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Genetic diversity and some biological characteristics of antibacterial yeasts isolated from natural honey and beeswax in Son La province

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Abstract. Yeast living in honey, an environment with high sugar content (up to 70 %, w/v), normally shows good resistance to the high level of osmotic pressure; they are of high potential for application in many fields. There were not many studies on the genetic diversity and biological characteristics of yeast from honey in Viet Nam. This study aims to (1) evaluate the genetic diversity of antibacterial yeast isolated from natural honey and beeswax in Son La province by RAPD (Random Amplified Polymorphic DNA) and (2) study some biological characteristics of them. The research results may contribute to the scientific basis for screening yeast strains applied in different fields such as bioethanol and probiotic production. Sixty-eight yeast strains were isolated from natural honey and beeswax collected in Son La. Among them, twenty-one strains showed antibacterial activity against at least a tested bacterium including *Escherichia coli*, *Bacillus subtilis*, and *Serratia marcescens*. These yeast strains were genetically distinct in the RAPD analysis using M13 and $(GTG)_5$ primers. Evaluation of yeast growing in the medium containing high glucose concentration (30 - 40 %, w/v) or high ethanol concentration (5 - 10 %, v/v) has shown some yeast strains that can tolerate high osmotic pressure and high ethanol concentration for different applications. YC.8 and YC.61 strains exhibited relatively good survival rates in two phases of digestion and have a wide pH range (2 - 7). YC.8 strain expressed the most potential for human or animal probiotics.

Keywords: antibacterial, natural honey, RAPD, probiotic, yeast.

Classification numbers: 1.2.1, 1.2.5, 3.1.2.

1. INTRODUCTION

Yeast is a group of unicellular eukaryotic microorganisms that have a wide range of applications in many fields such as alcoholic beverages, food processing, bioethanol production, etc. Yeast is normally found on the surface of different flowers and fruits, on the leaves and branches of many plants, and especially found in sugar-rich materials (fruit juice, sugar beet, sugar cane, etc.) [1]. Osmotic-tolerant yeast has been found in some harsh environments as

seawater [2] and honey [3]. Yeast living in such an environment may show potential application in the food processing, pharmacy, and chemical industries [2, 4].

Honey is a natural product that contains high sugar content (fructose: 38 %; glucose: 31 %; sucrose: 1 %; maltose: 7 %) and low water activity (about 17 %) [5, 6]. Due to its high nutritional value and some medicinal properties, honey has been used directly in confectionery, beverages, food processing, etc., or in the treatment of surface infection, wound healing, cough, and intestinal infection [6]. An environment with low water activity and high osmotic pressure causes stress for microorganisms living in honey. Therefore, one that can live in honey often exhibits special tolerability characteristics. Research articles since 2000 have reported the isolation of yeast and mold from honey [3, 7, 8]; some investigated honey yeast as a valuable enzyme producer [4], as probiotics for humans and livestock [9], or as a producer of alcohol in bio-ethanol production [10]. Antibacterial property has been reported in lactic acid bacteria isolated from honey [11]; however, there is no report on this activity in honey yeast.

Son La is a mountainous province in the Northwest region of Viet Nam, where the climate and soil are suitable for diverse plants. A large natural forest area with many species of flowering plants creates favorable conditions for honey bees to develop and produce high-quality honey. In this study, we report preliminary results in studying genetic diversity and biological characteristics of yeast strains with antibacterial activity isolated from natural honey and beeswax in two districts (Moc Chau and Yen Chau) of Son La province. This result may create a premise for screening new yeast strains with the potential for different applications.

2. MATERIALS AND METHODS

2.1. Materials

Materials: Natural honey and beeswax samples were collected in March and October 2020 at different locations in Moc Chau and Yen Chau districts, Son La province, Viet Nam. Samples were stored in sterile glass bottles with tight-fitting lids.

The chemicals used in this study were purchased from Merck (Germany) and Bioneer (South Korea); all of those were of analytical grade.

