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Research

Screening and Identification of Potential Indigenous Yeasts Isolated During Fermentation of Wine Coffee

Anggita Bella Siez Kanita and Yoga Dwi Jatmiko*

Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Veteran Street, Malang, East Java 65145, Indonesia
*Corresponding author: jatmiko_yd@ub.ac.id

ABSTRACT

Wine coffee is a fermented coffee product that involves yeast as the fermentative agent which has potency as probiotics. This study aimed to determine the potency of yeast isolated from wine coffee fermentation and to identify the yeast species with the best probiotic properties. This study comprised three main steps: coffee fermentation, yeast isolation, and probiotic characterization. A series of probiotic tests were carried out, including resistance tests at low pH (pH 2, 3, & 4) and bile salts (0.5% & 2%), antimicrobial activity tests, antibiotic resistance tests, hemolytic activity tests, and species identification based on the ITS rDNA sequence. The data obtained were analyzed using One-way ANOVA ($p \leq 0.05$) and continued with the Tukey test. A total of 25 yeast isolates were isolated and purified. Nine isolates (A2, B1, B3, C3, D4, D5, E2, E3 & E5) had the highest tolerance to pH 2 and 2% bile salts with survival rates were more than 100% and 90%, respectively. Nine isolates were resistant to all tested antibiotics, and only isolate A2 exhibited a pathogenic characteristic (β-hemolysis). Three isolates (B3, E3 & E5) could inhibit the five indicator pathogens, with the highest inhibitory activity shown by isolating E3 against *Bacillus cereus* by 68 AU/mL. The isolate E3 was selected as the best yeast with probiotic properties identified as *Pichia kudriavzevii* with 100% similarities towards strain iwate20191107.

Key words: ITS rDNA, *Pichia kudriavzevii*, probiotics, wine coffee, yeast

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INTRODUCTION

Yeast is eukaryotic unicellular microorganism that has been widely explored for applications in the health and food industries. Currently, yeast is widely applied in the fermentation process of food industries, including in manufacturing bread, tape, yogurt, wine, and other types of fermented foods (Willaert, 2017). In addition to playing a role in the fermentation process, yeast also provides good benefits for human health, one of the benefits is probiotics (Moslehi-Jenabian *et al*., 2010). Probiotics are live microorganisms that, when consumed in sufficient quantities will provide health benefits for the host (Staniszewski & Kordowska-Wiater, 2021).

Some health benefits derived from yeast as a probiotic have been demonstrated, including prevention and treatment of diarrheal diseases, anti-inflammatory, increasing immune response, and inhibiting enteric pathogenic bacteria (Moslehi-Jenabian *et al*., 2010). Probiotic yeasts are naturally more resistant to antibiotics than probiotic bacteria, so probiotic yeasts are considered more effective in treating diarrhea (Tomičić *et al*., 2016). In the early 1920s, it was known that a type of yeast that had the potential as a probiotic was *Saccharomyces boulardii*, isolated from lychee fruit in Indochina by Henri Boulard (Staniszewski & Kordowska-Wiater, 2021). Since the 1970s, *S. boulardii* in clinical studies has been indicated to be safe and non-pathogenic (McFarland, 2010). Various studies on *S. boulardii* as a probiotic focused on human medicine have shown significant success. *S. boulardii* restored the integrity of the intestinal epithelium of the GI tract in patients undergoing HIV-1 treatment (Villar-García *et al*., 2015).

So far, studies on yeast isolation from fermented

coffee beans are still rare. Coffee is one of the largest commodities in Indonesia. Robusta coffee is one type of coffee that is widely cultivated in Indonesia. Currently, the coffee industry produces processed coffee, which is quite popular among the public, whose manufacturing process is fermented, namely wine coffee. Fermentation in the manufacture of wine coffee is conducted many times, so it is expected that there will be yeast involvement in wine coffee (Sulaiman *et al*., 2021).

The character of probiotic yeast has not been explored much in wine-coffee fermentation. Previous studies isolated *Saccharomyces* sp. in coffee fermented with a wet process and used it as a starter culture (Pereira *et al*., 2014). Meanwhile, regarding probiotic yeast isolated from fermented wine coffee, there is still no further research, even though wine coffee has excellent potential as a functional food product. Based on the description above, there is a need for a more in-depth analysis of yeast isolated during the fermentation of wine coffee that could have the potential as probiotics.

MATERIALS AND METHODS

Coffee fermentation and sampling

The type of coffee used in this study was Robusta. Robusta coffee cherries were obtained from Sukapura Village, Sukapura District, Probolinggo Regency, East Java. The coffee cherries used were red and were picked directly from the farm. The first stage was sorting the coffee cherries by soaking them in water for 30 min followed by selecting the submerged coffee cherries. Fermentation was performed by placing 250 g of coffee cherries into 15 jars (labels A1, A2, A3, B1, B2, B3, C1, C2, C3, D1, D2, D3, E1, E2 & E3). These fifteen jars consisted of five jars with different letters and each jar had three replications. The fermentation was carried out in a tightly closed plastic jar, which was placed in a dark room at ambient temperature for 50 days. After reaching a particular day of fermentation (days 21, 28, 34, 39, 43, 46, 48 & 49), the coffee cherries were moved outdoors in a closed container and not exposed to direct sunlight (black gauze protection) for seven hours (between 9 am – 4 pm). Sampling of fermented coffee was conducted five times during the fermentation period (days 0, 21, 28, 43, & 49). Samples from jars A1, A2, and A3 were collected before fermentation was carried out (day 0). Jar B1, B2, and B3 were collected after 21 days of fermentation. Furthermore, jars C1, C2, and C3 were collected after 28 days of fermentation. Then, jars D1, D2, and D3 were sampled after 43 days of fermentation. Last, jars E1, E2, and E3 were collected after 49 days of fermentation. The fermented coffee cherries in the jars that had been collected were used for the next procedure.

Yeast isolation and characterization

A sample of 25 g of fermented coffee cherries from each jar was added to 225 mL of 0.85% NaCl. The mixture was homogenized using a stomacher for 15 min (250 r.p.m). Next, serial dilution was carried out up to 10^{-6}. Then, 0.1 mL of the suspension was placed into a Petri dish containing Yeast Extract Peptone Dextrose (YEPD) agar (containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 15 g/L agar, and chloramphenicol 500 mg/L) by the spread plate method and incubated at 30 °C for 48 h. Then the growing yeast colonies were counted by the Total Plate Count method. The dominant yeast colonies with different morphological characters (size, shape, & color) were purified by the quadrant streak technique in YEPD agar. A total of purified 25 isolates were subculture on slant YEPD agar as a stock culture for further testing. The isolates were then stained using methylene blue to observe the cell morphology using a microscope (1000× magnification) (Moreira *et al*., 2013).

