BIODIVERSITAS
Volume 24, Number 7, July 2023
Pages: 4126-4135

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d240751

Isolation and identification of osmophilic yeasts from Indonesian honeys collected from South Kalimantan, Banten, and East Java Provinces

MEILIA PRIHARTINI, SYOFI ROSMALAWATI♥, CATUR SRIHERWANTO♥♥, ANIS HERLIYATI MAHSUNAH

Research Centre for Applied Microbiology, National Research and Innovation (BRIN). Jl. Raya Jakarta-Bogor Km 46, Cibinong, Bogor 16911, West Java, Indonesia. Tel.: +62-21-31924703, Fax.: +62-21-3911-789, ♥email: syofi.rosmalawati@brin.go.id, ♥♥catur.sriherwanto@brin.go.id

Manuscript received: 12 June 2023. Revision accepted: 26 July 2023.

Abstract. *Prihartini M, Rosmalawati S, Sriherwanto C, Mahsunah AH. 2023. Isolation and identification of osmophilic yeasts from Indonesian honeys collected from South Kalimantan, Banten, and East Java Provinces. Biodiversitas 24: 4126-4135.* Certain yeasts possess the remarkable ability to thrive in the challenging conditions characterized by high sugar levels and low water activity commonly found in honey. However, limited research has been conducted on yeasts isolated from honey in Indonesia. To address this gap, honey samples were collected from three provinces in Indonesia: South Kalimantan, Banten, and East Java. A total of ten isolates were obtained and subjected to comprehensive morphological, physiological, and biochemical characterization. Among these isolates, four (M 1.4, M 1.6, M 1.8, and M 2.4) were selected for molecular identification using ITS1 and ITS4 primers. Through sequencing of the Internal Transcribed Spacer (ITS) region and performing homology analysis using BLAST on NCBI, it was determined that the M 1.4 and M 1.8 isolates belong to the *Zygosaccharomyces* genus with a percentage identity of ≥97%. The M 2.4 isolate was classified as the *Schizosaccharomyces* genus with a percentage identity of ≥98%, while the M 1.6 isolate was identified as *Zygosaccharomyces siamensis* with a percentage identity of ≥99%. All four isolates exhibited osmophilic characteristics and demonstrate significant potential as valuable microorganisms for future investigations.

Keywords: Honey, ITS, molecular identification, osmophilic, yeast

INTRODUCTION

Honey exhibits a complex composition characterized by a diverse array of chemical constituents encompassing sugars, polyphenols, flavonoids, hydroxymethylfurfural, hydrogen peroxide, methylglyoxal, proteins, enzymes, vitamins, and minerals (Mohammed 2022). Moreover, honey displays distinctive physical attributes encompassing color, aroma, taste, and conductivity (Mohammed 2022). Sugars constitute a significant proportion, accounting for 95-99% of the dry-weight component of honey, with fructose and glucose as primary monosaccharides constituting approximately 60% of the total sugar content (Rao et al. 2016). The notable abundance of sugars in honey contributes to its elevated osmotic pressure (osmolarity), low pH, and presence of antimicrobial agents, collectively constraining the microbial community within honey (Silva et al. 2017; Snowdon and Cliver 1996).

Honey has been observed to support the growth of various microorganisms, such as yeast and spore-forming bacteria (Snowdon and Cliver 1996). Notably, yeast is the prevailing microbial constituent, with an estimated density of approximately 100 colony-forming units per gram (cfu/gram) (Snowdon and Cliver 1996). Investigations have identified several yeast species commonly found in honey, including *Debaryomyces hansenii*, *Zygosaccharomyces rouxii*, *Zygosaccharomyces mellis*, *Aureobasidium pullulans*, and *Cryptococcus uzbekistanensis* (Sinacori et al. 2014).

Yeast, a member of the fungal group, undergoes asexual reproduction through budding or fission, without involving a sexual phase either internally or on the fruiting body (Kurtzman et al. 2011). Yeast's contribution to various industries is significant, thanks to its capacity for fermenting substrates and generating beneficial products like vinegar, baked goods, cheese, sausages, and more (Kurtzman et al. 2011). Its applications extend to the production of ethanol, enzymes, single-cell proteins, and other simple metabolites, along with its role in animal feed production (Kurtzman et al. 2011; Alnaimy and Habeeb 2017).

Observation of physical, physiological, and biochemical traits is employed to identify yeasts, including tests involving fermentation, assimilation of sugars and nitrogen compounds, as well as the examination of their sexual and asexual forms. These phenotypic assessments, although time-consuming and requiring expertise for result interpretation, serve as a means for yeast identification. Nevertheless, they lack the ability to differentiate yeasts at the intraspecific level during fermentation processes. Alternatively, molecular DNA-based techniques provide enhanced discrimination, sensitivity, and autonomy from gene expression and environmental influences. As a result, they prove more effective than traditional phenotypic methods for yeast identification and characterization (Ceccato-Antonini 2022).

Utilizing the Polymerase Chain Reaction (PCR) technique and sequencing specific genes like the Internal

Transcribed Spacer (ITS) region within the ribosomal DNA (rDNA) is crucial for molecular identification of yeasts (Pincus et al. 2007). The ribosomal DNA (rDNA) subunit region comprises conserved domains and variable domains with species-specific characteristics. Therefore, various rDNA regions serve as universal PCR target primers or primers specific to genera and species (Pincus et al. 2007). Despite the recent advancements in molecular identification methods, it is highly recommended to combine morphological and molecular data whenever possible for fungal identification purposes (Raja et al. 2017).

Studies on molecular identification of honey-isolated yeasts have been reported. For example, fifteen yeast strains, classified as *Yarrowia lipolytica*, *Candida magnolia*, and *Starmerella magnoliae*, were isolated and identified from a lime honey sample obtained from Poland. The identification process involved the utilization of three distinct methods, namely biochemical analysis, MALDI TOF/MS (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry), and identification based on rDNA sequences (Ziuzia et al. 2023). Five yeasts were isolated from honeys in Brazil, and were molecularly identified as *Zygosaccharomyces mellis*, *Pseudozyma* sp., *Sympodiomycopsis* sp., *Aureobasidium pullulans*, and *Sporisorium elionuri*. The identification involved analyzing the molecular sequences of the D1/D2 region of the large subunit (LSU) rRNA gene and the internal transcribed spacer (ITS) region (Rodrigues et al. 2018). It is worth noting that the molecular identification of yeasts present in Indonesian honeys has still received limited attention. Therefore, this research aimed to isolate yeast strains from honey samples collected in three Indonesian provinces, namely South Kalimantan, Banten, and East Java. The main objective was to identify osmophilic yeasts that could serve as valuable genetic resources for the future development and exploration of innovative bioproducts. To achieve this, a comprehensive approach was employed, encompassing morphological, physiological, and biochemical characterization methods, along with molecular identification through sequencing the Internal Transcribed Spacer (ITS) region.