Medium: The medium used in the isolation and cultivation of yeast strains is the modified Hansen medium (G90) with the following components (g/L): Glucose, 90; KH_2PO_4, 3; $MgSO_4.7H_2O$, 2; Peptone, 10; Agar, 20; pH 5. The liquid medium contains no agar.

RAPD-PCR primers: M13 (5’ GAGGGTGGCGGTTCT 3’) and $(GTG)_5$ (5’ GTGGT GGTGGTGGTG 3') [12].

Testing microorganisms: *Escherichia coli* B and *Bacillus subtilis* 168 were purchased from Novagen, USA; *Serattia marcescens* VTCC-10141 was purchased from Vietnam Type Culture Collection, Institute of Microbiology and Microbiology, Hanoi National University.

2.2. Methods

Isolation of yeast: The honey and ground beeswax samples were diluted in sterilized saline solution (0.9 % NaCl) to reach the concentrations of 10^{-3}, 10^{-4}, 10^{-5} (v/v for honey and w/v for beeswax), and then 200 µL of each dilution was spread on G90 agar plates and incubated at 32 °C for 48 h. Colonies with different morphology were selected and streaked to new G90 agar plates, incubated at 32 °C for 48 hours, and then stored at 4 °C for further studies [13].

Total DNA extraction: Yeast strains were cultivated in G90 broth at 32 °C for 48 hours, and yeast cells were collected by centrifuge and suspended in 600 µL of lysis buffer. After incubating at 65 ^{o}C for 1 hour, the lysates were extracted twice with chloroform: isoamyl alcohol (24:1) solution and centrifuged at 13,000 rpm for 15 minutes. DNA from the aqueous phase was precipitated in two volumes of cold ethanol (supplemented with CH_3COOK 5M pH 5) for 2 hours at -20 °C, washed with 70 % ethanol, and air-dried before being suspended in 30 µL of TE buffer [14].

RAPD-PCR reaction: Application reactions contained 10 µL of master mix, 1 µL of primer, 1 µL of a total DNA sample, and 8 µL of water to reach a final volume of 20 µL. The thermocycler was set with 40 cycles (94 ºC for 60 seconds, 45 ºC for 20 seconds, and 72 ºC for 120 seconds), starting with an initial denaturation at 95 ºC for 60 seconds and finishing with a final elongation at 72 ºC for 5 minutes [15].

Electrophoresis method: The PCR products were separated on 1.5 - 2.0 % agarose gel; electrophoresis was run at 70V and 80 mA for 80 minutes.

Bioinformatics method: The electrophoresis gels were photographed and analyzed by GelQuest, a software to determine the size of the electrophoresis bands. The data on the images were converted into binary data using MVSP software and analyzed the polymorphism of the yeast strains which was based on the Jaccard similarity coefficient and UPGMA method.

Determination of antibacterial activity using agar well diffusion method: The agar plate surface was inoculated by spreading a volume of testing microorganisms (*Escherichia coli* B or *Bacillus subtilis* 168 or *Serattia marcescens* VTCC-10141) over the entire agar surface. Then, holes were punched with a diameter of 10 mm, and 50 µL of the cell-free - culture broth at 48 hours (microbial biomass was removed by centrifuging at 8000 rpm for 10 minutes) was added into the hole. The agar plate was kept at 4 ^{o}C for 12 hours before being incubated at 37 ^{o}C for 24 hours. Then the antibacterial activity of each yeast strain was evaluated by the size of the inhibition zone [16]. Negative control (sterilized water) and positive control (ampicillin 10 mg/mL) were included in each tested plate.

Determination of yeast growth: G90 broth was used for the cultivation of yeast strains in an orbital shaker at 200 rpm at 32 ^{o}C. The OD_{600} of 0.1 was the initial inoculum. Cell density was determined by turbidity measurement (OD_{600}) after 48 hours of cultivation and used to evaluate the yeast growth. Each test was done in triplicate.