Yeast resistance towards lgow pH

The yeast isolates (two loopful) were subcultured in 10 mL of YEPD broth and incubated at 30 °C for 24 h. Then the cell density was equalized using a UV-Vis spectrophotometer (Shimadzu, North America) (λ=600 nm) to achieve an OD value of 2.15. After that, 5 mL of YEPD broth was prepared with three types of acidity levels (pH 2, 3, & 4). The pH was adjusted using 1 mol/L HCl. Then 10% of the yeast culture was inoculated into each YEPD broth and homogenized with a vortex, then incubated at 30 °C for 3 h. The yeast cell density was determined using a hemocytometer before and after incubation. These cell densities were used to determine the survival rate (Equation 1). This experiment was carried out in triplicates. Yeast isolates with a survival rate ≥ 100% were selected and used for the next test (Hu *et al*., 2018).

$$\text{Survival rate (\%)} = \frac{\text{Cell density at n-hours}}{\text{Cell density at 0 hour}} \times 100$$

Yeast tolerance test for bile salt

The selected yeast isolates (two loopful) were cultured in 10 mL YEPD broth, and incubated at 30 °C for 24 h. Then the cell density was equalized using a UV-Vis spectrophotometer (λ=600 nm) to achieve an OD value of 2.19. After that, 5 mL of YEPD broth was prepared with different concentrations

of bile salts, namely 0.5% and 2%. Then 10% yeast cultures were inoculated on each YEPD broth and homogenized with a vortex. Then, the cultures were incubated at 30 °C for 4 h. At the time of incubation at 0 and 4 h, 1 mL of yeast culture was taken, and the cell density was calculated using a hemocytometer. This treatment was carried out in triplicates. Yeast isolates with a survival rate of ≥ 90% were selected as isolates for the next test. The survival rate was calculated using Equation 1 (Hu *et al*., 2018).

Antimicrobial activity test

The selected yeast isolates were tested for antimicrobial activity against five pathogens: *Bacillus cereus*, *Salmonella* Typhimurium, *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*. Two loops of yeast isolates were cultured in 10 mL of YEPD broth and incubated at 30 °C for 24 hours. The yeast cell density was equalized using a UV-Vis spectrophotometer (λ=600 nm) to achieve an OD value of 2.15 and centrifuged (5804 R Eppendorf, Germany) at 10000 r.p.m for 10 min at 4 °C to obtain a cell-free supernatant (CFS). The supernatant was then adjusted to neutral (pH 7) using 1 mol/L NaOH. Then the supernatant was filtered with a Millipore membrane (Minisart, Germany) with a pore diameter of 0.22 µm (Azhar & Munaim, 2019).

The pathogenic bacteria were prepared in 10 mL Nutrient Broth (NB) and pathogenic yeasts were prepared in 10 mL YEPD broth, then incubated at 37 °C for 24 h. The cell density of the pathogenic cultures was equalized using a UV-Vis spectrophotometer (λ=600 nm) to achieve an OD value of 1.95. Furthermore, 0.1 mL of the pathogenic cultures were inoculated on the respective agar media using a spread plate technique. Then, 60 µL of yeast supernatant was placed on a blank disk (6 mm) and left at room temperature for 15 min. Then blank disk containing the supernatant was placed on the surface of the agar medium and incubated at 37 °C for 48 h. Next, the diameter of the inhibition zone around the colony was measured using a caliper (Azhar & Munaim, 2019). The inhibitory activity (AU/mL) was calculated using Equation 2 (Yulianti & Astuti, 2021).

$$\text{Inhibitory activity (AU/mL)} = \frac{(Lz - Ls)}{v}$$

Lz = diameter of the inhibition zone

Ls = diameter of the blank disk

v = supernatant volume

Antibiotic resistance test

Four types of antibiotics were used, namely streptomycin (10 µg), cefazolin (30 µg), erythromycin (15 µg), and aztreonam (30 µg). The four antibiotics represent four classes of antibiotics, namely streptomycin (aminoglycosides), cefazolin (cephalosporins), erythromycin (macrolides), and aztreonam (monobactams). The selected yeast isolates (two loopful) were inoculated into 10 mL of YEPD broth and incubated at 30 °C for 24 h. Then, the cell density was first equalized using a spectrophotometer (λ=600 nm) to achieve an OD value of 2.15. Afterward, 0.1 mL of yeast cultures were inoculated on YEPD agar using a spread plate technique. Then, the disks of different antibiotics were placed on the agar surface and incubated at 30 °C for 48 h. This experiment was performed in triplicates. The diameter of the inhibition zone formed was measured using a caliper, and then grouped into resistance, intermediate/moderate, and sensitive according to CLSI guidelines (Azhar & Munaim, 2019).

Hemolytic activity test

The selected yeast isolates were inoculated in a blood agar plate (5% sheep blood) and incubated at 30 °C for 48 h. Then, the presence of a clear zone around the colony was observed. The α-hemolysis reaction was indicated by the presence of a greenish color in the colony, β-hemolysis was indicated by the presence of a clear zone around the colony, and γ-hemolysis was indicated by the absence of neither a greenish color nor a clear zone (Moradi *et al*., 2018).

Molecular identification of the selected yeast

The yeast isolate with the best probiotic characteristics was identified based on Internal Transcribed Spacer (ITS) rDNA sequences. The total genomic DNA of the selected yeast isolate was extracted using Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, California). The DNA of the selected yeast isolate was amplified based on the ITS rDNA sequence using a Thermal Cycler (Eppendorf, Germany) with a primer of ITS 1 (5´-TCC GTA GGT GAA CCT GCG G-3´) and ITS 4 (5´-TCC TCC GCT TAT TGA TAT GC-3´). Next, the amplicon was analyzed by using 1.5% agarose gel electrophoresis and running at 100 volts for 30 min. The DNA bands formed were observed with a UV Transilluminator (EBOX VX2, France). Then the ITS rDNA sequence was sequenced at 1st Base

Laboratories, Malaysia (Siddiquee *et al*., 2010). The nucleotide sequences were analyzed using Sequence Scanner version 1.0 and Bioedit version 7.2, then the DNA sequences were aligned with the GenBank collection using the Basic Local Alignment Search Tool (BLAST) algorithm. The phylogenetic tree was constructed using MEGA 11 software with the Neighbor-Joining method and the Tamura-Nei model with 1000x bootstraps (Jatmiko *et al*., 2012).

Data analysis

The data obtained from the results of the yeast resistance test to low pH, the tolerance test to bile salts, and the antimicrobial activity test were analyzed using One-way Analysis of Variance (ANOVA) with SPSS for Windows version 26 with a significant level of 95% ($p \leq 0.05$). If there was a significant difference, the analysis was continued using the Tukey test.

