable fermentation temperature.

To determine a suitable time for fermentation, an inoculum of 5 x 10^7 yeast cells/mL was used. Fermentation was carried out at 28 °C for 6 - 12 - 18 - 24 days.

** Measurement of yeast growth*

Hansen broth was used for culturing yeast strains in an orbital shaker at 180 rpm and a specific temperature for 36 hours. Cell density was determined every 6 hours using the Neubauer counting chamber.

To determine a suitable pH value, Hansen broth containing 100 g/L glucose was adjusted pH by 0,1M citric acid to reach pH 4.0 - 4.5 - 5.0 - 5.5 - 6.0 before inoculating and incubating in an orbital shaker at 30 ^{O}C, 180 rpm.

To determine a suitable sugar concentration, Hansen broth containing different concentrations of glucose (30 - 60 - 90 - 120 - 150 g/L) was used at pH 4.3, incubating in an orbital shaker at 30 ^{O}C, 180 rpm.

Different temperatures (25 - 28 - 32 - 35 ^{O}C) were set to the determination of a suitable temperature for the growth of yeast strains in Hansen broth containing 60 g/L glucose at pH 4.3.

** Screening of yeast strains*

Preselected strains were given scores at 5 criteria with different maximum scores (Max. score) such as growth (Max. score of 6), ABV (Max. score of 10), FE (Max. score of 6), °Brix decreased (Max. score of 6) and sensory properties (Max. score of 10). Growth was measured after 80 hours of incubation at 28 ^{O}C in a fermentation medium with an initial ^{O}Brix of 25° ± 1°, pH 4.0, and inoculum of 5 x 10^7 yeast cells/mL. In each parameter, the strain that showed the greatest result was given the maximum score, and other strains were given scores based on the ratio to the greatest [16]. The strain that had the highest total score was selected for further experiments.

** ITS sequencing and classification of yeast strain*

Genomic DNA from a selected strain was extracted using the i-genomic BYF DNA extraction mini kit (iNtRON Biotechnology, Inc., Korea) following the manufacturer's protocol.

To amplify the ITS region of the selected strain, PCR reactions were performed using a 2X solution of master mix (iNtRON Biotechnology, Inc., Korea) and a pair of primer ITS1(5'-TCCGTAGGTGAACCTGCGG-3')-ITS4(5'-CCTCCGCTTATTGATATGC-3'). PCR was done in 30 cycles (94 °C - 30 seconds, 50 °C - 20 seconds 72 °C - 1 minute), with an initial denaturation at 94 °C for 5 minutes and a final extension at 72 °C for 5 minutes.

All PCR products were purified using the MEGAquick-spin^{TM} plus fragment DNA purification kit (iNtRON Biotechnology, Inc., Korea) following the manufacturer's protocol. The amplicon was sequenced from both ends by the Sanger sequencing method. The sequence was compared with the available data in the GenBank database at the NCBI using the BLASTN tool. The phylogenetic tree was constructed by MEGA-X software (version 10.2.1) using the maximum likelihood method. The topology of the tree was tested by performing bootstrap resampling from 1,000 replicates [17].

2.2. Results and discussion

2.2.1. Isolation of yeast strains from natural honey and beeswax from Son La province

Seventy-two yeast strains were isolated from 6 samples of honey and beeswax in Son La based on various features of colony and cell. They were classified into 29 distinct groups based on the morphological similarity of the colony (the colony size and color; the margin, the glossiness, and the elevation of the colony). This result may give initial information on the diversity of yeast in natural honey and beeswax from Son La province. It is much higher diversity compared to the study of Mukti et al. (2019) in Bangladeshi honey, which isolated 8 yeast strains and classified them into 7 distinct groups based on morphological physiological, and genetic characteristics [18].

2.2.2. Screening of yeast strain

Six yeast strains named from NT94 to NT99 were prescreened based on 5 criteria (growth, ABV, FE, °Brix decreased, and sensory properties). Figure 1 and Table 1 show the determined parameters for the growth and fermentation activity of these six strains. The strain NT95 received the greatest total score of 35.41 (Table 2) and was chosen for

further experiments. The result in Table 2 shows the fermentation product of this strain contained about 5% ABV and got the highest sensory score (13.51 ± 0.19) based on TCVN 3217-79 [15].