Osmo-tolerance and ethanol tolerance test: Yeast strains were cultured in Hansen broth containing glucose concentrations of 10, 20, 30, and 40 % (w/v) for the osmotolerance test [17]; or containing ethanol concentrations of 5 and 10 % (v/v) for the ethanol tolerance test. The beginning inoculum was OD_{600} of 0.1. Cultivation was done in an orbital shaker at 32 ^{o}C, 200 rpm. Cell density was determined by turbidity measurement (OD_{600}) after 48 hours of cultivation and used to evaluate the growth of all yeast strains under different stress conditions. Each test was in triplicate.

Measurement of yeast growth at different pH: Yeast strains were incubated in an orbital shaker at 32 ^{o}C and 200 rpm in a G90 broth with a pH range from 2 to 8. The growth of yeast strains was evaluated based on OD_{600} after 48 hours of cultivation [17]. Each test was in triplicate.

In vitro two-phase digestion method [18]

- The first phase: Dilute yeast culture broth (100 µL) was added to 900 µL solution of 0.1 M HCl pH 2 to a density of 10^4 cells/mL and incubated for 90 minutes at 37 ^{o}C [18]. Then, 60 µL of the mixture was spread on a G90 agar plate, incubated at 32 ^{o}C for 48 hours, and counted

for colonies forming. The control was at the same concentration of yeast culture which was added to the same volume of the sterilized saline water. Each test was in triplicate.

- Second phase: 100 µL of culture from the first phase was added to 900 µL of 2 % bile saline in 1M $NaHCO_3$ to a density of 10^3 cells/mL and incubated for 3 hours at 37 ℃ [18]. And then, 60 µL of the mixture was spread on a G90 agar plate, incubated at 32 ^oC for 48 hours, and counted for colonies forming. The control was at the same concentration of yeast culture which was added to the same volume of the sterilized saline water. Each test was in triplicate.

2.3. Theoretical background

Microorganisms living in harsh conditions have their way to thrive their environments. Yeast living in honey often shows especially tolerant characteristics to the condition of low water activity and high osmotic pressure.

3. RESULTS AND DISCUSSION

3.1. Isolation and screening for antibacterial yeasts from natural honey and beeswax

Table 1. Antibacterial activity of isolated yeast strains

Isolated strain	Diameter of inhibition zone against testing bacteria (mm)		
	E. coli	*B. subtilis*	*S. marcescens*
YC.1	-	15.78 ± 0.05	-
YC.2	-	15.64 ± 0.05	12.18 ± 0.16
YC.4	-	13.51 ± 0.12	
YC.7	14.21 ± 0.05	16.14 ± 0.12	11.40 ± 0.17
YC.8	-	13.74 ± 0.06	11.89 ± 0.10
YC.10	12.23 ± 0.06	-	-
MC.11	10.94 ± 0.12	-	-
MC.12	12.07 ± 0.11	-	-
MC.13	-	12.59 ± 0.03	12.89 ± 0.04
MC.15	12.40 ± 0.00	-	-
MC.16	12.65± 0.05	-	-
MC.17	14.74 ± 0.06	-	11.03 ± 0.25
MC.20	-	13.60 ± 0.01	12.84 ± 0.07
MC.30	11.57 ± 0.05	-	-
MC.37	14.70 ± 0.70	-	-
MC.38	-	11.47 ± 0.14	-
MC.45	-	12.51 ± 0.02	-
MC.48	-	11.64 ± 0.07	-
YC.60	10.97 ± 0.15	-	-
YC.61	10.57 ± 0.11	-	-
YC.64	13.78 ± 0.03	-	-

Note: (-) No clear zone appeared

After two sampling sessions, 68 yeast strains were isolated from 6 samples of natural honey and beeswax collected in 02 districts (Moc Chau and Yen Chau) in Son La province. The yeast density in these six samples of honey and beeswax was approximately 31 - 88 × 10^2 CFU/g. Among these 68 yeast strains isolated, 21 strains were recorded with antibacterial activity against 1, 2, or all three testing microorganisms (*E. coli, B. subtilis,* and *S. marcescens*) (Table 1).

