Evaluation of Indigenous *Pichia kudriavzevii* from cocoa fermentation for a probiotic candidate

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Abstract. *Wulan R, Astuti RI, Rukayadi Y, Meryandini A. 2021. Evaluation of Indigenous Pichia kudriavzevii from cocoa fermentation for a probiotic candidate. Biodiversitas 22: 1317-1325.* Currently, probiotics are becoming a concern along with a healthy lifestyle awareness. Besides bacteria, yeast can be used as a probiotic candidate with specific functional properties. Research on yeast as a probiotic is still limited (except for *Saccharomyces boulardii*). Previous research has isolated yeasts from the cocoa fermentation process. This study aimed to evaluate the probiotics properties and antioxidant activity of yeast strains isolated from spontaneous cocoa fermentation in Sukabumi, Indonesia. Previous research has isolated 23 yeast strains from spontaneous cocoa (*Theobroma cacao* L.) fermentation. Of the 23 yeast strains isolated from cocoa fermentation, 22 strains showed negative hemolysis as an indicator of non-pathogenic properties. Ten yeast strains were able to grow at 37 °C and 41 °C, pH 3, 0.5% bile salts, had autoaggregation ability (63.99-95.33%), and co-aggregation with *S. typhimurium* ATCC14028 (>80%), as character requirement for probiotic candidates. The genetic identification of the ten yeast strains showed that they were 99% identical to *Pichia kudriavzevii*. Based on its antioxidant activity, the *P. kudriavzevii* 2P10 metabolites had the highest percentage of inhibition (68.51%) against DPPH free radicals and resistance to H_2O_2 oxidative stress up to 3 mM. It can be concluded that *P. kudriavzevii* 2P10 is a promising probiotic candidate for functional foods and health purposes.

Keywords: Antioxidant, cocoa fermentation, *Pichia kudriavzevii*, probiotics, yeast

INTRODUCTION

Probiotics are increasingly popular, along with healthy gut awareness. Routine probiotics consumption can improve gut microbiota balance to prevent dysbiosis, boosting immunity, and lowering the risk of certain metabolic systemic diseases. Medical experts often recommend probiotics to treat various diseases, especially diarrhea (Sanders et al. 2018; Aponte et al. 2020). Probiotics are defined as live microorganisms when administered in adequate amounts, confer a health benefit to the host (Hill et al. 2014). Probiotics are widely used in industries, including foods, dietary supplements, and pharmaceuticals (Sanders et al. 2018).

The most commonly used probiotics are lactic acid bacteria, *Lactobacillus* and *Bifidobacterium*. The presence of yeast in the human gastrointestinal tract as mycobiome indicates that the yeasts have many beneficial effects on their host. *Saccharomyces, Malassezia*, and *Candida* are yeasts found in the human gastrointestinal tract (Nash et al. 2017). *Saccharomyces cerevisiae var. boulardii* is the only yeast that has been studied extensively for probiotic properties, and it was clinically tested to be effective in preventing *Clostridium defficile* infection (Carstensen et al. 2018).

Yeast is a eukaryotic microorganism that is known to be safe for use in various food fermentation processes. Several previous studies have conducted the isolation and characterization of probiotic yeast from different fermented foods, including kefir, cheese, frozen idli batter, and fermented cocoa. These yeasts could grow at 37 °C, tolerate the pH of gastric acid and bile salts, have the ability to auto aggregate and co-aggregation with pathogenic microorganisms (Chelliah et al. 2016; Gut et al. 2019; Menezes et al. 2019; Hsiung et al. 2020). Probiotic yeast also has functional benefits, one of which is antioxidant activity. Probiotic *S. boulardii* NCYC 3264 has been shown to have antioxidant activity (Datta et al. 2017). *Pichia kudriavzevii* FY05 has biological activity on the heavy metals binding capacity and antioxidant activity in-vivo (Banwo et al. 2020).

The majority of research on probiotics usually relates to bacteria, especially lactic acid bacteria. There is a lack of information about probiotic yeast (except *S. boulardii*). The cocoa fermentation process involves various microbial growth, i.e., filamentous fungi, yeast, lactic acid bacteria, and acetic acid bacteria. Therefore, the cocoa fermentation process can be a potential yeast probiotic source (Ardhana and Fleet 2003). Previous research has isolated microbes from the cocoa fermentation process in Sukabumi,

Indonesia, for the cocoa fermentation starter (Berutu et al. 2017). However, the probiotics properties of these yeasts have not been carried out. This study aimed to evaluate the probiotics properties and antioxidant activity of yeast strains isolated from spontaneous cocoa fermentation in Sukabumi, Indonesia.

MATERIALS AND METHODS

Procedures

Yeast subculture and morphological characterization

A total of 23 lyophilized yeast strains (1P4, 4B2, 2P1, 2P10, 3B2, 1B3, 2P6, 2P4, 0P1, 2P8, 2P7, 0P2, 2P2, 3B1, 1B2, 2B3, 3B1, 2P9, 1P3, 4B1, NHC1, NHC2, and NHC3) from cocoa fermentation process were grown in YPD broth medium (1% yeast extract, 2% peptone, and 2% dextrose) and incubated aerobically in a shaker incubator 100 rpm at room temperature (28 °C) for 24 hours. After incubation, yeast was purified. Yeast morphology was observed using a light microscope (LED Olympus CX-23) without staining. Morphological characters were carried out by observing cell shape and budding cells.