RESULTS AND DISCUSSION

Characterization of yeast isolates from fermented wine coffee

A total of 25 yeast isolates were isolated during the fermentation period of wine coffee production consisting of five isolates from each sample: A (before fermentation), B (21 days of fermentation), C (28 days of fermentation), D (43 days of fermentation), and E (49 days of fermentation). The highest yeast cell number was found in sample D, which was 1.47×10^6 CFU/g. This can be occurred due to differences in the fermentation duration of each sample (Table 1).

Table 1. Yeast total cell number in each sample

Sample	Fermentation time (days)	Yeast Total Cell Number (CFU/g)
A	0	1.26×10^6
B	21	2.57×10^5
C	28	1.01×10^6
D	43	1.47×10^6
E	49	5.60×10^5

There were four groups of yeast colony morphological characteristics. A total of eight isolates had a colony shape of round, convex, shiny, all-round, and white. Then, ten isolates had round, thin flat, rough colonies, with complete edges and white. Furthermore, four isolates had an irregular colony shape, thin flat, rough, thorough edges, and white. Then, the three isolates were round, thin flat, rough, thorough edges, and white. The yeast isolates had an average colony diameter of 3-10 mm. In general, yeasts have a colony morphology of round shape, thorough edges, shiny, smooth surface, convex elevation, and thick white or cream. However, some yeast isolates also had irregular colony shapes with flat elevations (Sulmiyati *et al*., 2019). The yeast colony diameter in general ranges from 1.5-10 mm (Vulin *et al*., 2014).

The cellular characterization through cell staining showed 25 yeast isolates have three different cell shapes: round (four isolates), oval (14 isolates), and apiculate (seven isolates) (Figure 1). The representative isolates with three distinctive cell shapes are shown in Figure 1. The oval-shaped yeasts were found in all samples (A, B, C, D, & E), the round shape was only found in samples A, C, and D, while the apiculate shape was found in samples B, D, and E. The yeast with oval shapes that have been found in all samples indicated that they play a role in the fermentation process.

The shape of yeast cells and the presence or absence of budding is an important first step to ensure the yeast's characteristics. In general, yeasts have four cell shapes: round, oval, apiculate (oval with a pointed tip), and elongated (Figure 2). Yeasts with round or oval cell shapes generally have round or oval budding shapes, while yeasts with apiculate or elongated cell shapes generally have long budding shapes (Knop, 2011). The size of the yeast cells that were isolated had an average length of about 2-7 µm. In general, yeast cell sizes vary widely, with a length of about 1-40 µm and a width of about 1-10 µm (Zakhartsev & Reuss, 2018).

Yeast resistance in acidic conditions

Microorganism ability to survive in acidic conditions is one of the important requirements as a probiotic property. Good probiotics can survive at a pH range of 2-3 because this is a general standard often used to test the resistance of a probiotic microorganism to acid (Kim *et al*., 2019). After incubation for three hours at pH 2, 3, and 4, the ten yeast isolates had Survival Rate (SR) values of ≥100%. The SR even can reach ≥200% at pH 3 and 4 (Figure 3). The yeast isolates that were able to survive at pH 2 were isolate A2 (152.02%), isolate B1 (192.55%), isolate B3 (185.70%), isolate C3 (159.03%), isolate D4 (170.78%), isolate D5 (258.92%), isolate E1 (190.90%), isolate E2 (223.61%), isolate E3 (168.76%), and isolate E5 (174.15%). The isolate D5 was a yeast isolate with the highest SR.

The difference in pH significantly affected the growth of yeast. This was indicated by the significant difference in the resistance of yeast isolates at pH 2, 3, and 4. The SR of each isolate was

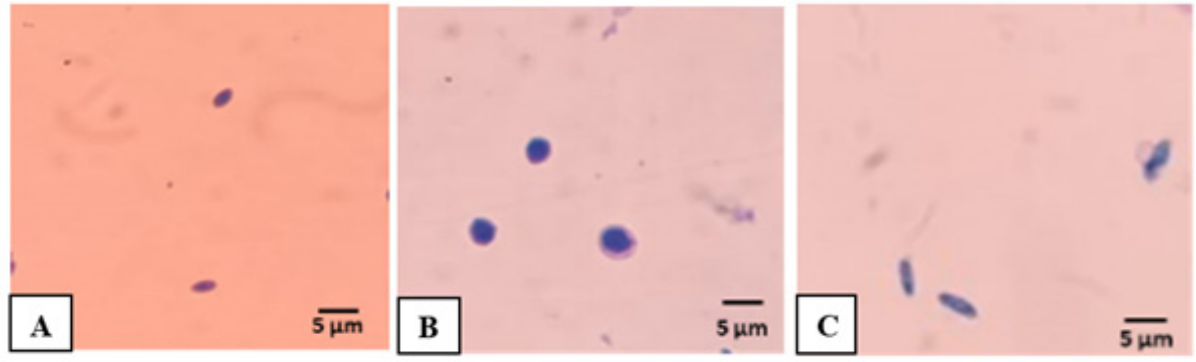

Fig. 1. Yeast cell morphology. (A) oval: isolate A1, (B) round: isolate C5, and (C) apiculate: isolate B5

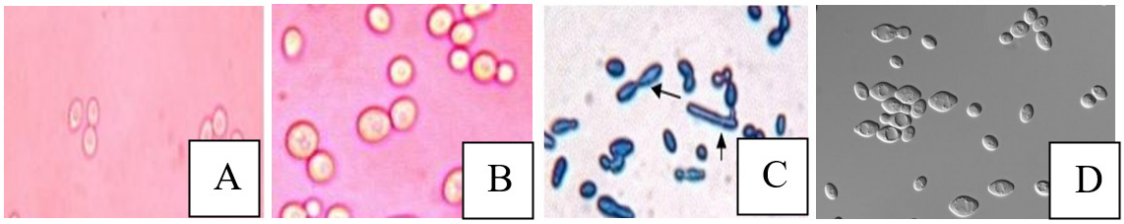

Fig. 2. Yeast cell morphology from the references. (A) Oval, (B) round, (C) elongated (Mukti *et al*., 2019), and (D) apiculate (Chang *et al*., 2012)

influenced by the type of isolate and the ability of the isolate to survive in acidic conditions for a certain period. This result was similar to a previous study that all yeast isolated from fermented cocoa could survive and grow well at pH 3 (Wulan *et al*., 2021). In another study, yeast isolated from kefir products had an SR of 97% after being incubated for 8 h at pH 2 (Lara-Hidalgo *et al*., 2017). Yeasts can survive at low pH due to alteration mechanisms in their cell walls (Ullah *et al*., 2013). This involves the induction mechanism of the cell wall integrity (CWI) gene and the general stress response (GSR) pathway. The reduction of pKA activity causes the release of GSR, which in turn instructs the cells to reprogram gene expression to adapt to low pH (Lucena *et al*., 2015). In addition, Ca^{2+} signaling is required for yeast to adapt to a low pH. This influx of Ca^{2+} plays a role in calcineurin activation (Lucena *et al*., 2020). The adaptive responses through the CWI pathway are needed under low pH conditions (Ullah *et al*., 2013).