Strain YC.7 showed antibacterial activity against all three types of testing microorganisms; strains YC.1, YC.2, MC.7, MC.17, and MC.37 showed good antibacterial activity with a diameter of the clear zone of more than 14 mm; strains YC.2, YC.8, MC.13, and MC.20 could inhibit two testing microorganisms, *B. subtilis* (Figure 1) and *S. marcescens*. Honey is traditionally believed to have good medicinal properties (antiseptic, treatment of intestinal diseases and upper respiratory tract infections, etc.). Kwakman *et al.* in 2011 reported the antibacterial activity of Revamil honey is due to the presence of Defensin-1 (a small peptide in bee venom glands, which has an antibiofilm activity to destroy the capsule of bacteria) and H_2O_2 accumulated in honey [19]. However, the research group also confirmed that these active ingredients are not present in manuka honey (a type of honey believed to have high antibacterial activity); a small part of the antibacterial activity of manuka honey may be due to the active ingredient 1,2-dicarbonyl methylglyoxal (MGO) [19]. The antibacterial activity detected from yeast strains isolated from natural honey and beeswax in this study may demonstrate a role for yeast to contribute partly to these medicinal properties. The discovery of yeasts with antibacterial activity in honey showed the potential of these yeast strains in the production of probiotic products for humans and animals.

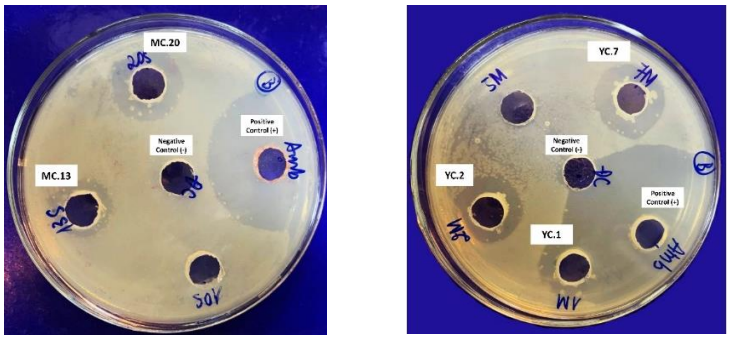

Figure 1. Antibacterial activity of some isolated yeast strains against *Bacillus subtilis*
Note: (-) negative control: sterilized water; (+) positive control: ampicillin

3.2. Genetic diversity of antibacterial yeast isolated from natural honey and beeswax

Figure 2 shows a genetic relationship tree of 21 antibacterial yeast strains based on the UPGMA method. After RAPD-PCR reactions and electrophoresis, the analysis showed 186 bands with different sizes (ranging from 300 bp to 4300 bp); all bands are polymorphic. This genetic tree showed that each yeast strain differed from the other strains from 15 – 100 %; almost all of them differed from 82 – 100 % (Figure 2). At 15 % similarity, 21 yeast strains were divided into 18 groups: group 1 (YC.1 and YC.2), group 2 (MC.48), group 3 (MC.45), group 4 (MC.20), group 5 (MC.11), group 6 (YC.4), group 7 (YC.8), group 8 (YC.7), group 9 (YC.10), group 10 (MC.17), group 11 (MC.30), group 12 (MC.12), group 13 (MC.15 and MC.16), group 14 (MC.13), group 15 (MC.37 and MC.38), group 16 (YC.60), group 17 (YC.64) and group 18 (YC.61).

The similarity between strains was low, indicating that these antibacterial yeast strains may belong to many different genera and species, showing much potential for exploitation and practical application.