Hemolytic activity

The pathogenicity of yeast was screened by hemolysin production (Gut et al. 2019). The 3 μL yeast culture (24 hours) in YPD broth medium was spotted onto blood sheep agar and incubated at 37 °C for 48 hours. *Lactobacillus rhamnosus* MC7 was used as a negative control. *Klebsiella pneumoniae* ATCC13773 and *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) ATCC14028 were used as the positive control. The presence of a clear zone around the colony indicated hemolysin production.

Yeast growth at human body temperature

Colonies of yeast strains were streaked onto a YPD agar and incubated at room temperature (28 °C), human body temperature (37 °C), and 41 °C (for another application i.e., poultry probiotics) aerobically for 24 hours. A room temperature (28 °C) was used as a control.

Bile salt and acid tolerance

Yeast resistance tests to bile salts and acid were carried out according to Greppi et al. (2017) with some modifications. Yeast strains were cultured in YPD medium at 28 °C for 18 h and used as a starter. Bile salt tolerance analysis: 1% of yeast culture starter was inoculated in the YPD broth medium with 0.5% bile salt (HIMEDIA) and incubated at 37 °C. The OD_{600} absorbance was measured after 5 hours using a spectrophotometer (Jenway 7206, United Kingdom) at λ 600 nm. *L. rhamnosus* MC7 in MRS+0.5 bile salt was used as a positive control. Acid tolerance analysis: 1% of yeast culture starter was inoculated in the YPD broth medium at pH 3 (adjusted with 1 M HCl) and incubated at 37 °C. The absorbance of the OD_{600} was measured after 3 hours of incubation. *L. rhamnosus* MC7 in MRS pH 3 was used as a positive control.

Autoaggregation assay

Autoaggregation assay was performed by following the method of Fadda et al. (2017) with some modifications. Yeast strains were incubated in YPD broth at 28°C aerobically in shaker 100 rpm for 24 hours. Yeast culture was centrifuged at 4000 rpm for 15 min at 28 °C, washed twice, and resuspended in 3 ml Phosphate Buffered Saline (PBS), vortexed for 30 s, and incubated for 24 h at 37 °C. The surface suspension was carefully removed after 0 ,2 , and 4 hours incubation. After that, the absorbance of the OD_{600} was measured using a spectrophotometer (Jenway 7206, UK). *L. rhamnosus* MC7 was also used as a positive control. Percentage of autoaggregation was calculated as follows:

% Autoaggregation = $(1 - (A_t / A_0))$ x 100%

Where: OD is OD_{600nm} before incubation (0 hour) and OD_t is OD_{600nm} after 2 hours or 4 hours incubation

Co-aggregation assay

The co-aggregation ability test was carried out based on Ladha and Jeevaratnam (2018). Yeast strains were grown in YPD broth for 24 hours at 28 °C. Pathogenic bacteria (*S. typhimurium* ATCC14028) was cultured in NB (nutrient broth) medium for 24 hours at 37 °C. A total of 2 ml of each culture was centrifuged separately (4000 rpm, 10 minutes) at room temperature. Furthermore, each cell pellet was washed twice and resuspended in PBS pH 7.2. The 4 ml mixture consisted of 2 ml of yeast cell suspension and 2 ml of *S. typhimurium* ATCC14028 cell suspension was incubated for 4 hours at 37 C. After that, the absorbance of the OD_{600} was measured using a spectrophotometer (Jenway 7206, United Kingdom). *L. rhamnosus* MC7 was also used as a positive control. The co-aggregation percentage is calculated using the following formula.

% Co-aggregation = $(1 - (A_t / A_0))$ x 100%

Where: OD is OD_{600nm} before incubation (0 hour) and OD_t is OD_{600nm} after 2 hours or 4 hours incubation.

Molecular Identification of yeast strains based on sequence Internal Transcribed Spacer (ITS) regions

Yeast strains were identified through molecular analysis of the ITS gene region. Yeasts were culture on YPD broth for 18 h at 28 °C. According to the protocol, the DNA was extracted with The YeaStar Genomic DNA Kit™ (Zymo Research, United States). The concentration and purity of the genomic DNA were quantified using the nanodrop spectrophotometer (MaestroGen, Taiwan). ITS region was amplified using the Polymerase Chain Reaction (Applied Biosystems™ 2720 Thermal Cycler Thermo Fisher, United States) with the primers ITS1 forward (5′ TCCGTAGGT GAACCTGCGG-3′) dan primer ITS4 reverse (5′-TCCTCCGCTTATTGATATGC-3′). The amplification was performed in a total reaction volume of 50 μL containing 25 μL GoTaq Green® Master Mix 123 (Promega), 5 μL of 10 pmol ITS1 primers, 5 μL of 10 pmol ITS4 primers, 4 μL DNA template (100 ng/μL), and 11 μL nuclease-free water. The PCR conditions were as follows: Pre-denaturation at 94 °C for 5 minutes, 35 cycles of

denaturation at 94 ℃ for 30 s, annealing at 49 ℃ for 30 s, elongation at 68 ℃ for 1 minute, post-elongation at 68 ℃ for 20 s, and post-PCR at 15 ℃ for 5 minutes. Following gel electrophoresis and EtBr staining, the amplification results were visualized using a UV transilluminator. The PCR product was sent for sequencing to a sequencing services company. The results obtained were analyzed using *Seqtrace* software. The sequences were then compared to the National Center for Biotechnology Information (NCBI) GenBank (https://www.ncbi.nlm.nih. gov) using the BLAST.n program. Construction of phylogenetic tree was constructed using MEGA 6.0 software by neighbor-joining with 1000× bootstrap replications (Tamura et al. 2013)