MATERIALS AND METHODS

Yeast isolation

Three honey samples were used in this study. The samples were obtained from the Agriculture Service of South Barito Regency, South Kalimantan Province (M 1); Lebak Regency, Banten Province (M 2); and Malang City, East Java Province (M 3). South Kalimantan's wild honey was obtained directly from the forest, while Banten honey was sourced from the local community, and Malang honey underwent industrial processing. Each honey sample (1 mL) was pipetted into 50 mL of yeast extract peptone dextrose broth (YPDB) and incubated in a shaker incubator at 28 ± 2°C for 24-72 hours. The resulting culture was serially diluted by pipetting 1 mL into 9 mL of sterile distilled water, reaching a dilution of 10^{-7}. The 10^{-6} and 10^{-7}-diluted cultures were each inoculated onto yeast extract peptone dextrose agar (YPDA) media in petri dishes using the spread plate technique. Cultures were then incubated for 24-72 hours at 28 ± 2°C. The entire study was conducted in two repetitions. Colonies exhibiting distinct characteristics such as colony color, shape, edge shape, elevation, and surface were observed and recorded. Colonies with different characteristics were isolated and inoculated onto YPDA media using the streak plate technique. Cultures were incubated for 24-72 hours at 28 ± 2°C. The resulting colonies were examined under a microscope (Olympus CX 41, Japan) to confirm purity, and pure colonies were subsequently inoculated onto YPDA slant media for further analysis. Both YPDA and YPDB were prepared according to Xue et al. (2023).

Identification of colony and cell morphology

Colony and cell morphology were observed under a microscope (Olympus CX 41, Japan) using 48-hour-old yeast culture grown on YPDA. The colony morphology, including texture, shape, color, elevation, surface, and margin, was recorded.

Biochemical identification

Biochemical identification involved fermenting carbohydrates (glucose, sucrose, and maltose) and conducting growth assays in 60% glucose media. For the carbohydrate fermentation test, a basal fermentation medium was prepared by mixing 4.5 g of yeast extract and 7.5 g of peptone in 1 L of demineralized water. To this solution, 4 mL of Bromothymol Blue stock solution was added per 100 mL of basal fermentation medium, followed by the addition of a 2% sugar solution. The medium was then sterilized using an autoclave at 121°C for 15 minutes. For the growth test in 60% glucose media, 22.5 g of agar was dissolved in 1% yeast infusion solution and supplemented with 600 g of glucose. The medium was sterilized using an autoclave at 121°C for 15 minutes. The carbohydrate fermentation test was carried out by inoculating 48-hour-old isolates into each fermentation medium and incubating them at 28 ± 2°C for 7 days. Simultaneously, the growth test in 60% glucose media was conducted by inoculating 48-hour-old isolates onto streak plates containing 60% glucose media, followed by incubation at 28 ± 2°C for 2 days.

Yeast DNA extraction

Yeast DNA extraction was performed using the PrepMan™ Ultra Sample Preparation Reagent kit (ThermoFisher Applied Biosystems, USA). For each 48-hour yeast isolate, 1 loopful was suspended in 60 µL of PrepMan™ Ultra Sample Preparation Reagent (Applied Biosystems, USA) in a 2 mL microtube. The sample was vortexed for 10-30 seconds and heated at 100ºC for 10 minutes using a heat block. Subsequently, the sample was cooled to room temperature (28 ± 2°C) for 2 minutes and then centrifuged at 15,000 rpm, 15ºC for 3 minutes. The resulting supernatant was transferred into a new microtube and stored at 4°C in a refrigerator for further analysis.

The concentration and purity of yeast DNA were determined using a NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). A total of 1 µL of DNA sample from each isolate was analyzed for concentration and purity at a wavelength ratio of 260/280. A ratio of approximately 1.8 indicated pure DNA, as assessed by the NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific, USA).

DNA amplification was carried out using the Polymerase Chain Reaction (PCR) technique with the Ex Taq DNA polymerase (Takara, Japan) kit. A PCR mix, comprising 50 µL, was prepared to amplify the ITS rDNA region from each yeast DNA sample. The PCR mix contained 0.25 µL Ex Taq DNA polymerase (Takara, Japan) PCR kit, 5 µL 10x buffer (Takara, Japan), 5 µL 25 mM $MgCl_2$ (Takara, Japan), 4 µL 25 mM dNTP (Takara, Japan), 2 µL forward primer ITS 1 (5'-TCC GTA GGT GAA CCT GCG G-3'), 2 µL reverse primer ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3'), 29.75 µL NFW (Biotechnology grade, 1st BASE, Singapore), and 2 µL of each sample. The ITS region, which is identical to the primer region, was utilized for partial sequencing within the range of 600 to 800 bp. DNA amplification involved 30 cycles with initial denaturation at 95°C for 2 minutes, denaturation at 95°C for 30 seconds, annealing at 55°C for 20 seconds, extension at 72°C for 1 minute, post-extension at 72ºC for 5 minutes, and a final hold stage at 4°C. The reaction was performed using a PCR Thermal Cycler (Takara, Japan).

The PCR product was subjected to electrophoresis (Mupid-exU, Advance, Japan) analysis to determine the expected target DNA band length. A 1% gel electrophoresis was prepared by dissolving 0.3 g of agarose (Promega, USA) in 30 mL of 0.5× TAE buffer (Invitrogen, USA) and heating it for 90 seconds. To the warm agarose, 1 µL of SYBR™ Safe DNA Gel Stain (Invitrogen, USA) was added, mixed thoroughly, and poured into the gel tray until it solidified. For each sample, 5 µL was mixed with 1 µL of loading dye (Thermo Scientific, USA) and loaded into the agarose well. Additionally, 4 µL of a 1 kb DNA ladder (Thermo Scientific, USA) was loaded into the agarose well. Electrophoresis was conducted for 40 minutes at a voltage of 50 V. The DNA bands were visualized using a UV-transilluminator (FAS-IV, Nippon Genetics, Japan). The PCR products, characterized by distinct bands (not smears), and free from contaminants with an approximate size of 600 base pairs (bp), were subjected to sequencing using the services provided by PT Genetika Science Indonesia.

Phylogenetic tree construction

The obtained sequences were edited using Chromas Pro 1.7.5 software (Technelysium Pty Ltd., Australia). The resulting FASTA data were subsequently analyzed for homology against reference sequences available in GenBank, employing the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). From the obtained results, the five reference sequences exhibiting the highest percentage of identity, query coverage values, and the lowest E-value were selected and downloaded in FASTA format. Each isolate, along with its respective reference sequence, was then utilized to construct a phylogenetic tree using MEGA11 software (Tamura et al. 2021). The Neighbor-Joining Tree method was employed, and bootstrap analysis with 1,000 iterations was performed.