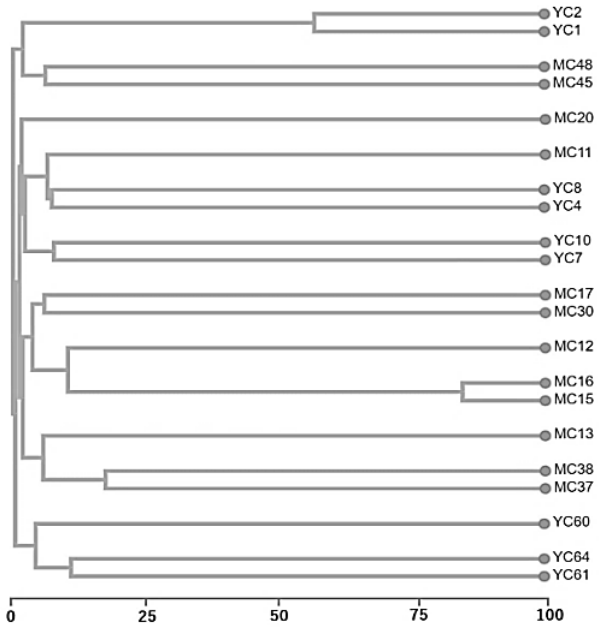

Figure 2. Genetic relationship tree of antibacterial yeast strains

Note: The numbers on the bar show the degree of similarity (%) between yeast strains.

3.3. Some biological characteristics of antibacterial yeast isolated from natural honey and beeswax

3.3.1. Growth of antibacterial yeasts

The growths of 21 antibacterial yeast strains were evaluated by turbidity (OD_{600}) in G90 broth after 48 hours of cultivation (Data not shown). Among 21 antibacterial yeast strains, 17 of them showed good growth (the value of OD_{600} was over 10 after 48 hours of cultivation in G90 broth). Other strains showed medium growth with the value of OD_{600} ranging from 6 to 10. Strain YC.8 and MC.13 were the best growth ability with OD_{600} values of 11.88 and 12.17, respectively.

3.3.2. Growth of antibacterial yeasts at high concentrations of sugar

The growth of antibacterial yeast strains in high sugar concentration media was checked after 48 hours of cultivation in a Hansen medium containing glucose concentrations of 100, 200, 300, and 400 (g/L). Most strains were able to grow at a high sugar concentration medium (400 g/L glucose) reaching an OD_{600} value over 5 compared to the initial OD_{600} of 0.1. Strains YC.1, YC.2, YC.8, MC.15, MC.17, MC.20, MC.48, and YC.60 achieved OD_{600} over 10 in a medium containing 400 g/L of glucose (Table 2). Strain YC.8, MC15, MC.48, and YC.60 showed great potential for industrial starters since they could grow at a wide range of substrate concentrations and tolerate high osmotic pressure (400 g/L glucose, Figure 3).

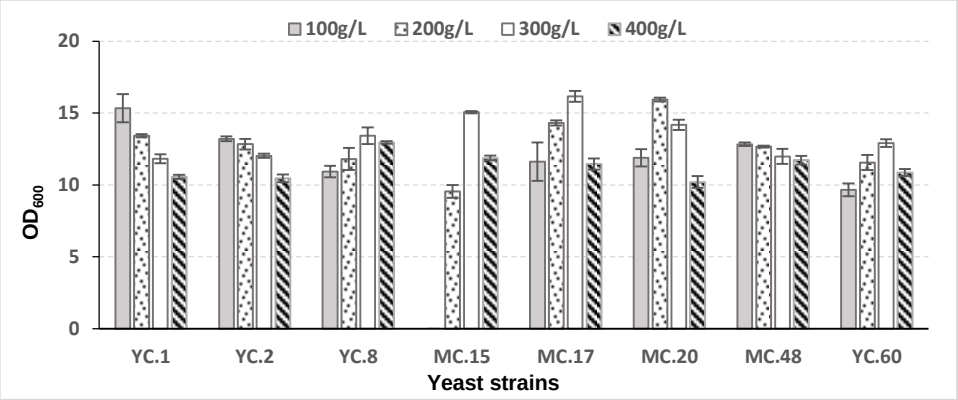

Figure 3. Effect of high concentrations of glucose (100 - 400 g/L) on the growth of eight promising yeasts strains