Antioxidant activity

The antioxidant activity was measured by the capacity of reduction of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical using the method described by Andrianto et al. (2015) with some modifications. Yeasts were cultured in YPD broth (yeast extract 0.5%, 1% peptone, and 1% dextrose) for 18 h and 48 h 150 rpm (start OD= 0,1). The supernatant of each yeast culture was harvested by centrifugation at 5000 rpm for 15 min and filtered using a syringe filter of 0.45 μm (Shahat 2018). The supernatant of yeast was added into a mixture (0.9 ml) of 0.4 mM DPPH solution, 20% methanol aqueous solution, and Tris-HCl buffer solution pH 7.4. Samples were incubated in a dark room for 20 minutes. Absorbance was measured at 522 nm. Ascorbic acid was used as standard. The inhibitory activity was calculated from the following equation:

$$\text{DPPH radical scavenging (\% inhibition)} = \left(1 - \frac{A_1}{A_0}\right) x\ 100\%$$

Where:
A_0 =the absorbance of the mixture without sample
A_1=the absorbance of the mixture with a sample

Spot test on H_2O_2 oxidative stress

The spot test on H_2O_2 oxidative stress was carried out using the spot method (Yusuf 2020). Yeast strains 0P2, 1P4, and 2P10 were cultured in YPD broth (yeast extract 0.5%, 1% peptone, and 1% dextrose) for 48 hours. The yeast culture was spotted after 48 hours of incubation. The spot process was started by adjusting the OD_{600} of each culture to an OD_{600} of 1, then diluting by serial dilution of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} using YPD broth medium in a sterile microplate well (Nunc 96). A total of 2 μL of each dilution and culture OD_{600}=1 was spotted on agar plate YPD medium (yeast extract 1%, 2% peptone, and 1% dextrose) without the addition of H_2O_2 (control) and YPD agar with various concentrations of H_2O_2 (1 mM, 2 mM, and 3 mM). Plates were incubated for 24 hours at 28 ℃.

RESULTS AND DISCUSSION

A total of 23 yeast strains have been isolated from the cocoa fermentation process in previous research. There are two groups of yeast-based on their morphology (Figure 1).

The first morphology was cream-white colonies yeast, round, convex elevation, entire edge, and opaque (Figure 1.A). There were 19 yeast strains included in the first morphological group (1P4, 4B2, 2P1, 2P10, 3B2, 1B3, 2P6, 2P4, 0P1, 2P8, 2P7, 0P2, 2P2, 3B1, 1B2, 2B3, 3B1, 2P9, and 1P3). Figure 1.C showed that the yeast cells' morphology is ovoid-elongate measuring 5-10 μm, had a vacuole in the center of the cell, and formed a bud (Figure 1.C). Other yeast strains (NHC1, NHC2, and NHC3) showed cream-white colonies, irregular shape, umbonate elevation, undulate edges, and opaque not transparent (Figure 1.B). Yeast on Figure 1.D showed oval/ ovoid cell shape, measuring 5-8 μm, had a vacuole in the middle of the cell, and formed a bud. Morphology of the 4B1 strain was not observed because of its positive hemolysis ability.

Hemolytic activity

All yeast strains have been tested for their hemolysis ability against red blood cells on blood agar. Among 23 yeast strains, 22 yeast strains did not show hemolysis ability. Only one yeast strain (4B1) showed beta-hemolysis ability, so it was discontinued in the following probiotic yeast selection process. Yeasts strains and negative control of *L. rhamnosus* MC7 did not form clear zones on blood agar. The clear zone was visible around the *K. pneumoniae* ATCC13773 colony (positive control), which showed beta-hemolytic. *S. typhimurium* ATCC14028 showed alpha-hemolysis ability (Figure 2). The 22 non-pathogenic yeast strains were further selected for probiotic, and observed their growth at human body temperature.

Yeast growth at human body temperature

A total of 19 yeast strains were able to grow well at various temperatures, i.e., 28 ℃ (control), 37 ℃, and 41 ℃ by forming visible thick colonies on YPD agar medium after 24 hours incubation (Figure 3). However, three yeast strains (NHC1, NHC2, and NHC3) did not grow well at 37 ℃ and 41℃. It indicated they were not suitable for human probiotics.
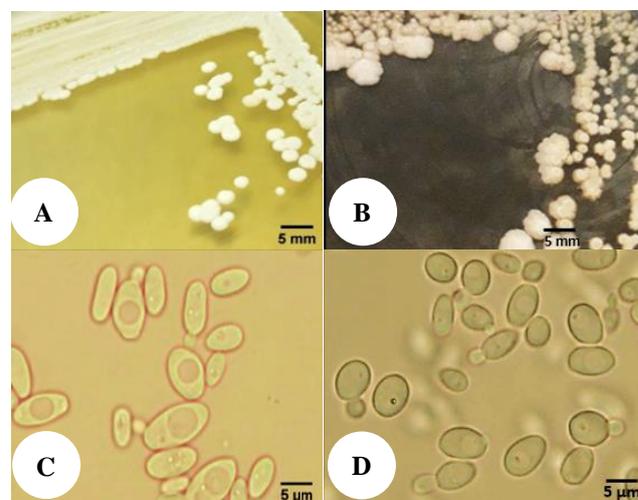