RESULTS AND DISCUSSION

Yeast isolation and morphological characterization

Fifty-one yeast isolates were obtained from three honey samples: 11 isolates (M 1.1-M 1.11) from South Barito Regency, South Kalimantan Province; 24 isolates (M 2.1-M 2.24) from Lebak Regency, Banten Province; and 16 isolates (M 3.1-M 3.16) from Malang City, East Java Province. The isolates were selected and grouped based on morphological characterization of the colonies and cells, resulting in the identification of 10 distinct types of isolates: M 1.4, M 1.5, M 1.6, M 1.8, M 1.9, M 2.4, M 3.1, M 3.2, M 3.11, and M 3.15. Table 1 presents the morphological characteristics of the isolates. Isolates M 1.4, M 1.6, M 1.8, and M 3.1 exhibited similar colony and cell morphology, including friable texture, white cream colony color, raised elevation, wrinkled colony surfaces, ovoid cell shape, and asexual budding reproduction. The colony texture varied between friable and butyrous, while the color of all isolates was white cream (Figure 1). Among the 10 yeast isolates, 9 exhibited budding type of sexual reproduction, while only one isolate, M 2.4, exhibited fission asexual reproduction (Figure 2). This type of asexual reproduction through fission is a characteristic specific to yeasts of the genera *Schizosaccharomyces*, *Dipodascus*, *Trichosporon*, and several other genera (Kurtzman et al. 2011).

Physiological and biochemical identification

Physiological and biochemical identification tests were conducted on the yeast isolates, including fermentation tests with three types of carbohydrates (glucose, sucrose, and maltose) and yeast growth tests in 60% glucose media. The fermentation test is an important tool for characterizing and identifying yeast isolates, as it involves the conversion of carbohydrates, such as sugar and starch, into alcohol or acids, detecting acids and gases produced during sugar fermentation (Kali et al. 2015; Maicas 2020). Table 2 presents the results of the fermentation tests conducted on the 10 yeast isolates. Based on the data, it was observed that all isolates were able to ferment the three tested sugars within 7 days. According to Kurtzman et al. (2011), yeasts that can ferment the substrate within 7 days are classified as strongly positive. Notably, isolate M 2.4 exhibited fermentation of all three sugars within 24 hours after inoculation, indicated by gas production filling the Durham tube and a color change in the fermentation medium from green to yellow.

Table 1. Identification of morphological colony and cell of yeast isolates

Isolate	Colony morphology					Cell morphology	
	Texture	Color	Elevation	Surface	Margin	Shape	Asexual reproduction
M 1.4	Friable	White cream	Raised	Wrinkled	Undulate	Ovoid	Budding
M 1.5	Butyrous	White cream	Raised	Smooth	Entire	Ovoid	Budding
M 1.6	Friable	White cream	Raised	Wrinkled	Irregular	Spherical to ovoid	Budding
M 1.8	Friable	White cream	Raised	Wrinkled	Undulate	Ovoid	Budding
M 1.9	Butyrous	White cream	Raised	Smooth	Entire	Ovoid	Budding
M 2.4	Butyrous	White cream	Raised	Smooth	Entire	Ovoid to elongated	Fission
M 3.1	Friable	White cream	Raised	Wrinkled	Entire	Ovoid	Budding
M 3.2	Butyrous	White cream	Raised	Smooth	Entire	Ovoid	Budding
M 3.11	Butyrous	White cream	Raised	Smooth	Entire	Ovoid	Budding
M 3.15	Butyrous	White cream	Raised	Smooth	Entire	Ovoid to ellipsoidal	Budding

Note: Observation of colony and cell morphology was carried out on isolates inoculated on YPDA media after 24-48 hours of incubation at 28 ± 2°C