Table 2. Growth of antibacterial yeasts at high concentrations of sugar

Strain	OD_{600} after 48 hours of cultivation in liquid media containing different concentrations of glucose			
	100 g/L	200 g/L	300 g/L	400 g/L
YC.1	15.33 ± 0.98	13.43 ± 0.12	11.83 ± 0.31	10.57 ± 0.15
YC.2	13.21 ± 0.17	12.84 ± 0.37	12.03 ± 0.14	10.47 ± 0.26
YC.4	12.64 ± 0.61	14.21 ± 1.12	11.67 ± 0.24	6.54 ± 0.35
YC.7	15.87 ± 0.50	12.49 ± 0.42	11.52 ± 0.58	9.05 ± 0.13
YC.8	10.93 ± 0.40	11.81 ± 0.76	13.43 ± 0.58	12.96 ± 0.08
YC.10	9.74 ± 0.55	12.53 ± 0.85	14.11 ± 0.36	7.65 ± 0.05
MC.11	15.73 ± 0.58	15.768 ± 0.61	11.85 ± 0.19	9.33 ± 0.19
MC.12	-	6.57 ± 0.42	11.64 ± 0.70	7.83 ± 0.14
MC.13	6.00 ± 0.03	6.94 ± 0.08	9.50 ± 0.03	7.87 ± 0.31
MC.15	-	9.55 ± 0.45	15.07 ± 0.08	11.87 ± 0.17
MC.16	15.13 ± 0.94	12.74 ± 0.54	5.56 ± 0.30	8.92 ± 0.32
MC.17	12.39 ± 0.36	14.32 ± 0.18	16.16 ± 0.38	11.46 ± 0.38
MC.20	11.90 ± 0.60	15.94 ± 0.14	14.17 ± 0.36	10.21 ± 0.41
MC.30	7.18 ± 0.24	12.22 ± 0.47	8.75 ± 0.52	5.67 ± 0.40
MC.37	11.54 ± 0.74	13.03 ± 0.18	6.36 ± 0.40	-
MC.38	11.31 ± 0.53	10.97 ± 0.92	11.76 ± 0.27	7.93 ± 0.24
MC.45	15.77 ± 0.95	11.98 ± 0.22	8.65 ± 0.15	-
MC.48	12.83 ± 0.12	12.67 ± 0.07	11.99 ± 0.53	11.72 ± 0.31
YC.60	9.67 ± 0.45	11.56 ± 0.53	12.91 ± 0.27	10.87 ± 0.24
YC.61	17.9 ± 0.44	13.17 ± 0.75	12.52 ± 0.43	8.56 ± 0.39
YC.64	16.26 ± 0.30	14.86 ± 0.56	13.97 ± 0.25	7.80 ± 0.28

Note: (-) not done or no replication

Ortiz-Muñizet *et al.* reported *Saccharomyces cerevisiae* ITV-01 isolated from sugar cane molasses has shown that optimal sugar concentration for the growth of this strain was in the range of 100 - 200 g/L glucose [20]. Compared to that, our isolated yeast strains from honey and beeswax showed more osmotolerant since most of them showed optimal growth at 200 - 300 g/L glucose (13/21 strains, Table 2). This result proved the origin of these yeast strains from the osmotic environment (honey and bee wax).

The osmotic pressure of the medium has a great influence on the growth and fermentation efficiency of yeast [21]. Therefore, yeast strains that show the ability to grow well in the medium with high sugar concentration are those that are expected to become good producers in bioethanol production and the food industry [22, 23].