Figure 1. Yeast morphology on YPD with 2% agar: A. 1P4 colonies at 72 h, B. NHC3 colonies at 72 h, C. Ovoid-elongate cell shape (1P4 at 24 h), D. Ovoid cell shape (NHC3 at 24 h)
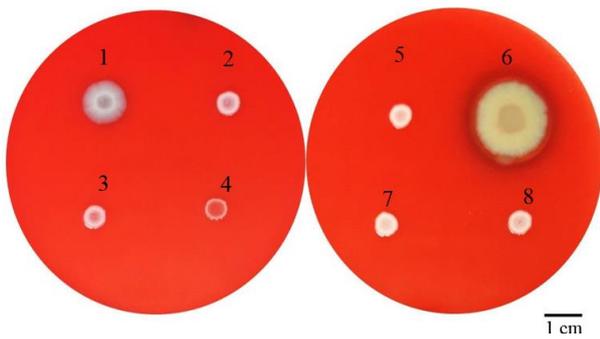
Figure 2. Hemolytic activity of yeast in blood sheep agar incubated at 37 ºC for 48 hours. (1) *S. typhimurium* ATCC14028 (K+), (2) 2P8, (3) 2P6, (4) *L. rhamnosus* MC7 (K-), (5) 3B1, (6) *K. pneumoniae* ATCC13773 (K+), (7) 0P1, (8) 1B3. *S. typhimurium* ATCC14028= α- hemolysis, *K. pneumoniae* ATCC13773 =β- hemolysis, other strains = γ- hemolysis

Bile salt tolerance

The results showed that all of the yeast strains grew better on YPD+0.5% bile salt than the control *Lactobacillus rhamnosus* MC7 on MRS+0.5% (Figure 4) after 5 hours incubation. Due to their growth ability in

0.5% bile salts and different morphological characters, 16 yeast strains (1P4, 4B2, 2P1, 2P10, 3B2, 1B3, 2P6, 0P1, 2P8, 0P2, 2P2, 3B1, 1B2, 2B3, 3B1, 1P3, and NHC2) were selected for their tolerance in the pH 3.

Yeast tolerance to pH 3

The result showed that 15 yeast strains had better growth at pH 3 than positive control *L. rhamnosus* MC7. Only the NHC2 yeast strain had lower growth than *L. rhamnosus* MC7 positive control (Figure 5). Twelve of the selected yeasts were selected for the autoaggregation and the co-aggregation assay with *S. typhimurium* ATCC14028.

Autoaggregation ability and co-aggregation ability

Table 1 shows the autoaggregation and co-aggregation abilities of 12 yeast with *S. typhimurium* ATCC14028. There was 3 yeast strain (2P8, 2P6, and 1B3) had better autoaggregation than positive control *L. rhamnosus* MC7. All of the tested yeast strains had higher co-aggregation than positive control *L. rhamnosus* MC7. This co-aggregation of bacteria and yeast sediments was also observed using a light microscope (Figure 6).
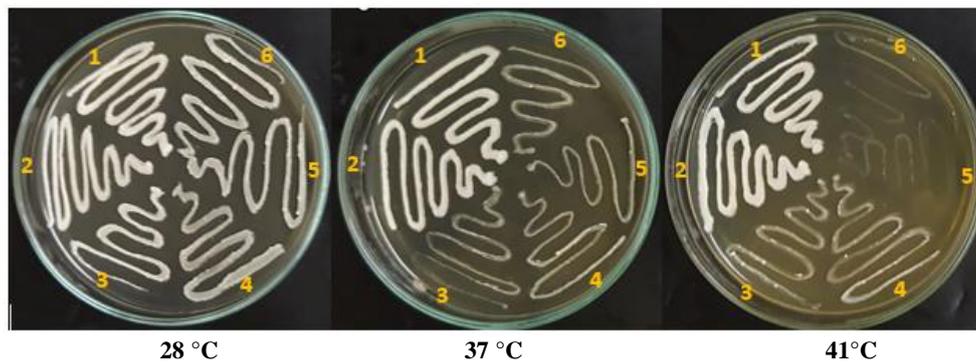