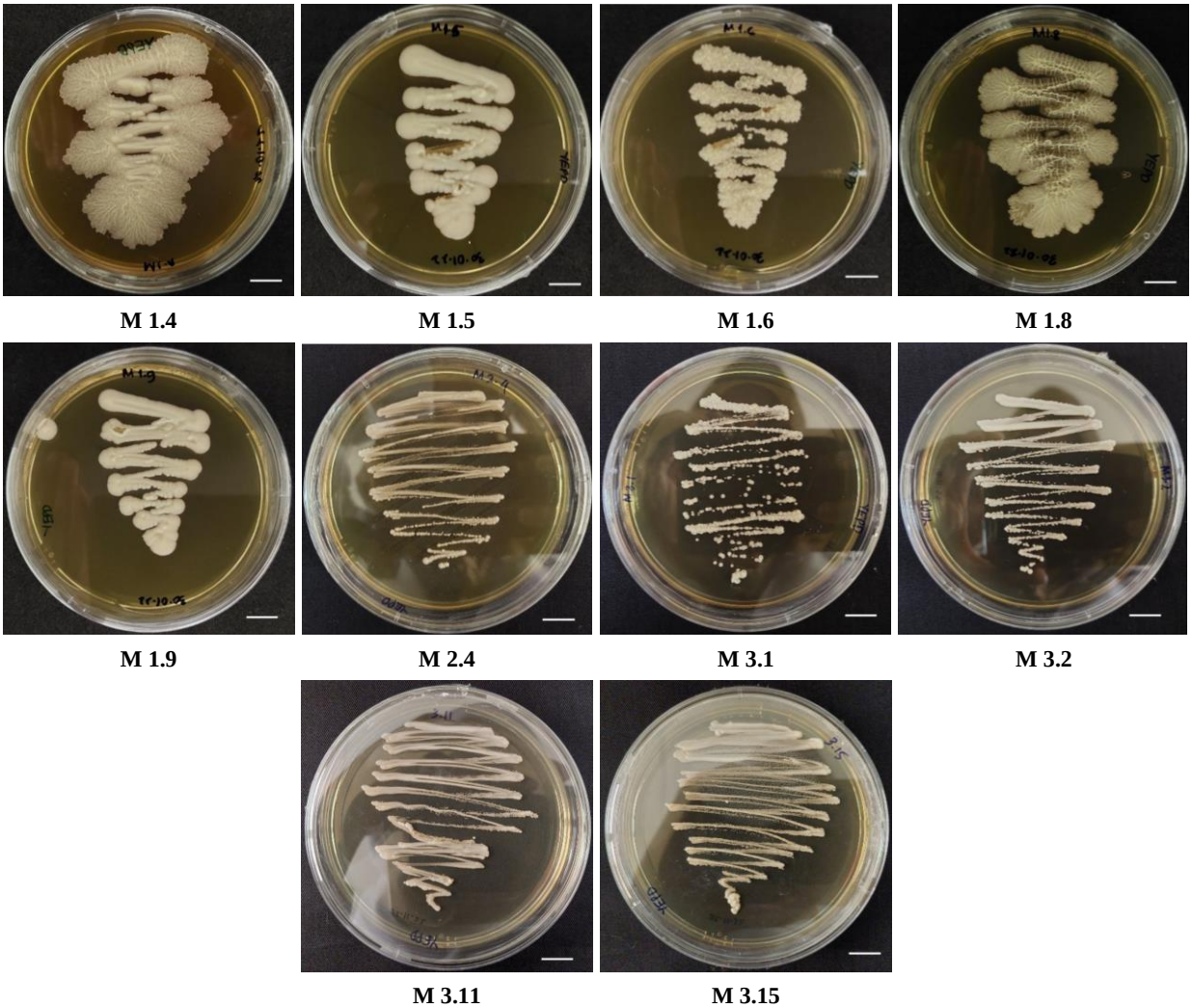

Figure 1. Yeast isolates from honey samples from South Kalimantan (M 1.4, M 1.5, M 1.6, M 1.8, and M 1.9), Banten (M 2.4) in, and East Java (M 3.1, M 3.2, M 3.11, and M 3.15) provinces (after 7-day incubation at 28 ± 2°C on YPDA media; the white horizontal bars indicate 1 cm)

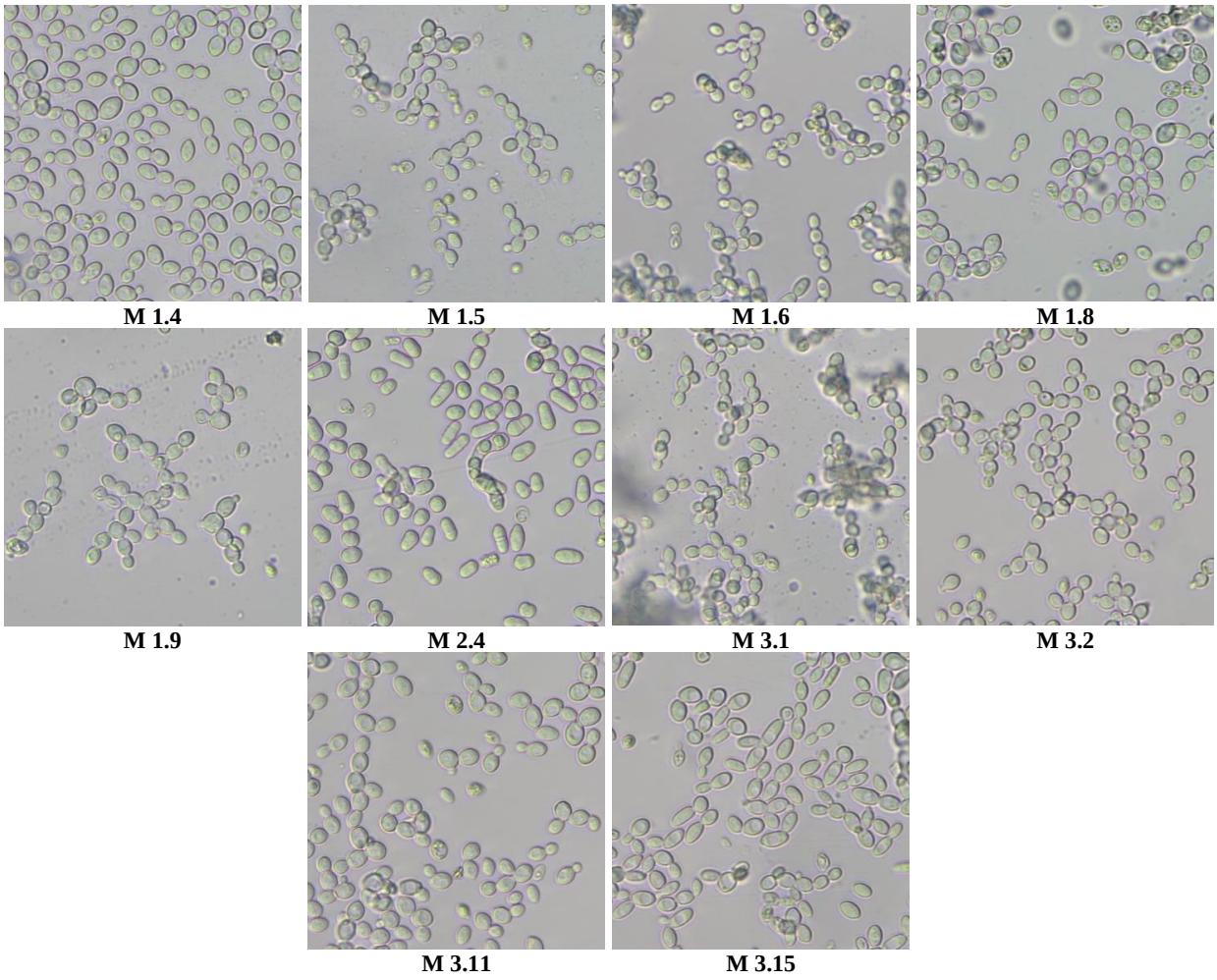

Figure 2. Cell morphology of yeast isolates from honey from South Kalimantan (M 1.4, M 1.5, M 1.6, M 1.8, and M 1.9), Banten (M 2.4) in, and East Java (M 3.1, M 3.2, M 3.11, and M 3.15) provinces (after 24-hour incubation on YPDA media at 28 ± 2°C; 100× magnification)

Table 2. Biochemical identification of yeast isolates.

Isolate	Fermentation of carbohydrate test			60% glucose media
	Glucose	Sucrose	Maltose	
M 1.4	+	+	+	+
M 1.5	+	+	+	+
M 1.6	+	+	+	+
M 1.8	+	+	+	+
M 1.9	+	+	+	+
M 2.4*	+	+	+	+
M 3.1	+	+	+	+
M 3.2	+	+	+	+
M 3.11	+	+	+	+
M 3.15	+	+	+	+

Note: *) M 2.4 exhibited rapid fermentation, especially in maltose, within 24 hours, while all the other isolates underwent sugar fermentation within 7 days.

Other physiological and biochemical tests were conducted by cultivating isolates on a 60% glucose medium. The objective of this test was to determine the yeast strains capable of surviving in an environment with high osmotic pressure due to high sugar concentration. In this study, all yeast isolates exhibited growth in the 60% glucose medium during an incubation period of 24-96 hours at 28 ± 2°C (Table 2).