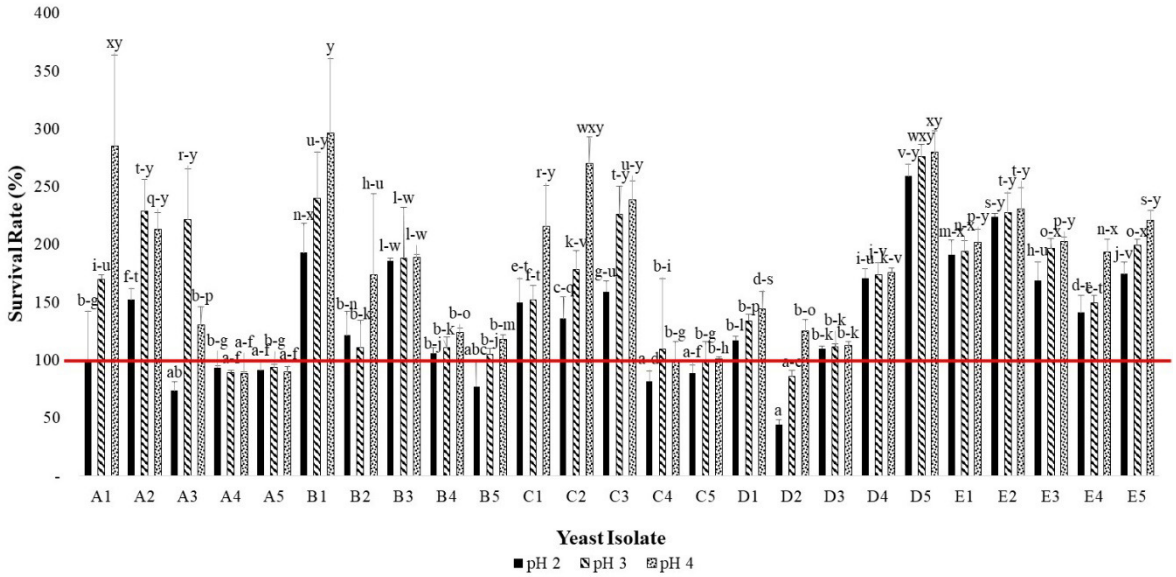

Fig. 3. Yeast isolate tolerance to acidic conditions. Different notations indicate a significant difference among treatments with a significance level of $p \leq 0.05$

Tolerance of yeast isolate to bile salt

Bile salts are intensely toxic to the cell membranes of microorganisms (Lennars & Lane, 2013). Tolerance to bile salts is one of the important properties of probiotics. This characteristic determines a microorganism's ability to survive in the digestive tract so that it has a functional role as a probiotic (Ruiz *et al*., 2013). The optimal concentration of bile salts in the human digestive tract ranges from 0.3% - 2% (Helmy *et al*., 2019). The results showed that most yeast isolates had a high tolerance for bile salts. Nine yeast isolates after being incubated for 4 h, had SR of ≥90% in the presence of 0.5% and 2% bile salt concentrations. One isolate with an SR of below 90%, namely isolate E1 (80.71%) in the concentration of 2%. The highest SR value was shown by isolate B1 which was 137.90% at 2% of bile salt (Figure 4).

The difference in the concentration of bile salts had a significant effect on the growth of yeast isolates. This was indicated by the significant difference in the resistance of yeast isolates at bile salt concentrations of 0.5% and 2%. Nine yeast isolates (A2, B1, B3, C3, D4, D5, E2, E3, & E5) were able to survive and grow in the presence of 2% bile salt. This was in line with previous research that yeast isolated from fermented food products has a high tolerance against bile salts with a survival rate of ≥99% and remains survive at a concentration of 2% after being incubated for 4 h (Helmy *et al*., 2019). This indicated that the selected yeast isolates were potential probiotic candidates. Yeast can overcome the effects of bile salts by producing the enzyme of bile salt hydrolases (BSH) or bile salt hydrolases derived from the gut microbiome. This BSH minimizes the toxic effects of conjugated bile (Alkalbani *et al*., 2022).

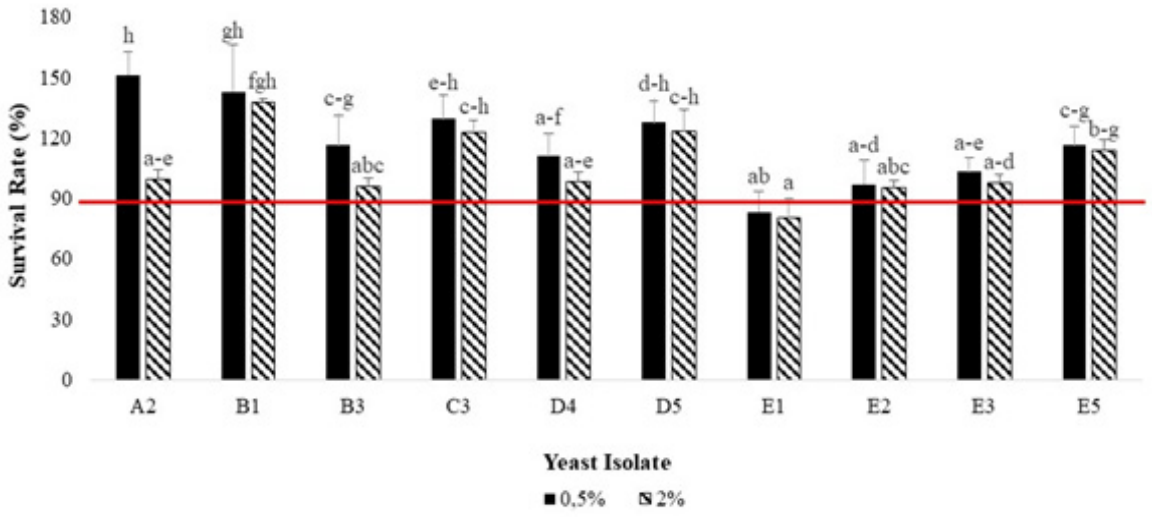

Fig. 4. The tolerance of yeast isolates to bile salts. Different notations indicate a significant difference among treatments with a significance level of $p \leq 0.05$