Figure 3. Yeast growth on ypd 2% agar media after 24 hours incubation: (1) 2P9, (2) 1P3, (3) NHC1, (4) NHC2, (5) NHC3, (6) NHC3
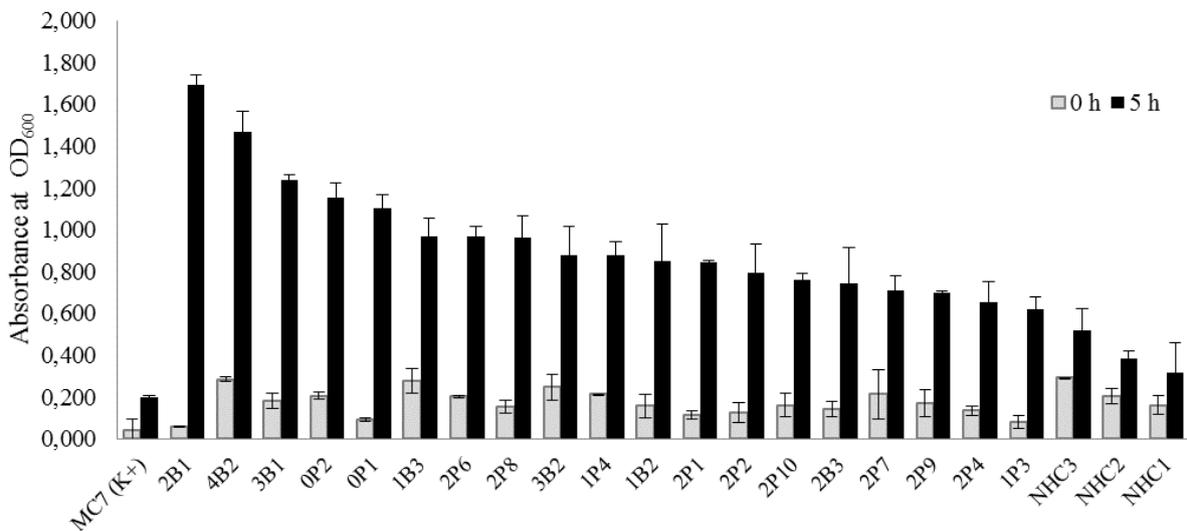

Figure 4. The growth of yeast strains in the YPD medium (0.5% bile salt) after 0 h (grey) and 5 h (black) incubation. *L. rhamnosus* MC7 was used as the positive control
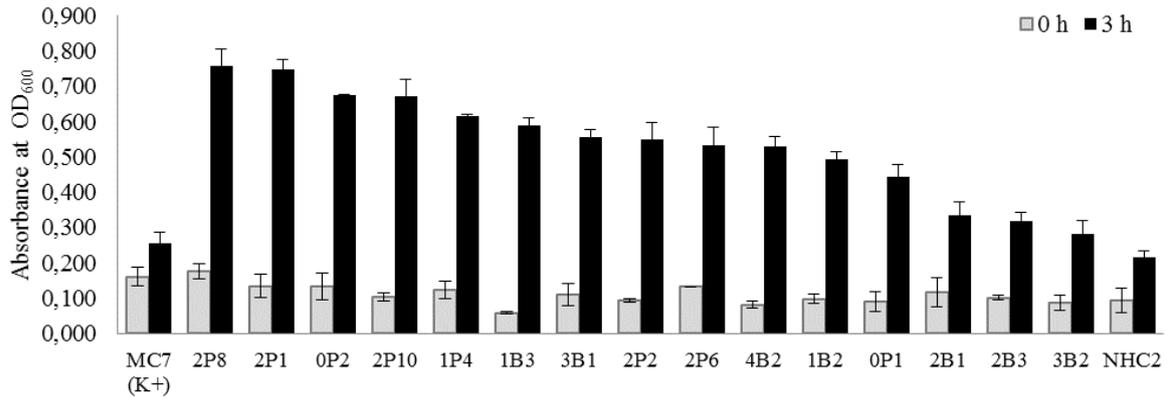
Figure 5. The growth of yeast strains in the YPD medium at pH 3 after 0 h (grey) and 3 h (black) incubation. *L. rhamnosus* MC7 was used as a positive control

Molecular identification

A total of 10 yeast strains were identified based on their ITS region sequence. All yeast strains collected from the cocoa fermentation process were closely related (>99%) to *P. kudriavzevii* / MK394162.1. Phylogenetic analysis was used to confirm the close relationship between yeast strains based on their ITS sequence (Figure 7). Based on the phylogenetic tree, all yeasts were confirmed closely related and were located in the same branch as *P. kudriavzevii* / MK394162.1. However, strains 1P4, 2P1, and 3B1 formed their separate branch.

Antioxidant activity

Three strains of yeast (0P2, 1P4, and 2P10) were determined for their antioxidant activity using the DPPH free radicals. Previous analysis showed that these strains had better autoaggregation and co-aggregation ability with *S. typhimurium* than control positive *L. rhamnosus* MC7. The antioxidant activity of yeast extracellular metabolites in the supernatant was presented in Figure 8. The antioxidant activity of *P. kudriavzevii* 0P2, 1P4, and 2P10 at 48 hours (stationer phase) was higher compared to 18 hours (log phase). The inhibitory percentage of three yeast strains against DPPH at 48 hours, ranging from 59.67 to 68.51% (Figure 8).

The antioxidant capacities of yeast strain supernatants were not significantly various, ranging from 21.44-22.73 ppm, equivalent to ascorbic acid at 48 hours (stationary phase) (Table 2).