Based on the phenetic identification comparison, four yeast isolates, namely M 1.4, M 1.6, M 1.8, and M 2.4, were selected for molecular identification. Isolates M 1.4, M 1.6, and M 1.8 were chosen due to their distinctive colony characteristics of friable texture, wrinkled surface, and undulate or irregular margin, setting them apart from the other isolates. Isolate M 2.4 was selected because of its distinct fission reproduction. Additionally, isolate M 2.4 exhibited a faster fermentation rate for glucose, sucrose, and maltose within 24 hours compared to the other isolates, particularly in maltose fermentation.

Molecular identification

The molecular identification process was performed on yeast isolates M 1.4, M 1.6, M 1.8, and M 2.4. Yeast DNA was isolated using the PrepMan kit (Applied Biosystems, USA), and the purity of the DNA was assessed using a NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific, USA), followed by gel electrophoresis. The

isolated DNA from the four yeast isolates was then subjected to amplification in the ITS region using PCR with forward primer ITS1 and reverse primer ITS4. The results of this amplification are depicted in Figure 3. As shown in the figure, the DNA bands of the four yeast isolates had sizes ranging from 600 to 800 base pairs, which align with the approximate length of 700 base pairs (Fajarningsih 2016). Subsequently, the PCR products were sequenced by PT Genetika Science Indonesia.

The four yeast isolate sequences were processed using ChromasPro software to obtain FASTA data for each sequenced isolate. Subsequently, molecular identification was performed by comparing the isolate's FASTA data with sequences in the GenBank database using BLAST (Basic Local Alignment Search Tool) at NCBI (National Center for Biotechnology Information) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The results are presented in Table 3. To identify the yeast species, several criteria were considered based on Petti et al. (2008) in the Clinical and Laboratory Standard Institute and Buehler et al. (2017). These criteria included: (i) a sequence identity value of ≥99%, (ii) a separation of at least 1% from the closest species, (iii) a sequence coverage of ≥90% for matching sequences, and (iv) the matching sequence being published in a peer-reviewed journal or submitted by ATCC. For genus-level identification, all of these criteria were used, except the sequence identity threshold was ≥97% and the separation criteria from the closest species was not required (Petti et al. 2008). Based on these criteria, five sequences were selected for each isolate (Table 3), and one sequence (*Debaryomyces hansenii*) was chosen as the outgroup. Phylogenetic tree construction was performed using MEGA11, employing the Neighbor-Joining Tree construction method with a bootstrap value of 1,000. The resulting phylogenetic tree of yeast isolates is presented in Figure 4. The tree construction results include bootstrap values at each node, indicating the number of times the same tree was formed when resampling the dataset, as explained by Ojha et al. (2022). A bootstrap value of 100% or 95% indicates accurate results. However, bootstrap values below 50% are not considered reliable for constructing phylogenetic trees (Ojha et al. 2022).

Based on homology analysis (Table 3), it is known that isolates M 1.4 and M 1.8 were identified as yeast isolates belonging to the genus *Zygosaccharomyces*, and isolate M 2.4 was identified as a yeast isolate belonging to the genus *Schizosaccharomyces*. Moreover, isolate M 1.6 was identified as *Zygosaccharomyces siamensis*. The homology analysis revealed that isolates M 1.4 and M 1.8 share a percent identity of ≥97% with the genus *Zygosaccharomyces*, confirming their classification within this genus. Additionally, the construction of the phylogenetic tree (Figure 4) indicated that isolates M 1.4 and M 1.8 have a bootstrap value of 100%, indicating a close relationship between the two isolates. Furthermore, both isolates exhibited a bootstrap value of 95% with the yeast species *Zygosaccharomyces rouxii* strain IFO:1814 (AB302830.1), demonstrating their genetic similarity to *Z. rouxii*. A comparison of the morphological and biochemical characteristics of the two isolates with *Z. rouxii* in the literature is presented in Table 4.

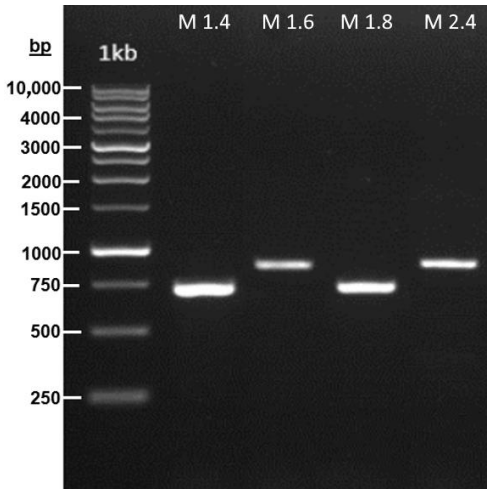

Figure 3. Electrophoresis of PCR products of yeast isolate DNA M 1.4, M 1.6, M 1.8, and M 2.4

Isolates M 1.4 and M 1.8 share similar characteristics with *Z. rouxii*, as illustrated in Table 4, particularly in terms of sexual reproduction through budding, their ability to ferment glucose, sucrose, and maltose, as well as their ability to grow in a 60% glucose medium. This corresponds to the natural environment in which the yeast isolates were sourced, specifically honey. In a similar study, *Z. rouxii* has been identified within green honey samples, showcasing its remarkable osmotolerant trait characterized by an enhanced capacity to withstand elevated sugar concentrations (Ullah et al. 2023). *Zygosaccharomyces rouxii* is known for its involvement in food spoilage, particularly in products with high sugar and/or salt content, low pH, and those containing weak organic acids (Escott et al. 2018), including mix base for ice cream (Iacumin et al. 2022), concentrated apple juice (Wang and Sun 2020) and concentrated grape juice (Rojo et al. 2019). Additionally, *Z. rouxii* is frequently employed in the production of fermented foods such as soy sauce and miso (James and Stratford 2011).

Isolate M 1.6 exhibits a percent identity value of ≥99% with the yeast species *Zygosaccharomyces siamensis* and demonstrates a bootstrap value of 80% with the same species (Figure 4). Therefore, isolate M 1.6 is identified as *Zygosaccharomyces siamensis*, indicating a close genetic relationship with this species. A comparison of the morphological and biochemical characteristics between M 1.6 and *Z. siamensis* isolates from the literature is presented in Table 4. Based on Table 4, it is evident that there are similarities between M 1.6 and *Z. siamensis* isolates, particularly in their ability to ferment glucose and maltose. However, in this study, isolate M 1.6 exhibited sucrose fermentation on day 6, whereas *Z. siamensis* did not ferment sucrose (Saksinchai et al. 2012). Phenetic identification methods often have a relatively high error rate; thus, molecular identification is necessary to confirm the results. Additionally, *Z. siamensis* is classified as an osmophilic yeast, having extreme tolerance to high sugar concentration (osmotolerance) (Solieri 2021). Therefore, this yeast is suspected to contribute to food spoilage processes (Saksinchai et al. 2012). This study further

supports this correlation, as isolate M 1.6, identified as *Z. siamensis*, was isolated from honey samples with exceptionally high sugar levels and low water activity values. These conditions signify elevated osmotic pressure.