3.3.3. Growth of antibacterial yeasts at high concentrations of ethanol

Yeast strains used in the production of alcoholic beverages are often able to tolerate the ethanol that they produce [24]. In this experiment, the ability of antibacterial yeast strains to tolerate high concentrations of ethanol was evaluated. Results showed that: At an ethanol concentration of 5 % (v/v) and an initial OD_{600} value of 0.1, there were 20 out of 21 strains recorded an increase in cell density after 48 h cultivation in G90 broth although the increases were very less compared to the control samples (at 0% ethanol). Strain YC.8, MC.15, YC.60, YC.61, and YC.64 were the best tolerant since they could grow with a value of OD_{600} over 4 which were 50 % less than those of the controls after 48 h of cultivation in G90 broth (Figure 4). The research conducted by Silva *et al*. on the biological activities of yeast strains isolated from honey and pollen, including alcohol tolerance, showed that in YEP medium adding 5 % (v/v) ethanol after 24 hours of culture, the OD_{600} values of the studied yeast strains ranged from 0.35 to 1.4; by adding 10 % (v/v) ethanol, the studied yeast strains have OD_{600} values in the range of 0.1 - 1.3 [25]. Compared to this reported data, most of the yeast strains in our study show similar growth except the five strains (YC.8, MC.15, YC.60, YC.61, and YC.64); those showed 3 to 4 times better growth than what reported by Silva *et al*. [25].

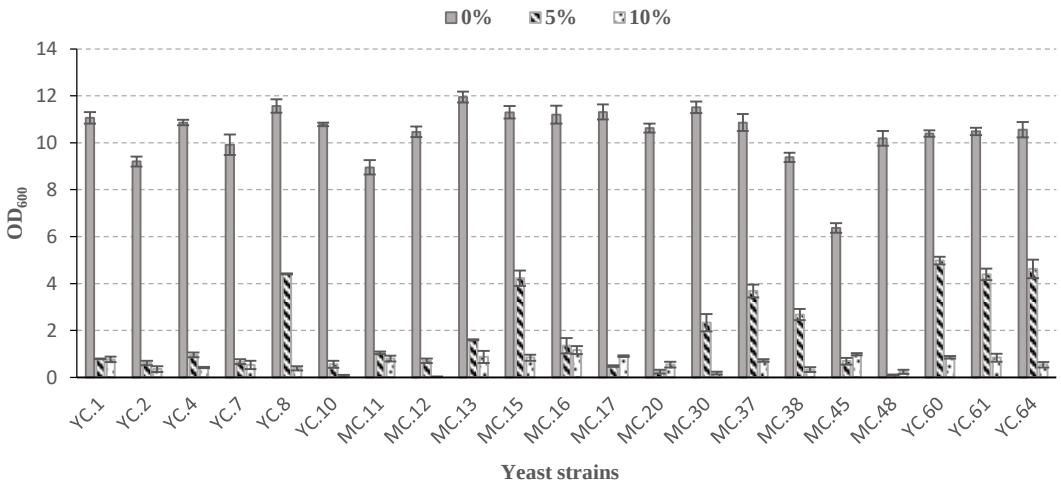

Figure 4. Growth of antibacterial yeasts at a high concentration of ethanol (5 - 10 %).

When ethanol concentration increased to 10 % (v/v), only two yeast strains including MC.13, and MC.16 reached OD_{600} value over 1 after 48 hours of cultivation in G90 broth (Table

3). Among 21 antibacterial yeast strains, 19 of them have OD_{600} values above 0.2 after 48 hours of cultivation, so these strains had limited growth in the medium containing an ethanol concentration of 10 % (v/v). This result is similar to what Silva *et al.* [25] reported on yeast strains isolated from honey and pollen. It also proves that the yeast in honey and beeswax is not a good candidate for alcoholic fermentation. Other experiments recorded that none of these 21 antibacterial yeast strains could produce more than 2 % of ethanol in Hansen medium containing 150 g/L glucose after 4 days of fermentation (data not shown).

3.3.4. Growth of antibacterial yeasts at different pH and their survival in vitro two-phase digestion

Humans and monogastric animals have two digestive phases: gastric and intestinal phases. Microorganisms that survive in these two phases of digestion may be used as probiotics. *In vitro*-two phases digestion experiments were carried out for 21 strains of antibacterial yeasts and the results showed that: At the first phase in the digestive system (pH 2), only two strains of yeast YC.8 and YC.61 expressed more than 50 % of survival rate (Figure 5). Meanwhile, in the second phase (having 2 % bile salt), almost all strains survived well; among 21 strains, seven show survival rates of less than 50 % (Figure 5).