Table 1. Fermentation products' parameters and sensory scores of 6 preselected strains

Data are the means of triplicate fermentations ± S.D

Strain name	°Brix decreased	ABV (%)	FE (%)	Sensory score
NT99	1.5 ± 0.42	0.3 ± 0.00	57.45 ± 10.2	12.23 ± 0.56
NT98	6.1 ± 0.55	4.7 ± 0.18	92.50 ± 4.0	11.93 ± 0.82
NT97	2.5 ± 0.71	2.5 ± 0.05	80.38 ± 4.7	9.33 ± 0.07
NT96	3.1 ± 0.60	2.5 ± 0.16	83.16 ± 5.4	9.73 ± 0.07
NT95	6.7 ± 0.37	5.0 ± 0.12	86.27 ± 4.5	13.51 ± 0.19
NT94	3.6 ± 0.47	2.0 ± 0.18	81.92 ± 4.3	10.75 ± 0.84

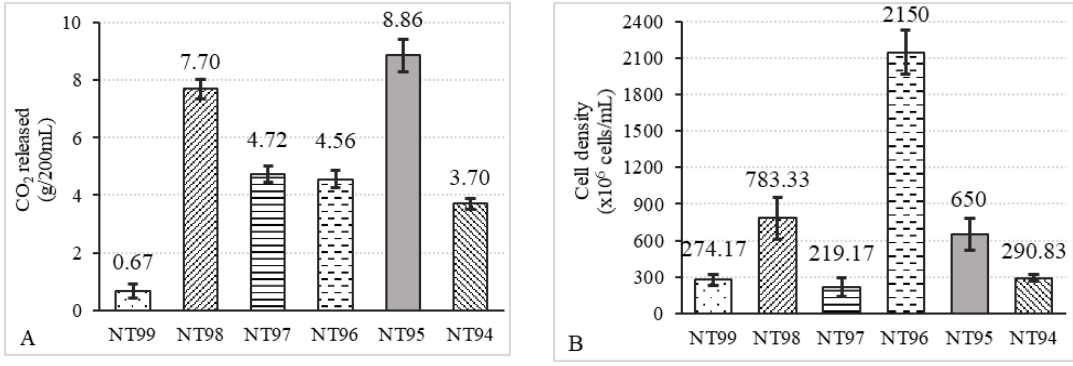

Figure 1. Total CO_2 released after 22 days of mead fermentation (A) and cell density after 80 hours of growth in fermentation medium (B) of 6 preselected strains

Data are the means of triplicate experiments ± S.D

Table 2. Given scores of 6 preselected strains

Criteria	Max. score	Strain name					
		NT99	***NT98***	***NT97***	***NT96***	***NT95***	***NT94***
Growth[a]	6	0.77	2.19	0.61	6.00	1.81	0.81
ABV[b]	10	0.60	9.31	4.95	4.95	10.00	3.96
FE[b]	6	3.73	6.00	5.21	5.40	5.60	5.32
°Brix decreased[b]	6	0.45	5.44	2.25	2.72	6.00	3.21
Sensory properties[b]	12	10.86	10.60	8.29	8.64	12.00	9.55
Total score	***40***	***16.41***	***33.54***	***21.31***	***27.71***	***35.41***	***22.85***

[a]*Growth was measured after 80 hours of incubation in honey-must.* [b]*ABV, FE, decrease °Brix and sensory properties for each strain were calculated from Table 1*

2.2.3. Identification of strain NT95

The colony and cell morphology of yeast strain NT5 are shown in Figures 2 and 3. After 120 culture hours on agar medium, the colony of strain NT95 had a diameter of 2.8 - 3.5 mm, white color, rough and shiny surface (Figure 2). After 72 culture hours on agar medium, cells of strain NT95 showed the size ranging from (2.8 – 4.3 x 3.4 – 4.9) µm, the shape of a round or short oval under light and electron microscope (Figure 2); cells often gathered in clusters of cells, reproduced by multipolar budding.