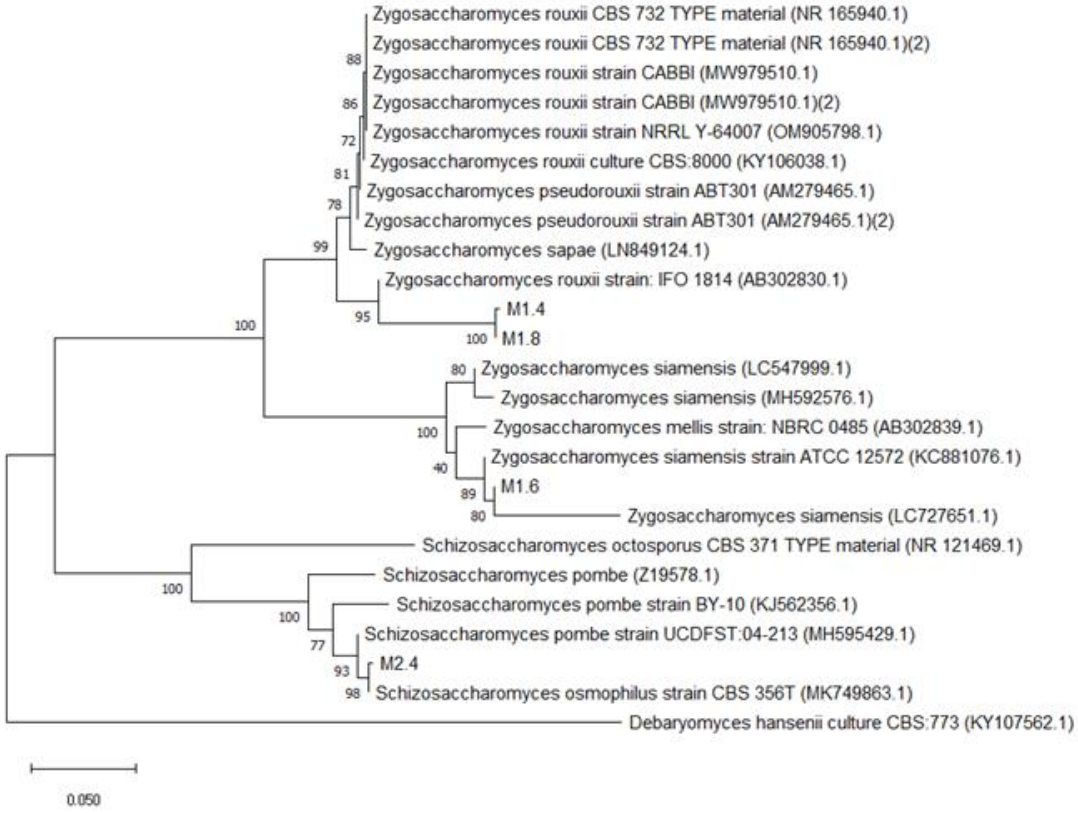

Figure 4. Phylogenetic tree of yeast isolates M 1.4, M 1.6, M 1.8, and M 2.4 with neighbor-joining and 1,000 bootstraps

Table 3. Homology analysis of yeast isolates using BLAST (Basic Local Alignment Search Tool) of NCBI

Description	Max score	Total score	Query cover	E value	Per. ident	Acc. Len	Accession
M 1.4							
Zygosaccharomyces pseudorouxii strain ABT301	1227	2375	100%	0.0	97.76	721	AM279465.1
Zygosaccharomyces rouxii CBS 732 TYPE material	1216	2353	100%	0.0	97.36	835	NR_165940.1
Zygosaccharomyces rouxii culture CBS:8000	1216	2356	100%	0.0	97.35	721	KY106038.1
Zygosaccharomyces rouxii strain CABBI	1216	2353	100%	0.0	97.36	836	MW979510.1
Zygosaccharomyces sapae strain M21 clone copy_1	1120	2184	95%	0.0	96.23	687	LN849124.1
M 1.6							
Zygosaccharomyces siamensis	1441	1441	100%	0.0	99.62	1640	LC547999.1
Zygosaccharomyces siamensis strain ATCC 12572	1430	1430	99%	0.0	99.49	799	KC881076.1
Zygosaccharomyces siamensis	1417	1417	100%	0.0	99.11	1207	LC727651.1
Zygosaccharomyces siamensis	1362	1362	94%	0.0	99.60	772	MH592576.1
Zygosaccharomyces mellis strain: NBRC 0485	1306	1306	99%	0.0	96.61	820	AB302839.1
M 1.8							
Zygosaccharomyces pseudorouxii strain ABT301	1232	2369	100%	0.0	97.90	721	AM279465.1
Zygosaccharomyces rouxii CBS 732 TYPE material	1221	2347	100%	0.0	97.50	835	NR_165940.1
Zygosaccharomyces rouxii strain CABBI	1221	2347	100%	0.0	97.50	836	MW979510.1
Zygosaccharomyces rouxii strain NRRL Y-64007	1221	2347	100%	0.0	97.50	836	OM905798.1
Zygosaccharomyces rouxii strain: IFO 1814	1221	2345	95%	0.0	99.70	683	AB302830.1
M 2.4							
Schizosaccharomyces pombe strain UCDFST:04-213	1354	1354	100%	0.0	98.70	885	MH595429.1
Schizosaccharomyces osmophilus strain CBS 356T	1351	1351	100%	0.0	98.57	814	MK749863.1
Schizosaccharomyces pombe strain BY-10	1253	1253	100%	0.0	96.22	844	KJ562356.1
Schizosaccharomyces pombe	1125	1125	99%	0.0	92.80	7865	Z19578.1
Schizosaccharomyces octosporus CBS 371 TYPE material	366	366	43%	3×10^{-96}	86.78	992	NR_121469.1

Table 4. Comparison of morphological, physiological, and biochemical characters of yeast isolates M 1.4, M 1.6, M 1.8, and M 2.4 with references