Antimicrobial activity of the selected yeasts

Antimicrobial activity is essential for probiotics and is one of the functional requirements of probiotics. The higher the antimicrobial activity produced by a microorganism, the better the microorganism is as a probiotic candidate (Fijan, 2016). Based on the results, three yeast isolates (B3, E3, & E5) could inhibit all tested pathogens (Figure 5). The highest inhibitory activity was exhibited by isolate D5, which was 93 AU/mL in inhibiting *Staphylococcus aureus*, but isolate D5 was not able to inhibit *Escherichia coli*. The isolate E3 was a potential isolate shown by the inhibitory activity against all tested pathogens compared to other yeast isolates. The antimicrobial activity values of isolate E3 were 41 AU/mL against *Candida albicans*, 53 AU/mL (*Staphylococcus aureus*), 50 AU/mL (*Salmonella* Typhimurium), 53 AU/mL (*Escherichia coli*), and 68 AU/mL (*Bacillus cereus*).

Previous research showed that yeast could inhibit *B. cereus* and *S. aureus* with an inhibition zone of 6 mm, and could inhibit *E. coli*, *Pseudomonas aeruginosa,* and *S*. Typhimurium with an inhibition zone of 8 mm (Fakruddin *et al*., 2017). In addition, another study also showed that *S. cerevisiae* isolated from fermented food in India could inhibit the growth of *Salmonella* sp. and *Pseudomonas* sp. with an inhibition zone of 16 mm (Syal & Vohra, 2013). This indicated that the selected yeast isolates were met as probiotic candidates. Yeast can inhibit the growth of pathogens by changing the pH of the media, competing directly for nutrients, as well as producing and secreting antimicrobial compounds such as mycocins (Muccilli & Restuccia, 2015). Mycocins or killer toxins an extracellular glycoprotein type that disrupts cell membrane function in pathogens by destroying 1,3-glucan, hence will induce cell death by osmotic lysis (Hatoum *et al*., 2012).

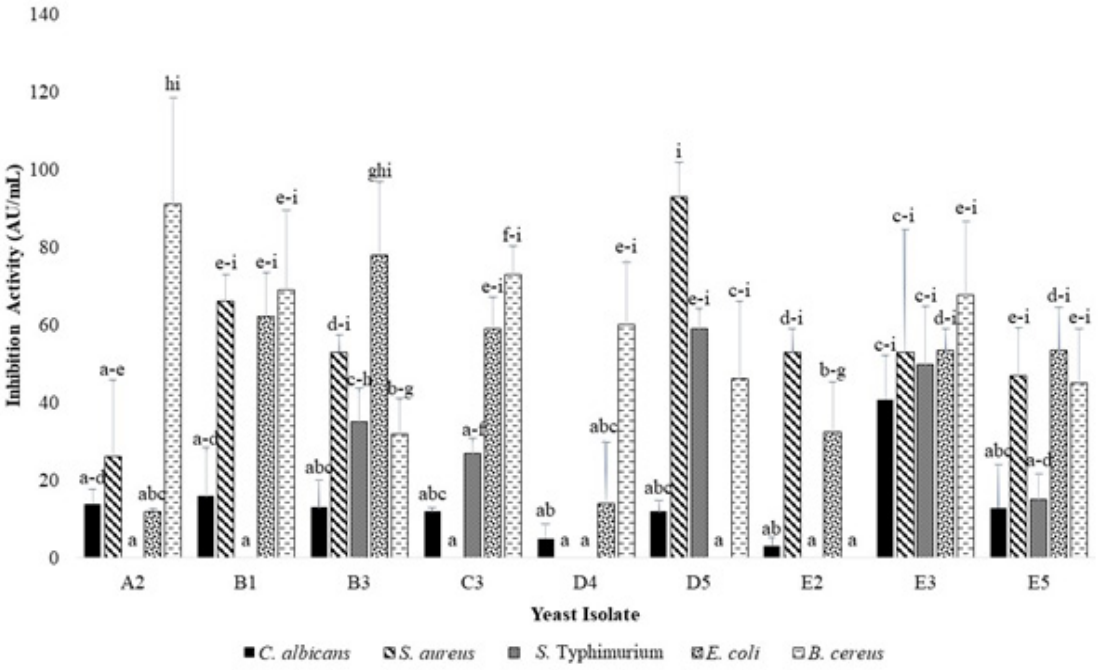

Fig. 5. The inhibitory activity of yeast isolates against tested pathogens. Different notation indicates a significant difference among treatments with a significance level of $p \leq 0.05$

Yeast resistance to antibiotics

Antibiotic resistance is a form of the ability of microorganisms to withstand the effects of certain drugs or antibiotics (Frieri *et al*., 2017). The existence of antibiotic resistance possessed by probiotics provides an opportunity to restore the gut microbiota after antibiotic treatment occurs (Gueimonde *et al*., 2013). All selected yeast isolates were resistant to all tested antibiotics (streptomycin 10 µg, cefazolin 30 µg, erythromycin 15 µg, & aztreonam 30 µg). The resistance of yeast isolates to this antibiotic can be seen by the absence of an inhibition zone formed around the antibiotic disc.

Previous studies on antibiotic resistance showed that probiotic yeasts were resistant to erythromycin, gentamicin, chloramphenicol, tetracycline, and ampicillin (Rodríguez *et al*., 2018). Other studies have also reported that *S. cerevisiae* was resistant to streptomycin, kanamycin, metronidazole, and vancomycin (Kim *et al*., 2019). Researchers have rarely carried out the resistance of yeast isolates to cefazolin and aztreonam antibiotics, but the results of this test showed that yeast isolates were also potentially resistant to cefazolin and aztreonam antibiotics. A microbe can become resistant to antibiotics because the microbe naturally has resistance genes inherited from previous generations (Pereira *et al*., 2018). So far, no studies have reported that antibiotic-resistant yeasts are capable of horizontal gene transfer (Kothari *et al*., 2019). In addition to resistance genes, cell wall structure also affects the resistance of yeast to antibiotics. Yeasts have cell wall structures including chitin, mannose, and glucans. Meanwhile, antibiotics are specifically designed to inhibit the synthesis of the peptidoglycan layer on the cell wall. Therefore, antibiotics do not affect yeast cell wall synthesis, so yeasts are resistant to antibiotics. This shows that probiotic yeast is relatively safe to be used at the same time as antibiotic therapy and can last for a long time in the digestive tract (Abid *et al*., 2022).