Yeast tolerance to H_2O_2 oxidative stress

Antioxidant activity of three yeast strains was also performed on the oxidative stress of H_2O_2 at various concentrations by spot test. Results showed that three selected yeasts grew well at 0 mM, 1 mM, and 2mM H_2O_2 concentrations, but their growth was inhibited in 3 mM of H_2O_2. The best growth in H_2O_2 was obtained in *P. kudriavzevii* 2P10 (Figure 9). The results were consistent with the 48-hour inhibitory percentage of supernatant against DPPH, where *P. kudriavzevii* 2P10 had the highest antioxidant activity among the three yeast strains.
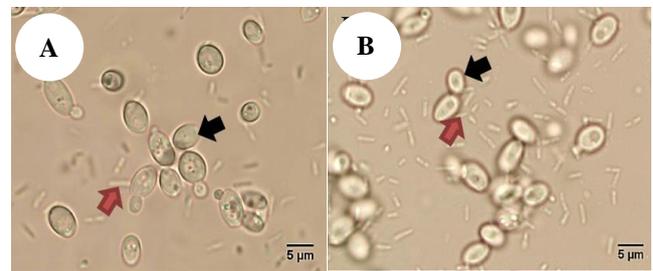

Figure 6. Co-aggregation of *P. kudriavzevii* with *S. typhimurium* ATCC14028 under the light microscope (Olympus CX23) at 1000× magnification. A= *P. kudriavzevii* 1P4 with *S. typhimurium* and B= *P. kudriavzevii* 2P10 with *S. typhimurium* ATCC14028
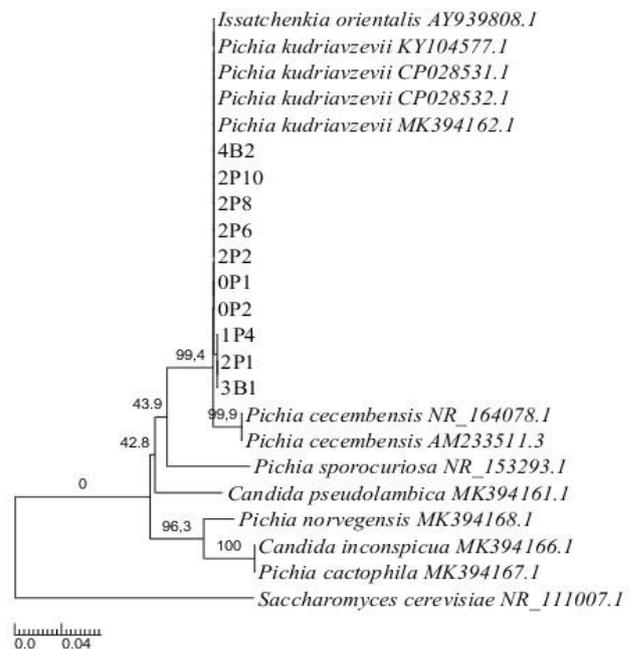

Figure 7. Neighbor-joining phylogenetic tree analysis of ITS yeast regions. Numbers at each node indicate the percentages of branch support of 1000 bootstrap replicates. *Saccharomyces cerevisiae* NR_111007.1 was used as an outgroup.
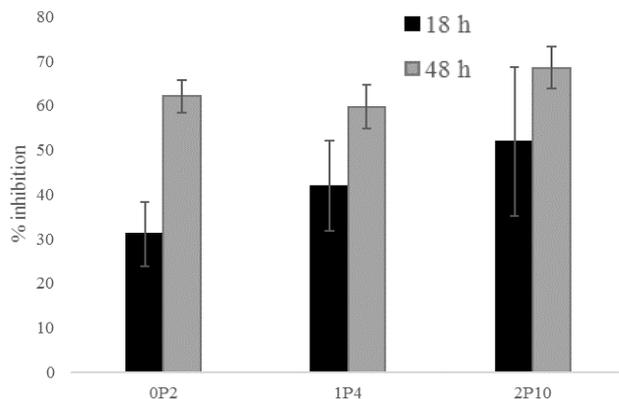
Figure 8. Antioxidant activity (%) of supernatants from three yeast strains at 18 h (black) and 48 h (grey) evaluated by DPPH reduction as ascorbic acid equivalent

Table 2. Antioxidant capacity of three selected yeast strains

Yeast strain	Ascorbic acid equivalent	
	Supernatant 18 h (ppm)	Supernatant 48 h (ppm)
0P1	17.79 ± 0.907	21.64 ± 0.189
1P4	19.16 ± 0.573	21.44 ± 0.375
2P10	20.45 ± 1.669	22.73 ± 0.250

Discussion

Pichia kudriavzevii is generally distributed in soil, fruits, and spontaneous fermentation processes (Kurtzman 2011). *P. kudriavzevii* usually was found in food fermentation processes, such as cheese, African fermented foods, and kimchi (Helmy et al. 2019; Johansen et al. 2019; Moon et al. 2014), and predominantly found in fermented cocoa beans (Delgado-Ospina et al. 2020). Rodríguez et al. (2018) reported that *P. kudriavzevii* (1200) isolated from the food environment could be a promising probiotic candidate.