Character		M 1.4	M 1.8	*Z. rouxii* [a)]	M 1.6	*Z. siamensis* [b)]	M 2.4	*S. osmophilus* [c)]
Colony morphology	Texture	Friable	Friable	Butyrous	Friable	Butyrous	Butyrous	Friable
	Color	White cream	White cream	White to cream	White cream	White	White cream	Cream to tan
	Elevation	Raised	Raised	Convex	Raised	Convex to raised	Raised	Not described
	Surface	Wrinkled	Wrinkled	Smooth	Wrinkled	Smooth	Smooth	Dull and rough to papillate
	Margin	Undulate	Undulate	Not described	Irregular	Entire	Entire	Undulate to lobate
Cell morphology	Cell Shape	Ovoid	Ovoid	Spherical to ovoid	Spherical to ovoid	Spherical to ellipsoidal	Ovoid to elongated	Globose, ovoid, irregular, elongated
	Asexual Reproduction	Budding	Budding	Budding	Budding	Not described	Fission	Fission
Physiology and biochemical character	Glucose fermentation	+	+	+	+	+(s)	+	+
	Sucrose fermentation	+	+	v	+	-	+	d
	Maltose fermentation	+	+	+/w	+	l and w/-	+	d
	60% glucose media	+	+	v	+	+	+	+

Note: +: strong positive, insert filled within 7 days; v: variable, some strains were positive, others were negative; w: weakly positive, the insert was not fully filled with gas (<1/3 = weak, >1/3=positive); −: negative; l: latent (longer than 7 days); s: slow; and d: delay. Reference: [a](James and Stratford 2011; Kurtzman et al. 2011; Sá-Correia et al. 2014), [b](Saksinchai et al. 2012), [c](Brysch-Herzberg et al. 2019)

The isolate M 2.4 exhibited a percentage identity value of >98% with the yeast genus *Schizosaccharomyces*, indicating its classification as a member of the genus. Furthermore, phylogenetic analysis revealed that isolate M 2.4 shared a bootstrap value of 93% with the yeast species *Schizosaccharomyces osmophilus*, suggesting a close genetic relationship between them. A comparative analysis of morphological, physiological, and biochemical characteristics between M 2.4 isolates and *S. osmophilus* (Table 4) revealed that both exhibited fission asexual reproduction and the ability to ferment glucose, sucrose, and maltose. Additionally, they demonstrated the ability to grow in 60% glucose media, indicating that isolate M 2.4 is an osmophilic/osmotolerant yeast. Conversely, *S. osmophilus* was unable to grow or displayed impaired growth under low osmotic pressure conditions (Brysch-Herzberg et al. 2019), which aligns with our study as isolate M 2.4 was isolated from honey samples with high osmotic pressure. Previous studies by Brysch-Herzberg et al. (2022) have established *S. osmophilus* as an obligate osmophilic yeast, dependent on substrates with consistently low water activity values for survival. According to their research, *S. osmophilus* primarily inhabits solitary bee combs and is rarely found in raisins. However, in a separate study by Brysch-Herzberg et al. (2022), *S. osmophilus* was discovered in 1 out of 386 honey samples collected from 43 countries, representing a prevalence of approximately 0.26%, thus highlighting its rarity. Within these isolates, a fission yeast *Schizosaccharomyces lindneri* sp. nov. has also been identified (Brysch-Herzberg et al. 2023). This finding corroborates our study, indicating the presence of *Schizosaccharomyces* yeast species in honey samples.

In conclusion, we obtained 51 yeast isolates derived from three distinct types of honey sourced from different regions in Indonesia: South Barito Regency, South Kalimantan; Lebak Regency, Banten; and Malang City, East Java. These yeast isolates underwent characterization based on their morphological, physiological, and biochemical traits, resulting in the selection of 10 isolates for further analysis. Subsequently, molecular identification was conducted on four chosen isolates. The findings revealed that isolates M 1.4 and M 1.8 were classified as belonging to the genus *Zygosaccharomyces*, while isolate M 2.4 was categorized as a member of the genus *Schizosaccharomyces*. Additionally, isolate M 1.6 was identified as a species of *Zygosaccharomyces siamensis*. All four isolates demonstrated osmophilic characteristics and will serve as valuable germplasm for future investigations.

ACKNOWLEDGEMENTS

We acknowledge the support provided by the Biotechnology Laboratory, National Research and Innovation Agency (BRIN), which greatly assisted in facilitating this study. We extend our thanks to Yusuf Sigit Ahmad Fauzan for generously providing the honey sample from South Kalimantan Province, Indonesia. We acknowledge and appreciate the significant efforts and contributions made by all of the authors to this study: Meilia Prihartini: Conducted the experiments, drafted the manuscript, performed data analysis, and contributed to the research methodology; Syofi Rosmalawati: Conducted the experiments, provided the samples, contributed to the research methodology, and assisted with editing; Catur Sriherwanto: Contributed to editing, proofreading, and formatting the article; and Anis Herliyati Mahsunah: Provided supervision throughout the research process.

REFERENCES

Alnaimy A, Habeeb M. 2017. Importance of yeast in ruminants feeding on production and reproduction. Ecol Evol Biol 2: 49-58. DOI: 10.11648/j.eeb.20170204.11.

Brysch-Herzberg M, Jia GS, Sipiczki M, Seidel M, Li W, Assali I, Du LL. 2023. *Schizosaccharomyces lindneri* sp. nov., a fission yeast occurring in honey. Yeast 2023: 1-17. DOI: 10.1002/yea.3857.

Brysch-Herzberg M, Jia GS, Seidel M, Assali I, Li, Du LL, Brysch-Herzberg M, Seidel M, Seidel M, Du LL, Assali I. 2022. Insights into the ecology of *Schizosaccharomyces* species in natural and artificial habitats. Antonie van Leeuwenhoek 115: 661-695. DOI: 10.1007/s10482-022-01720-0.

Brysch-Herzberg M, Tobias A, Seidel M, Wittmann R, Wohlmann E, Fischer R, Dlauchy D, Peter G. 2019. *Schizosaccharomyces osmophilus* sp. nov., an osmophilic fission yeast occurring in bee bread of different solitary bee species. FEMS Yeast Res 19: 38. DOI: 10.1093/femsyr/foz038.

Buehler AJ, Evanowski RL, Martin NH, Boor KJ, Wiedmann M. 2017. Internal transcribed spacer (ITS) sequencing reveals considerable fungal diversity in dairy products. J Dairy Sci 100: 8814-8825. DOI: 10.3168/JDS.2017-12635.

Ceccato-Antonini SR. 2022. Methods for the identification and characterization of yeasts from ethanolic fermentation. In: Microbiology of Ethanol Fermentation in Sugarcane Biofuels: Fundamentals, Advances, and Perspectives. Springer, Cham. DOI: 10.1007/978-3-031-12292-7_5.

Escott C, del Fresno JM, Loira I, Morata A, Suárez-Lepe JA. 2018. *Zygosaccharomyces rouxii*: Control strategies and applications in food and winemaking. Fermentation 4: 69. DOI: 10.3390/FERMENTATION4030069.