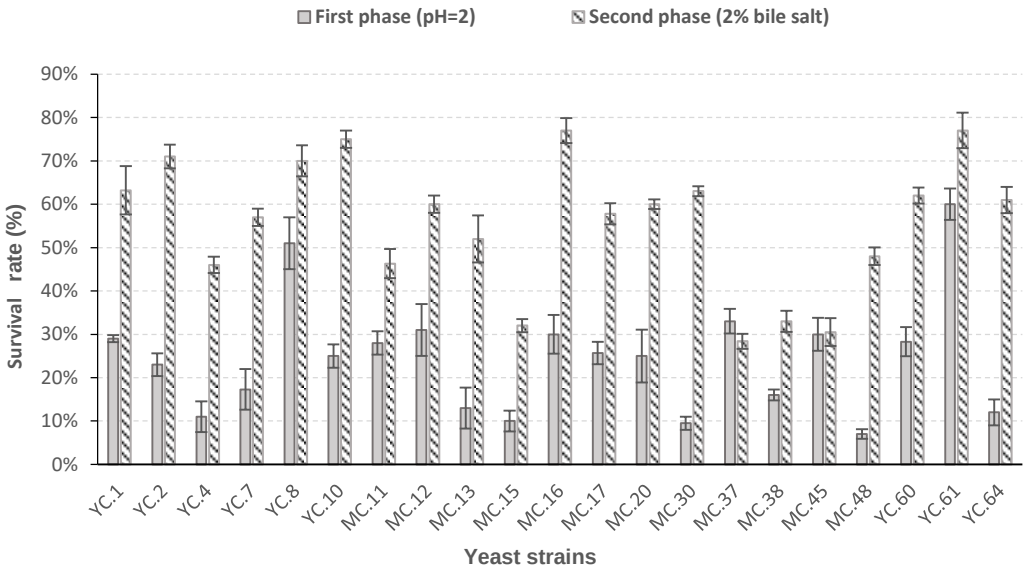

Figure 5. The survival rate of antibacterial yeasts in two-phase digestion (compared to the control)

The strains YC.1, YC.8, and YC.61 had a relatively wide range of pH for growth (pH 2 - 7) compared to the other yeast strains (data not shown), so these strains may adapt well to pH conditions in the digestive system of humans and animals. These strains need to be further evaluated in further experiments for probiotic production.

4. CONCLUSIONS

Yeast strains with antibacterial ability isolated from natural honey and beeswax in Son La province are highly genetically diverse, possibly belonging to different genera or species. Some yeast strains showed good antibacterial activity or inhibited the growth of 2 out of 3 testing

bacteria (YC.2, YC.7, YC.8, MC.13, and MC.20). Evaluation of other biological characteristics showed that some strains as YC.1. YC.2, YC.8, MC15, MC.17, MC.20, MC.48 and YC.60 showed good growth at 40 % glucose; they may be applied in alcohol production and the food industry where a high level of substrate concentration is used. Although most of these strains did not produce high levels of ethanol, some of them (YC.8, MC.15, YC.60, YC.61, and YC.64) showed good resistance to 5 % ethanol (3 to 4 times better than those reported by Silva *et al*. [25] on yeast strains isolated from honey and pollen. Strains YC.8 could inhibit the growth of two testing microorganisms (*B. subtilis* and *S. marcescens*), could grow at a wide pH range, showed more than 50 % survivability in the digestive system, and had high sugar tolerance. This strain has good potential in probiotic production for humans or animals.

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Authorship contribution statement. Pham Ngoc Anh contributed to the experiments and analysis on RAPD, and wrote the 1st draft of the manuscript; Ha Kieu Anh contributed to the experiments and data analysis on biological characteristics of yeast; Tran Thi Thuy supervised all experiments, did a formal analysis, and finalized the manuscript.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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