The main approach in selecting probiotic strains is non-pathogenic character. The pathogenicity of yeast strains can be evaluated from their hemolytic activity to lyse red blood cells by forming a clear zone on blood agar. Several pathogenic microorganisms, such as *S. typhimurium* and *Klebsiella pneumonia,* produce hemolysin. Hemolysin is an exoenzyme (protein) or toxin (non-protein) that causes hemoglobin to break down. The microorganisms which produce hemolysin can access iron bound to hemoglobin in red blood cells (Nayak et al. 2013). Hemolysis ability was divided into three groups: α-hemolysis, β-hemolysis, and γ-hemolysis. Alpha-hemolysin causes partial damage to red blood cells, beta-hemolysin damage/ lyse red blood cells completely, and gamma-hemolysis does not lyse red blood cells (negative) (Buxton 2005; Mogrovejo et al. 2020). A total of 22 yeast strains in this study were gamma-hemolytic, so they were categorized as non-pathogenic and safe to be used as probiotics. Menezes et al. (2019) reported that yeast probiotics isolated from fermented foods did not show hemolytic activity.

Table 1. The percentage of autoaggregation and co-aggregation after 2 h and 4 h incubation

| Yeast strains	Autoaggregation (%)		Coaggregation with *S. typhimurium* (%)	
	2 h	4 h	2 h	4 h
0P2	85.00 ± 0.50	88.97 ± 1.87	81.00 ± 0.14	89.93 ± 1.03
2P2	54.20 ± 11.89	67.24 ± 12.68	74.82 ± 3.62	87.56 ± 0.76
2P1	75.04 ± 13.07	82.66 ± 1.86	85.08 ± 0.78	87.35 ± 4.21
2P10	62.37 ± 6.11	86.45 ± 2.08	81.66 ± 2.98	87.19 ± 0.77
1P4	85.80 ± 3.45	86.24 ± 2.60	78.47 ± 2.69	86.80 ± 0.16
1B3	88.08 ± 6.26	93.42 ± 1.75	77.70 ± 2.30	$86.54 \pm 2,02$
4B2	61.23 ± 6.84	69.63 ± 5.06	79.21 ± 0.92	86.26 ± 0.31
1B2	57.56 ± 5.50	69.79 ± 1.08	65.99 ± 3.65	85.97 ± 2.05
3B1	55.72 ± 7.56	63.99 ± 1.31	75.19 ± 3.08	85.15 ± 1.20
2P8	76.98 ± 8.48	95.22 ± 1.40	76.79 ± 0.55	84.70 ± 2.23
2P6	88.70 ± 3.78	95.33 ± 1.21	75.61 ± 1.39	84.56 ± 3.11
0P1	67.46 ± 3.87	84.36 ± 1.55	79.06 ± 1.63	83.08 ± 2.58
MC7 (K+)	91.30 ± 4.39	91.93 ± 3.46	20.46 ± 4.39	29.64 ± 0.71
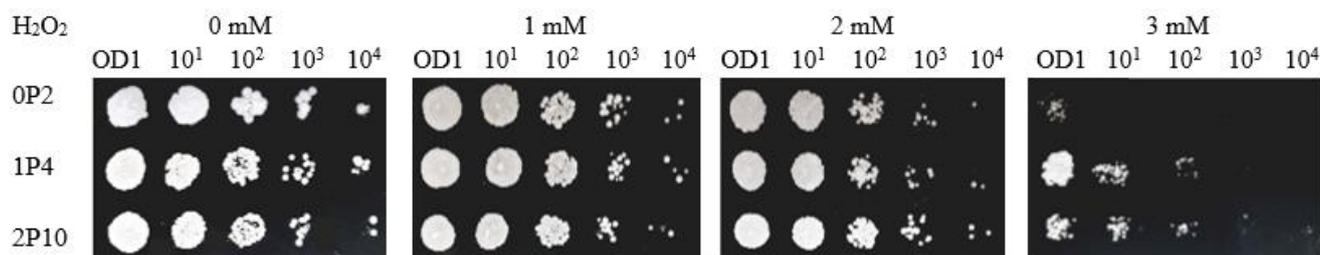
Figure 9. Growth of *P. kudriavzevii* 0P2, 1P4, and 2P10 against H_2O_2-induced oxidative stress

Probiotics must be able to grow at a human body temperature, 37 ºC. A total of 19 yeasts were able to grow well at 28 °C, 37 °C, and 41°C. This result was similar to the previous study that yeast strains isolated from cocoa fermentation in Southeast Sulawesi, Indonesia, can grow at 37 °C and 40 °C (Jamili et al. 2016). Hong et al. (2018) reported that the probiotic yeast *Pichia kudriavzevii* SJP-SNU grew well at 37 ºC. However, NHC1, NCH2, and NHC3 strains grew poorly at 37 ºC and 41 ºC, so they were not suitable for human probiotics. *P. kudriavzevii* RZ8-1 is thermotolerant yeast that can grow up to 42 °C. The genes encoding heat shock proteins (*ssq*1 and *hsp*90) from *P. kudriavzevii* RZ8-1 might play an essential role in the thermotolerance ability (Chamnipa et al. 2018).