Figure 2. Colony morphology of strain NT95

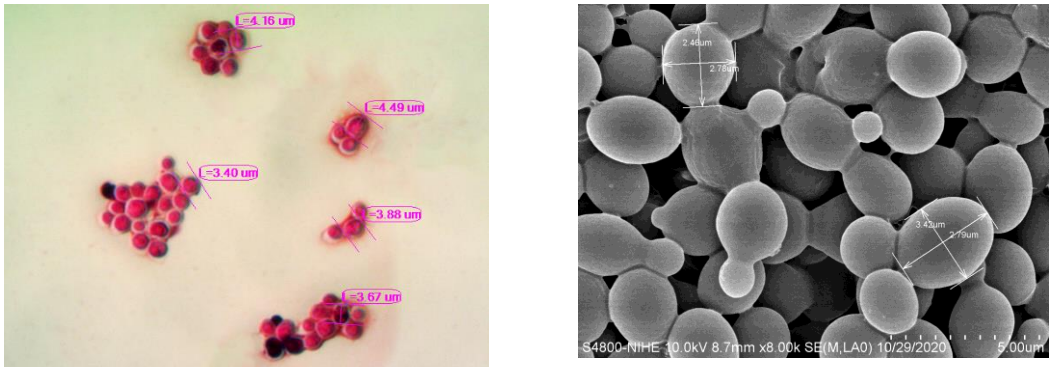

Figure 3. Cell morphology of strain NT95 under a light microscope (left) and scanning electron microscope (right)

ITS fragment of 766 bps was amplified from the total DNA sample of strain NT95 by PCR. The result from BLASTN of this ITS sequence in the NCBI database showed that ITS region of strain NT95 was more than 99% identical to that of *Zygosaccharomyces siamensis*.

The phylogenetic tree (Figure 4) shows different yeast strains which have ITS region closely related to the strain NT95. Based on this analysis, the NT95 was concluded that belong to the genus *Zygosaccharomyces* and named *Zygosaccharomyces* sp. NT95.

Zygosaccharomyces is a genus of very few yeast species found mostly in food spoilage and on the skin of fruit containing high sugar such as grape, in miso (a fermented soybean paste), in kombucha (a fermented tea beverage), balsamic and honey vinegar, etc. [19]. Due to the very similar morphology to *Saccharomyces* spp. of *Zygosaccharomyces* ssp., it is difficult to distinguish these two genera when they are present in grape must at the same time. Until using molecular markers such as ITS or D1/D2 sequence of ribosome RNA, one can separate *Zygosaccharomyces* spp. from *Saccharomyces* spp. in the family Saccharomycetacea [19]. Further studies to extend the amplicon fragment of the ITS region of the strain NT95 for comparison, and to identify more biochemical, and biophysical properties of this strain need to be done in order to confirm the species name of this strain NT95.

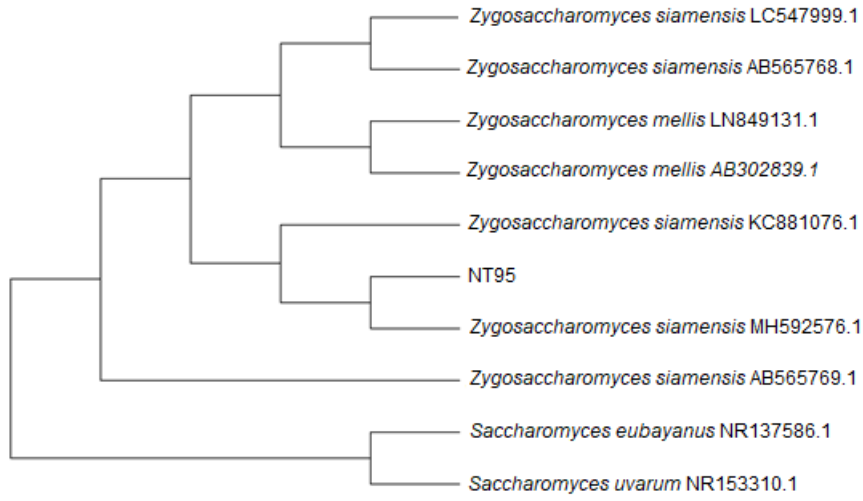

Figure 4. Phylogenetic tree of strain NT95 and related yeast strains based on the analysis of the ITS region

2.2.4. Growth of *Zygosaccharomyces* sp. NT95

Three experiments were designed to determine the effect of three factors including pH, sugar concentration, and temperature on the growth of *Zygosaccharomyces* sp. NT95. The cell density of each experiment was determined and shown in Figure 5.