Hemolytic activity of the selected yeast isolates

One of the conditions for microorganisms to be used as probiotic candidates is that they are non-pathogenic. The nature of the pathogenicity of a microorganism can be seen from its hemolytic activity on blood agar media (Ragavan & Das, 2017). Based on the results, yeast isolate A2 has hemolytic activity with the type of β-hemolysis which was indicated by the presence of a clear zone formed around the colony. This indicated that isolate A2 was a pathogenic yeast. Meanwhile, eight other yeast isolates, namely B1, B3, C3, D4, D5, E2, E3, and E5 had non-hemolytic activity (γ-hemolysis) indicated by the absence of a greenish color or clear zone around the colony (Table 2).

The results showed that the eight selected yeast isolates qualified as probiotic candidates. The type of β-hemolysis can completely lyse red blood cells in the blood, while γ-hemolysis does not involve the breakdown of red blood cells. The pathogenic yeasts can lyse red blood cells by producing hemolysin which will damage cell membranes. The type of γ-hemolysin completely breaks down red blood cells and hemoglobin to form a clear zone around the colony (Hanna & Noor, 2022).

Table 2. The results of the pathogenicity test of selected yeast isolates

Isolate	Hemolytic Activity		
	α-hemolysis	β-hemolysis	γ-hemolysis
A2	-	+	-
B1	-	-	+
B3	-	-	+
C3	-	-	+
D4	-	-	+
D5	-	-	+
E2	-	-	+
E3	-	-	+
E5	-	-	+

Molecular Identification of the Selected Yeast Isolate

The yeast isolate E3 was a yeast with the best probiotic potency. Some of the potencies are to prevent and treat infections in the digestive tract, able to increase antioxidant activity and body immunity (Ogunremi *et al*., 2015). The results of ITS rDNA sequencing showed that the isolate E3 was identified as *Pichia kudriavzevii* with a similarity level of 100% to *Pichia kudriavzevii* strain iwate20191107 (Figure 6).

Pichia kudriavzevii is a yeast commonly found on the skins of fruits, kimchi, wine, and cheese (Helmy *et al*., 2019). Most are also found in the fermentation of coffee and cocoa (Shankar *et al*., 2022*)*. So far, *P. kudriavzevii* iwate20191107 has only been found in the rumen of Japanese black calves in 2021 according to the National Center for Biotechnology Information. *Pichia* has also been used as a starter culture in coffee fermentation (Shankar *et al*., 2022). *Pichia kudriavzevii* isolated from fermented food products can be used as a promising probiotic candidate (Rodríguez *et al*., 2018). The characteristics of the probiotics possessed by *P. kudriavzevii* have been widely reported and proven to provide good benefits for the health of the body, one of which is the ability of antioxidant activity *in vivo* (Banwo *et al*., 2021). In addition, it can stimulate the growth of microflora in the digestive tract, so that the balance of microflora in the digestive tract can be maintained (Seftiono, 2017). *Pichia kudriavzevii* isolated from karish cheese has a relatively high tolerance to pH 2 and 2% bile salt (Helmy *et al*., 2019). *Pichia kudriavzevii* isolated from cocoa fermentation had inhibitory activity against *S.* Typhimurium (Wulan *et al*., 2021). *Pichia kudriavzevii* has been reported as a non-pathogenic yeast and resistant to erythromycin and streptomycin (Chelliah *et al*., 2016). Some of these studies have supported the results of this current study related to the characteristics of the probiotic yeast of *Pichia kudriavzevii* E3.

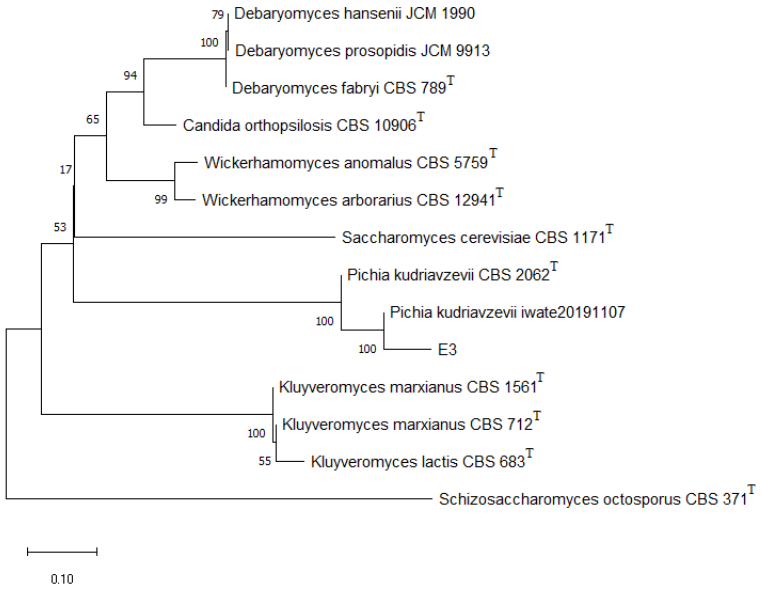

Fig. 6. Phylogenetic tree of E3 isolates and reference strains based on ITS rDNA sequences using the Neighbor-Joining method and the Tamura-Nei model with 1000x bootstraps.

CONCLUSION

Three yeast isolates (B3, E3, & E5) from the fermentation of wine coffee were qualified as probiotic candidates because they have a high tolerance to pH 2 and 2% bile salts with survival rates of ≥100% and ≥90% respectively, able to inhibit the pathogenic bacteria and yeast, resistant to all tested antibiotics, and non-pathogen. Moreover, yeast isolates E3 were selected as the best potential probiotic properties. The isolate E3 was identified as *Pichia kudriavzevii* with a similarity level of 100% to *Pichia kudriavzevii* strain iwate20191107. The potency of this isolate as a starter culture of wine-coffee fermentation is warranted to be elucidated.