Bile salts are antimicrobial compounds that can destroy bacterial membranes, denatured proteins, chelate iron, and calcium, and cause DNA damage in microorganisms (Urdaneta and Casadesús 2017). The optimum concentration of bile salts in the human intestinal tract ranges from 0.3% to 0.6%. A total of 22 yeast strains were able to grow in 0.5% bile salt. *P. kudriavzevii* QLB isolated from Karish cheese could survive and grow at a bile salt concentration of 0.0-2.0% (Helmy et al. 2019). The human digestive tract has a pH range from 2.4 to 8.0 (Oja and Maran 2015). The results in this study showed that all yeast strains could grow on YPD broth at pH 3. A study by (Hsiung et al. 2020) showed that yeast strains from food fermentation adapted and grew well at pH 3. Yeasts have different abilities to survive and grow at low pH. Generally, yeast has optimal growth at low pH (4.5-6.5), and yeast growth slows down at pH 2 (Samagaci et al. 2014). According to Chelliah et al. (2016), the probiotic yeast *P. kudriavzevii* KT000037 could grow in a pH range of 1.5-11.0 medium. Yeast from fermented cocoa is tolerant of low pH. Due to the cocoa fermentation process, it is carried out at a low pH (3.7-4.9) so that the yeast already could survive at low pH (Ardhana and Fleet 2003).

Autoaggregation is an indirect capability related to adherence and colonization of probiotics in the intestine to benefit the host (Chelliah et al. 2016). The ability of microorganisms to attach to the intestinal surface is essential in probiotic selection. In general, microorganisms' adhesion ability is an elaborate multistep process involving electrostatic interactions and hydrophobicity abilities, specific interactions between the microorganism cell wall's physical and chemical characteristics, and the intestinal mucosa (García-Cayuela et al. 2014; Menezes et al. 2019). Yeast autoaggregation percentage at 2 hours ranged from 54.20–88.70%, while at 4 hours ranged from 63.99-95.33%. *Pichia kudriavzevii* isolated from traditional soft cheese has autoaggregation percentage after 2 h ranged from 80.76%-87.32% (Merchán et al. 2020). The autoaggregation percentage was increased at 4 hours compared to 2 hours. Autoaggregation abilities are generally mediated by self-recognizing surface structures, such as surface-bound proteins and exopolysaccharides, and other macromolecules play a role in autoaggregation ability. The longer the contact time between the cell surfaces, the higher the autoaggregation percentage value (Tuo et al. 2013; Trunk et al. 2018).

The co-aggregation ability of yeast with *S. typhimurium* ATCC14028 was also evaluated. Co-aggregation with pathogenic bacteria is an alternative mechanism for probiotic culture to prevent mechanical colonization of pathogens into the intestine (Menezes et al. 2019). The co-aggregation ability of yeast with *S. typhimurium* ATCC14028 at 4 hours was higher than at 2 hours. The percentage of yeast co-aggregation with *S. typhimurium* ATCC14028 at 2 hours ranged from 65.99-81.00%, while at 4 hours ranged from 83.08-89.93%. The abilities of yeast cells to co-aggregate with pathogen bacteria were more excellent than 85%. These yeast strains can serve as potential probiotic candidates to prevent bacterial colonization at the mucosal intestine. The yeast cells that can co-aggregate with pathogen bacteria with capabilities more significant than 85% can serve as potential probiotic candidates who can help in preventing pathogen bacterial colonization at the mucosal intestine (Suvarna et al. 2018). The yeast cell wall is composed of two layers. The outer layer consists of a combination of mannose with protein (PPM/phosphopeptidomannan) or with lipids (LPM/phospholipomannan). The inner layer is composed of chitin and 1,3-β- and 1,6-β-glucan. In the cell wall mannose, there are lectin receptors that can be receptors for *S. typhimurium* and other bacteria adherence (Czerucka et al. 2007; Hirayama et al. 2012).

The inhibition percentage of yeast (*P. kudriavzevii* 0P2, 1P2, 2P10) supernatant against DPPH free radicals (59.67-68.51% was higher than the inhibition percentage of *P. kudriavzevii* cells isolated from cheese (26.89-48.92%) (Merchán et al. 2020). Yeast strains supernatant had a higher percentage of inhibition and antioxidant activity in 48 hours (log phase) than in 18 hours (stationary phase). It might indicate that antioxidant compounds were secondary metabolites secreted in the supernatant. Probiotics have cell components and secrete functional molecules, including antioxidants, enzymes, short-chain fatty acids, peptides, essential vitamins, and minerals, which provide health benefits to the host (Pereira et al. 2017).

High concentrations of H_2O_2 are toxic to yeast cells and can cause cell death. Martins and English (2014) show that the catalase activity of yeast increased during adaptation to sublethal H_2O_2 concentrations in nutrient-rich media such as YPD. Besides, there are also endogenous antioxidant compounds produced by probiotics: superoxide dismutase, glutathione dismutase, ascorbic acid, melatonin, and glutathione. These molecules protect the human body from high levels of oxygen radicals that cause damage to lipids, proteins, and DNA (Pereira et al. 2017).

In the present study, 23 yeast strains isolated from cocoa fermentation in Sukabumi were evaluated for their potential as probiotics. Ten strains of *Pichia kudriavzevii* showed good probiotic properties. The *P. kudriavzevii* 2P10 was the best selected potential candidate for probiotic yeast with non-pathogenic characteristics, 37 °C resistance, bile salt resistance, acid pH resistance autoaggregation, and co-aggregation ability against *S. typhimurium* ATCC14028.

This yeast strain also had antioxidant activity against DPPH free radicals with an inhibitory percentage of DPPH of 68.51% and grew in 3 mM H_2O_2 oxidative stress. *P. kudriavzevii* 2P1 could be a promising probiotic for functional food development and health purposes.

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