Based on the above results, a suitable condition for the growth of *Zygosaccharomyces* sp. NT95 was Hansen broth containing sugar of 60 - 90 g/L, pH 4.0 - 4.5 cultured at 28-32 °C for 24 hours. In this condition, the cell density reached 371.7 ± 9.6 to 374.1 ± 9.4 ($x10^6$ cells/mL) and the rate of death cell was below 5%. *Zygosaccharomyces rouxii* grew and reached the highest cell density at 30 °C and pH 4,0 - 6,0 [20]. *Zygosaccharomyces* sp. has been found in grape must and are known to grow slowly at low temperatures, produce acetic acid during the fermentation, and be tolerant to high salt or sugar concentrations [21]. Therefore, we decided to use 30 °C for the growth of *Zygosaccharomyces* sp. NT95.

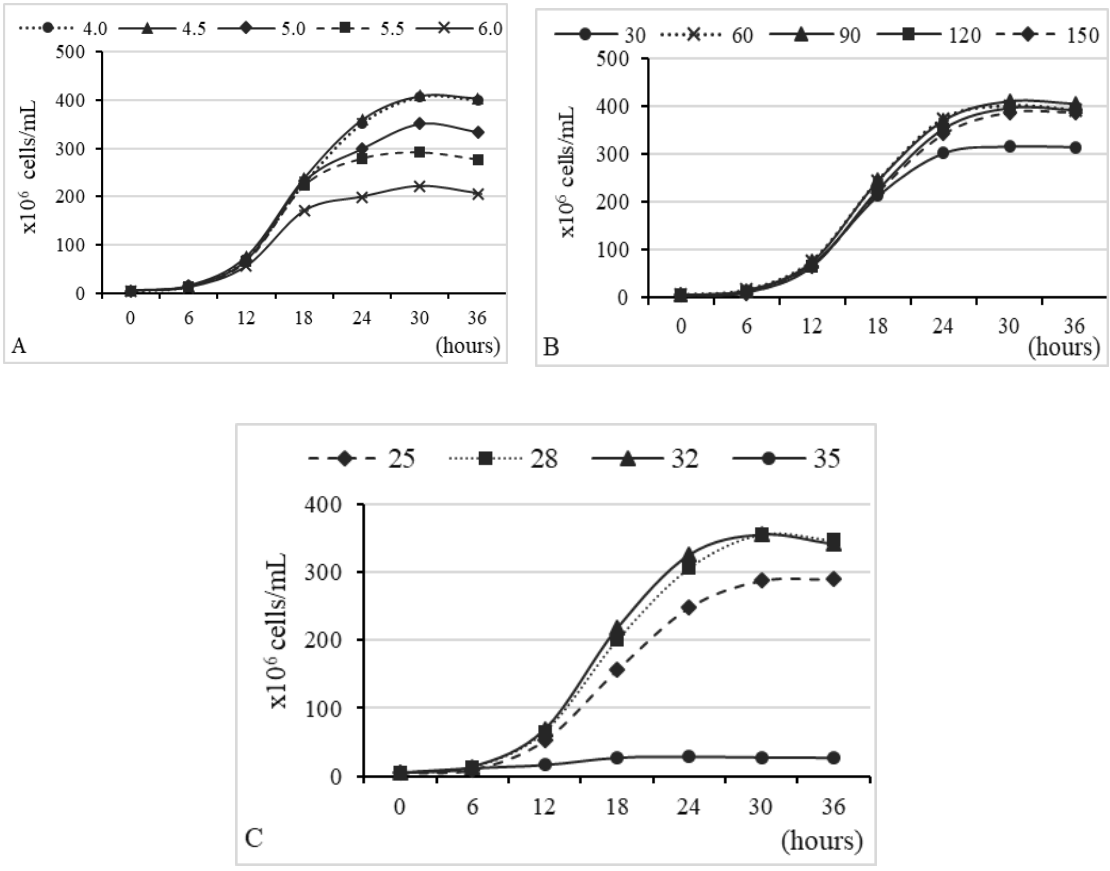

Figure 5. Effect of pH, sugar concentration (g/L), and culture temperature on growth of Zygosaccharomyces sp. NT95