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ETHICAL STATEMENT

Not Applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Abid, R., Waseem, H., Ali J., Ghazanfar, S., Ali, G.M., Elasbali, A.M. & Alharethi, S.H. 2022. Probiotic yeast *Saccharomyces*: Back to nature to improve human health. Journal of Fungi, 8(5): 444. https://doi.org/10.3390/jof8050444

Alkalbani, N.S., Osaili, T.M., Al-Nabulsi, A.A., Olaimat, A.N., Liu, S.Q., Shah, N.P., Apostolopoulos, V. & Ayyash, M.M. 2022. Assessment of yeasts as potential probiotics: A review of gastrointestinal tract conditions and investigation methods. Journal of Fungi, 8(4): 365. https://doi.org/10.3390/jof8040365

Azhar, M.A. & Munaim, M.S.A. 2019. Identification and evaluation of probiotic potential in yeast strains found in kefir drink samples from Malaysia. International Journal of Food Engineering, 15(7): 20180347. https://doi.org/10.1515/ijfe-2018-0347

Banwo, K., Alonge, Z. & Sanni, A.I. 2021. Binding capacities and antioxidant activities of *Lactobacillus plantarum* and *Pichia kudriavzevii* against cadmium and lead toxicities. Biological Trace Element Research, 199(2): 779–791. https://doi.org/10.1007/s12011-020-02164-1

Chang, C.F., Huang, L.Y., Chen, S.F., & Lee, C.F. 2012. *Kloeckera taiwanica* sp. nov., an ascomycetous apiculate yeast species isolated from mushroom fruiting bodies. International Journal of Systematic and Evolutionary Microbiology, 62: 1434-1437. https://doi.org/10.1099/ijs.0.034231-0

Chelliah, R., Ramakrishnan, S.R., Prabhu, P.R. & Antony, U. 2016. Evaluation of antimicrobial activity and probiotic properties of wild-strain *Pichia kudriavzevii* isolated from frozen idli batter. Yeast, 33(8): 385–401. https://doi.org/10.1002/yea.3181

Fakruddin, M., Hossain, M.N. & Ahmed, M.M. 2017. Antimicrobial and antioxidant activities of *Saccharomyces cerevisiae* IFST062013, a potential probiotic. BMC Complement Alternative Medicine, 17(1): 64. https://doi.org/10.1186/s12906-017-1591-9

Fijan, S. 2016. Antimicrobial effect of probiotics against common pathogens. In: V. Rao and L.G. Rao (Eds.). Probiotics and Prebiotics in Human Nutrition and Health. Intech Open Science, London. pp. 191-221.

Frieri, M., Kumar, K. & Boutin, A. 2017. Antibiotic resistance. Journal of Infection and Public Health, 10(4): 369–378. https://doi.org/10.1016/j.jiph.2016.08.007

Gueimonde, M., Sánchez, B., de los Reyes-Gavilán, C.G. & Margolles, A. 2013. Antibiotic resistance in probiotic bacteria. Frontiers in Microbiology, 4 : 202. https://doi.org/10.3389/fmicb.2013.00202

Hanna, M. & Noor, A. 2022. *Streptococcus* group B. StatPearls Publishing, London.

Hatoum, R., Labrie, S. & Fliss, I. 2012. Antimicrobial and probiotic properties of yeasts: From fundamental to novel applications. Frontiers in Microbiology, 3: 421. https://doi.org/10.3389/fmicb.2012.00421

Helmy, E.A., Soliman, S.A., Abdel-Ghany, T.M. & Ganash, M. 2019. Evaluation of potentially probiotic attributes of certain dairy yeast isolated from buffalo sweetened Karish cheese. Heliyon, 5(5): e01649. https://doi.org/10.1016/j.heliyon.2019.e01649

Hu, X.Q., Liu, Q., Hu, J.P., Zhou, J.J., Zhang, X., Peng, S.Y., Peng, L.J. & Wang, X.D. 2018. Identification and characterization of probiotic yeast isolated from digestive tract of ducks. Poultry Science, 97(8): 2902–2908. https://doi.org/10.3382/ps/pey152

Jatmiko, Y.D., Lopes, M.D.B. & Barton, M.D. 2012. Molecular identification of yeast isolated from dadih by RFLP-PCR and assessment on their ability in utilizing lactate. Microbiology Indonesia, 6(1): 30-34.

Kim, J.A., Bayo, J., Cha, J., Choi, Y.J., Jung, M.Y., Kim, D.H. & Kim, Y. 2019. Investigating the probiotic characteristics of four microbial strains with potential application in feed industry. PLoS ONE, 14(6): e0218922. https://doi.org/10.1371/journal.pone.0218922

Knop, M. 2011. Yeast cell morphology and sexual reproduction - A short overview and some considerations. Comptes Rendus - Biologies, 334(8–9): 599–606. https://doi.org/10.1016/j.crvi.2011.05.007

Kothari, D., Patel, S. & Kim, S.K. 2019. Probiotic supplements might not be universally-effective and safe: A review. Biomedicine and Pharmacotherapy, 111: 537–547. https://doi.org/10.1016/j.biopha.2018.12.104

Lara-Hidalgo, C.E., Hernández-Sánchez, H., Hernández-Rodríguez, C. & Dorantes-Álvarez, L. 2017. Yeasts in fermented foods and their probiotic potential. Austin Journal of Nutrition and Metabolism, 4(4): 1045.

Lennars, W.J. & Lane, M.D. 2013. Encyclopedia of biological chemistry. 2nd Ed. Academic Press, San Diego.

Lucena, R.M., Dolz-Edo, L., Brul, S., de Morais Jr, M.A. & Smits, G. 2020. Extreme low cytosolic ph is a signal for cell survival in acid stressed yeast. Genes, 11(6): 656. https://doi.org/10.3390/genes11060656

Lucena, R.M., Elsztein, C., Pita, W.D.B., de Souza, R.F., Júnior, S.D.S.L.P. & Junior, M.A.D.M. 2015. Transcriptomic response of *Saccharomyces cerevisiae* for its adaptation to sulphuric acid-induced stress. Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology, 108(5): 1147–1160. https://doi.org/10.1007/s10482-015-0568-2

McFarland, L.V. 2010. Systematic review and meta-analysis of *Saccharomyces boulardii* in adult patients. World Journal of Gastroenterology, 16(18): 2202–2222. https://doi.org/10.3748/wjg.v16.i18.2202

Moradi, R., Nosrati, R., Zare, H., Tahmasebi, T., Saderi, H. & Owlia, P. 2018. Screening and characterization of in-vitro probiotic criteria of *Saccharomyces* and *Kluyveromyces* strains. Iranian Journal of Microbiology, 10(2): 123–131.

Moreira, I.M.D.V, Miguel, M.G.D.C.P, Duarte, W.F, Dias, D.R. & Schwan, R.F. 2013. Microbial succession and the dynamics of metabolites and sugars during the fermentation of three different cocoa (*Theobroma cacao* L.) hybrids. Food Research International, 54(1): 9–17. https://doi.org/10.1016/j.foodres.2013.06.001

Moslehi-Jenabian, S., Pedersen, L. & Jespersen, L. 2010. Beneficial effects of probiotic and food borne yeasts on human health. Nutrients, 2(4): 449–473. https://doi.org/10.3390/nu2040449

Muccilli, S. & Restuccia, C. 2015. Bioprotective role of yeasts. Microorganisms, 3(4): 588–611. https://doi.org/10.3390/microorganisms3040588

Mukti, R.F., Chowdhury, M.M.K., & Uddin, M.A. 2019. Isolation and characterization of osmophilic fermentative yeasts from Bangladeshi honeys. Journal of Advanced Biotechnology and Experimental Therapeutics, 2(3): 127-133. https://doi.org/10.5455/jabet.2019.d35

National Center for Biotechnology Information. no date. *Pichia kudriavzevii iwate20191107 genes for SSU rRNA, ITS1, 5.8S rRNA, ITS2, LSU rRNA, partial and complete sequence.* URL https://www.ncbi.nlm.nih.gov/nuccore/LC633337.1 (accessed 7.28.22).