Fajarningsih ND. 2016. Internal transcribed spacer (ITS) as DNA barcoding to identify fungal species: A review. Squalen Bull Mar Fish Postharvest Biotechnol 11: 37-44. DOI: 10.15578/squalenv11i2213.

Iacumin L, Colautti A, Comi G. 2022. *Zygosaccharomyces rouxii* is the predominant species responsible for the spoilage of the mix base for ice cream and ethanol is the best inhibitor tested. Food Microbiol 102: 103929. DOI: 10.1016/jfm2021103929.

James SA, Stratford M. 2011. *Zygosaccharomyces* Barker (1901). In: Kurtzman CP, Fell JW, Boekhout T (eds). The Yeasts (Fifth Edition). Elsevier. DOI: 10.1016/B978-0-444-52149-1.00084-7.

Kali A, Srirangaraj S, Charles PMV. 2015. A cost-effective carbohydrate fermentation test for yeast using microtitre plate. Indian J Med Microbiol 33: 293-295. DOI: 10.4103/0255-0857154884.

Kurtzman CP, Fell JW, Boekhout T. 2011. The Yeasts, A Taxonomic Study. Elsevier, Amsterdam.

Maicas S. 2020. The role of yeasts in fermentation processes. Microorganisms 8: 1142. DOI: 10.3390/microorganisms8081142.

Mohammed MEA. 2022. Factors affecting the physicochemical properties and chemical composition of bee's honey. Food Rev Intl 38: 1330-1341. DOI: 10.1080/87559129.2020.1810701.

Ojha KK, Mishra S, Singh VK. 2022. Chapter 5 - Computational molecular phylogeny: concepts and applications. In: Singh DB, Pathak RK (eds). Bioinformatics Methods and Applications. Academic Press. DOI: 10.1016/B978-0-323-89775-4.00025-0.

Petti CA, Bosshard PP, Brandt ME, Clarridge JE, Feldblyum TV, Foxall P, Furtado MR, Pace N, Procop G. 2008. Interpretive Criteria for Identification of Bacteria and Fungi by DNA Target Sequencing; Approved Guideline. Clinical and Laboratory Standards Institute (CLSI), Wayne.

Pincus DH, Orenga S, Chatellier S. 2007. Yeast identification—past, present, and future methods. Med Mycol 45: 97-121. DOI: 10.1080/13693780601059936.

Raja HA, Miller AN, Pearce CJ, Oberlies NH. 2017. Fungal identification using molecular tools: A primer for the natural products research community. J Nat Prod 80: 756-770. DOI: 10.1021/acs.jnatprod.6b01085.

Rao PV, Krishnan KT, Salleh N, Gan SH. 2016. Biological and therapeutic effects of honey produced by honey bees and stingless bees: A comparative review. Rev Bras Farmacogn 26: 657-664. DOI: 10.1016/j.bjp.2016.01.012.

Rodrigues AMD, Pinheiro REE, Costa JA, Santos JTO, Poli JS, Rosa CA, Soares MJS, Muratori MCS, Nóbrega MMGP. 2018. Comparison between morphophysiological and molecular methods for the identification of yeasts isolated from honey. Intl Food Res J 25: 418-422.

Rojo MC, Cristiani M, Szerman N, Gonzalez ML, Lerena MC, Mercado LA, Combina M. 2019. Reduction of *Zygosaccharomyces rouxii* population in concentrated grape juices by thermal pasteurization and hydrostatic high pressure processing. Food Bioprocess Technol 12: 781-788. DOI: 10.1007/s11947-019-02251-9.

Sá-Correia I, Guerreiro JF, Loureiro-Dias MC, Leão C, Côrte-Real M. 2014. *Zygosaccharomyces*. In: Batt CA, Tortorello ML (eds). Encyclopedia of Food Microbiology (Second Edition). Academic Press. DOI: 10.1016/B978-0-12-384730-0.00364-5.

Saksinchai S, Suzuki M, Chantawannakul P, Ohkuma M, Lumyong S. 2012. A novel ascosporogenous yeast species, *Zygosaccharomyces siamensis*, and the sugar tolerant yeasts associated with raw honey collected in Thailand. Fungal Divers 52: 123-139. DOI: 10.1007/S13225-011-0115-Z.

Silva MS, Rabadzhiev Y, Eller MR, Ilia Iliev, Ivanova I, Santana WC, Silva MS, Rabadzhiev Y, Eller MR, Ilia Iliev, Ivanova I, Santana WC. 2017. Microorganisms in honey. In: Toledo VAA (eds). Honey Analysis. IntechOpen, London. DOI: 10.5772/67262.

Sinacori M, Francesca N, Alfonzo A, Cruciata M, Sannino C, Settanni L, Moschetti G. 2014. Cultivable microorganisms associated with honeys of different geographical and botanical origin. Food Microbiol 38: 284-294. DOI: 10.1016/j.fm.2013.07.013.

Snowdon JA, Cliver DO. 1996. Microorganisms in honey. Intl J Food Microbiol 31: 1-26. DOI: 10.1016/0168-1605(96)00970-1.

Solieri L. 2021. The revenge of *Zygosaccharomyces* yeasts in food biotechnology and applied microbiology. World J Microbiol Biotechnol 37: 96. DOI: 10.1007/s11274-021-03066-7.

Tamura K, Stecher G, Kumar S. 2021. MEGA11: Molecular evolutionary genetics analysis version 11. Mol Biol Evol 38: 3022-3027. DOI: 10.1093/molbev/msab120.

Ullah S, Abdul Wahabb R, Oyewusi HA, Huyop F. 2023. Exploring the microbial communities in green honey from Banggi Island, Sabah, Malaysia using amplicon sequencing analysis. Access Microbiol 000624-v1. DOI: 10.1099/acmi.0.000624.v1.

Wang H, Sun H. 2020. Assessment of different antimicrobials to inhibit the growth of *Zygosaccharomyces rouxii* cocktail in concentrated apple juice. Food Microbiol 91: 103549. DOI: 10.1016/j.fm.2020.103549.

Xue SJ, Li XC, Huang X, Liu J, Li Y, Zhang XT, Zhang JY. 2023. Diversity investigation of cultivable yeasts associated with honeycombs and identification of a novel *Rhodotorula toruloides* strain with the robust concomitant production of lipid and carotenoid. Bioresour Technol 370: 128573. DOI: 10.1016/j.biortech.2022.128573.

Ziuzia P, Janiec Z, Wróbel-Kwiatkowska M, Lazar Z, Rakicka-Pustułka M. 2023. Honey's yeast—New source of valuable species for industrial applications. Intl J Mol Sci 24: 7889. DOI: 10.3390/ijms24097889.