Note: A- the temperature was 30 °C, sugar concentration was 100 g/L; B- the temperature was 30 °C, pH was 4.3; C- pH was 4.3, sugar concentration was 60 g/L. Data are the means of triplicate experiments ± S.D

2.2.5. Mead fermentation by *Zygosaccharomyces* sp. NT95

The studies of Romano and Suzzi (1993) on the characterization of 29 *Zygosaccharomyces* spp. isolated from 12 wineries in Italy has shown some potential of some species in wine fermentation [21]. Unlike *Saccharomyces* spp. which grow and ferment glucose rapidly, species of *Zygosaccharomyces* can grow and ferment fructose better than glucose [21]. This may make the *Zygosaccharomyces* spp. have advantages in mead fermentation due to the high content of fructose in the honey medium. Three experiments were designed in mead fermentation to determine the effect of three factors (inoculum, temperature, and time) on the mead fermentation process (Table 3).

The product of mead fermentation at 28 °C for 18 days using 5 x 10^7 cells/mL inoculum got an ABV of 4.10 ± 0.14 % and a sensory score of 14.51 ± 0.15 in line with the aim of this study to produce low alcoholic mead. To save on production expenses, the mead fermentation is suggested that it only need to last at least 15 days, but should not exceed 20 days, depending on the target ABV. In comparison to the other experiments, this product had the greatest sensory score, reaching 14.51 ± 0.15. Although this score

only got the above-average level (based on TCVN 3217 - 79), the sensory of the product may be improved during the storage and the completion stage. *Zygosaccharomyces* spp. were known to do malolactic fermentation [21] which is a common process to improve taste and flavor during wine aging.

Table 3. Parameters of fermentation products and sensory scores

Data are the means of triplicate test ± S.D

Sample	°Brix decreased	ABV (%)	FE (%)	Sensory score
Inoculum (cells/mL):				
10^7	4.4 ± 0.24	2.63 ± 0.17	81.04 ± 3.20	13.18 ± 0.02
2.5 x 10^7	4.4 ± 0.17	2.83 ± 0.05	88.28 ± 9.47	13.53 ± 0.14
5 x 10^7	6.9 ± 0.41	4.70 ± 0.45	82.48 ± 1.89	14.32 ± 0.18
10^8	8.3 ± 0.35	4.45 ± 0.15	72.57 ± 2.13	12.72 ± 0.45
Temperature (°C):				
20	3.9 ± 0.12	2.87 ± 0.09	95.36 ± 1.82	13.60 ± 0.00
24	6.1 ± 0.20	4.80 ± 0.20	84.76 ± 0.23	13.62 ± 0.06
28	6.8 ± 0.14	4.83 ± 0.12	86.52 ± 4.10	14.27 ± 0.14
32	6.4 ± 0.05	4.85 ± 0.05	79.33 ± 0.47	13.24 ± 0.52
Time (days):				
06	3.6 ± 0.15	1.07 ± 0.05	58.81 ± 1.58	13.69 ± 0.27
12	4.8 ± 0.67	2.30 ± 0.24	63.70 ± 0.50	13.99 ± 0.15
18	5.6 ± 0.12	4.10 ± 0.14	81.25 ± 2.79	14.51 ± 0.15
24	6.7 ± 0.85	4.83 ± 0.17	85.55 ± 0.76	14.36 ± 0.21

3. Conclusions

Natural honey and beeswax from Son La province are the sources of diverse yeast in colonial and cellular morphological characteristics. A strain of *Zygosaccharomyces* sp. NT95 was selected and characterized for the production of low-alcohol mead. A suitable condition for the growth of *Zygosaccharomyces* sp. NT95 was Hansen broth containing sugar of 60 - 90 g/L, pH 4.0 - 4.5, and temperature of 30 °C. A suitable condition for the mead fermentation was at 28 °C, an inoculum of 5 x 10^7 cells/mL, and the fermentation should last 18 days. The mead product from this fermentation condition contained about 4.0 - 4.5% (v/v) alcohol and its sensory properties reached the above-average level. *Zygosaccharomyces* sp. NT95 showed a potential candidate as a starter culture for the mead production. Further studies need to be done on the characterization of *Zygosaccharomyces* sp. NT95 (classification to the species, malolactic fermentation, mead aging, and food safety) before the application of this strain for mead production.

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