Ogunremi, O.R., Agrawal, R. & Sanni, A.I. 2015. Development of cereal-based functional food using cereal-mix substrate fermented with probiotic strain - *Pichia kudriavzevii* OG32. Food Science and Nutrition, 3(6): 486-494.

Pereira, G.V.D.M., Soccol, V.T., Pandey, A., Medeiros, A.B.P, Lara, J.M.R.A, Gollo, A.L. & Soccol, C.R. 2014. Isolation, selection and evaluation of yeasts for use in fermentation of coffee beans by the wet process. International Journal of Food Microbiology, 188: 60–66. https://doi.org/10.1016/j.ijfoodmicro.2014.07.008

Pereira, G.V.D.M., Coelho, B.D.O., Júnior, A.I.M., Soccol, V.T. & Soccol, C.R. 2018. How to select a probiotic? A review and update of methods and criteria. Biotechnology Advances, 36(8): 2060–2076. https://doi.org/10.1016/j.biotechadv.2018.09.003

Ragavan, M.L. & Das, N. 2017. Molecular identification of probiotic yeast strains and their characterization. Asian Journal of Pharmaceutical and Clinical Research, 10(10): 339–343. https://doi.org/10.22159/ajpcr.2017.v10i10.20052

Rodríguez, P.F.-P., Arévalo-Villena, M., Rosa, I.Z. & Pérez, A.B. 2018. Selection of potential non-*Saccharomyces* probiotic yeasts from food origin by a step-by-step approach. Food Research International, 112: 143–151. https://doi.org/10.1016/j.foodres.2018.06.008

Ruiz, L., Margolles, A. & Sánchez, B. 2013. Bile resistance mechanisms in *Lactobacillus* and *Bifidobacterium*. Frontiers in Microbiology, 4: 396. https://doi.org/10.3389/fmicb.2013.00396

Seftiono, H. 2017. Penentuan aktivitas enzim mananase dari berbagai mikroorganisme di Indonesia dan peranannya dalam bidang pangan: Kajian pustaka (Determination of the activity of the mananase enzyme from various microorganisms in Indonesia and its role in the food sector: Literature review). Agrointek, 11(1): 14-20. https://doi.org/10.21107/agrointek.v11i1.2939

Siddiquee, S., Yusuf, U.K. & Zainudin, N.A.I.M. 2010. Morphological and molecular detection of *Fusarium chlamydosporum* from root endophytes of *Dendroblum crumenatum*. African Journal of

Biotechnology, 9(26): 4081-4090.
Shankar, S.R., Sneha, H.P., Prakash, I., Khan, M., Punil, K.H.N., Om, H., Basavaraj, K. & Murthy, P.S. 2022. Microbial ecology and functional coffee fermentation dynamics with *Pichia kudriavzevii*. Food Microbiology, 105: 104012. https://doi.org/10.1016/J.FM.2022.104012
Staniszewski, A. & Kordowska-Wiater, M. 2021. Probiotic and potentially probiotic yeasts—characteristics and food application. Foods, 10(6): 1306. https://doi.org/10.3390/foods10061306
Sulaiman, I., Erfiza, N.M. & Moulana, R. 2021. Effect of fermentation media on the quality of arabica wine coffee. IOP Conf Series: Earth and Environmental Science, 709(1): 012027. https://doi.org/10.1088/1755-1315/709/1/012027
Sulmiyati, F., Said, N.S., Fahrodi, D.U., Malaka, R. & Maruddin, F. 2019. The characteristics yeast isolated from commercial kefir grain. Hasanuddin Journal of Animal Science, 1(1): 26–37.
Syal, P. & Vohra, A. 2013. Probiotic potential of yeasts isolated from traditional indian fermented foods. International Journal of Microbiology Research, 5(2): 390–398. https://doi.org/10.9735/0975-5276.5.2.390-398
Tomičić, Z.M., Čolović, R.R., Čabarkapa, I.S., Vukmirović, D.M., Đuragić, O.M. & Tomičić, R.M. 2016. Beneficial properties of probiotic yeast *Saccharomyces boulardii*. Food and Feed Research, 43(2): 103–110. https://doi.org/10.5937/ffr1602103t
Ullah, A., Chandrasekaran, G., Brul, S. & Smits, G.J. 2013. Yeast adaptation to weak acids prevents futile energy expenditure. Frontiers in Microbiology, 4: 142. https://doi.org/10.3389/fmicb.2013.00142
Villar-García, J., Hernández, J.J., Güerri-Fernández, R., Gonzáles, A., Lerma, E., Guelar, A., Saenz, D., Sorli, L., Montero, M., Horcajada, J.P. & Freud, H.K. 2015. Effect of probiotics (*Saccharomyces boulardii*) on microbial translocation and inflammation in HIV-treated patients: A double-blind, randomized, placebo-controlled trial. Journal of Acquired Immune Deficiency Syndromes, 68(3): 256–263. https://doi.org/10.1097/QAI.0000000000000468
Vulin, C., Meglio, J.M.D., Lindner, A.B., Daerr, A., Murray, A. & Hersen, P. 2014. Growing yeast into cylindrical colonies. Biophysical Journal, 106(10): 2214–2221. https://doi.org/10.1016/j.bpj.2014.02.040
Willaert, R.G. 2017. Yeast biotechnology. Fermentation, 3(1): 6-8. https://doi.org/10.3390/fermentation3010006
Wulan, R., Astuti, R.I., Rukayadi, Y. & Meryandini, A. 2021. Evaluation of indigenous *Pichia kudriavzevii* from cocoa fermentation for a probiotic candidate. Biodiversitas, 22(3): 1317–1325. https://doi.org/10.13057/biodiv/d220331
Yulianti, S.E. & Astuti, D.I. 2021. Fermentation of tofu using consortium of lactic acid bacteria isolated from tofu whey as biocoagulant and bioperservation. Annales Bogorienses, **25(1)**: 15-27: https://doi.org/10.14203/ann.bogor.2021.v25.n1.15-27
Zakhartsev, M. & Reuss, M. 2018. Cell size and morphological properties of yeast *Saccharomyces cerevisiae* in relation to growth temperature. FEMS Yeast Research, 18(6): foy052. https://doi.org/10.1093/femsyr/foy052