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## Yeasts Isolated from Traditional Brem Bali Show Stress Tolerance Phenotype against Fermentation-Related Stresses

Audria Bayu Lenka

*Department of Biology, Faculty of Mathematics and Natural Sciences, Institut Pertanian Bogor, Bogor 16680, Indonesia*

Rika Indri Astuti

*Department of Biology, Faculty of Mathematics and Natural Sciences, Institut Pertanian Bogor, Bogor 16680, Indonesia, rikaindriastuti@apps.ipb.ac.id*

Sri Listiyowati

*Department of Biology, Faculty of Mathematics and Natural Sciences, Institut Pertanian Bogor, Bogor 16680, Indonesia*

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## **Yeasts Isolated from Traditional Brem Bali Show Stress Tolerance Phenotype against Fermentation-Related Stresses**

### **Cover Page Footnote**

The author thanked IPB Culture Collection for the preservation of all yeast isolates collection.

## Yeasts Isolated from Traditional Brem Bali Show Stress Tolerance Phenotype against Fermentation-Related Stresses

Audria Bayu Lenka, Rika Indri Astuti\*, and Sri Listiyowati

Department of Biology, Faculty of Mathematics and Natural Sciences, Institut Pertanian Bogor, Bogor 16680, Indonesia

\*E-mail: rikaindriastuti@apps.ipb.ac.id

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### Abstract

The search for microbes, primarily yeasts with unique characters such as the tolerance against fermentation-related stresses, is gaining significant interest nowadays. Traditionally made alcoholic beverages can be used as sources for such yeasts, given that during fermentation and storage, microbes may develop stress tolerance responses leading to naturally stress-tolerant yeast strains. In this study, we used an alcoholic beverage, that is, Brem Bali, as the source of potential yeast isolates. We isolated nine yeast isolates from two traditional Brem Bali products. All isolates showed tolerance against high glucose stress (40–50%) and sensitivity against high-temperature stress (37–50 °C). Notably, isolate BT5 showed tolerance phenotype against ethanol stress (up to 12.5%). Notably, the ethanol stress tolerance phenotype shown by isolate BT5 was unlikely correlated to the ability of the isolates in combating other stresses. Based on the internal transcribed spacer sequence, isolates BT2, BT5, and BT6 shared high similarity with *Wickerhamomyces anomalus* (98%). Further approaches may be needed to clarify the identity of these isolates. Based on our study, isolate BT5 bears potential as a fermentation agent based on its ability to combat high glucose and ethanol stresses.

**Keywords:** alcohol beverages, fermentation, non-conventional yeast, stress response, *Wickerhamomyces anomalus*

### Introduction

Yeasts have been widely applied in various fermentation reactions for various industrial purposes. *Saccharomyces cerevisiae* is popularly known as an industrial yeast for ethanol production relevant for biofuel, alcoholic beverage, and baking industries [1, 2]. However, several limitations have been found regarding the use of *S. cerevisiae* in fermentation, including its inability to use five-carbon sources, sensitivity to high-temperature, and inhibition by lignocellulosic hydrolysate [3]. Thus, the search for new potential industrial yeast isolates is gaining serious attention. Such isolates are needed to support the advancement and development of fermentation technology, which is also rapidly evolving.

During fermentation, yeasts are exposed to various fermentation-related stresses, including temperature, osmolarity, pH, solvent, etc [4]. These stresses may cause cellular damage to yeast cells and may compromise yeast viability, leading to a reduction in the fermentation rate. Thus, the ability of yeast isolates in combating fermentation-related stresses may serve as an important character of potential industrial yeasts.

In this study, we isolated yeast isolates from a traditional Indonesian fermented beverage popularly known as

Balinese rice wine or Brem Bali. Brem Bali is an alcoholic beverage made from the fermentation of black and white glutinous rice by a dry-yeast starter. *Saccharomyces fibuligera* and *Pichia anomala* are the main amyolytic and ethanologenic yeasts found in the starter, respectively [5, 6]. *Saccharomyces cerevisiae* (35 strains), *Candida glabrata* (six strains), *Pichia anomala* (three strains), and *Issatchenkia orientalis* (seven strains) are the main yeasts in rice wine fermentation [7]. However, no data are available regarding the ability of Brem Bali-derived yeasts in coping with fermentation stresses.

Brem Bali may be a potential source of yeast isolates that are tolerant to fermentation-related stresses. For this beverage, fermentation may occur in a closed fermentation chamber for six months, thus likely creating a specific environment that develops yeast community succession, including the corresponding stress-tolerant phenotypes. Therefore, this study aimed to isolate yeasts from Brem Bali that can deal with fermentation-related stresses, including high-temperature, osmolarity, and ethanol stresses.

### Material and Methods

**Isolates and Medium.** Yeasts were routinely maintained in a Yeast Peptone Dextrose Agar (YPDA) with

the following composition: 2% glucose, 2% peptone, 1% yeast extract, and 2% agar. All media for stress tolerance assay were prepared fresh prior their use.

**Isolation of Yeasts.** The Brem Bali products used in this study were produced by home industries in Bali, Indonesia. Yeast isolation was conducted using the YPDA medium as described previously [8]. Two Brem Bali products (DS and BT) were collected and used as samples. About 1 ml Brem Bali was serially diluted in saline solutions (0.85% (w/v) NaCl). The suspensions were then plated in YPDA by using the spread plate method. The plates were incubated at 30 °C for 2–3 days. Each yeast colony was purified in the same medium. The morphological characters of yeast isolates were observed by microscopy analysis [9].

**Stress Tolerance Assay.** Each yeast isolate was assayed for the ability to deal with osmotic stress induced by glucose and ethanol treatments by using the spot susceptibility assay [10]. One loop of yeast isolates was grown in the YPD broth medium for 24 h to prepare the sub-culture. The sub-culture was then inoculated in a new YPD broth medium at a starting optical density (OD)  $600 = 0.1$  and then incubated for the next 24 h at 30 °C. The particular culture was then adjusted to  $OD_{600} = 1$  and serially diluted by using the YPD sterile broth to  $10^{-4}$  dilution. Each suspension (2  $\mu$ L) was then spotted on top of the YPDA medium containing 30%, 40%, and 50% (w/v) glucose. For ethanol-induced osmotic stress treatment, yeast isolates were prepared the same as above, but the suspension was spotted on top of the YPDA medium containing 5%, 10%, and 12.5% (v/v) ethanol. The media were then incubated at 30 °C for 2–7 days. *S. cerevisiae* BY4741 was used as the control isolate. The yeast grown in the YPDA medium without any stress treatment was used as the control treatment.

**Molecular Analysis for Yeast Identification.** The selected yeast isolates were identified based on an internal transcribed spacer (ITS) sequence. Yeast genomic DNA was isolated by using YeaStar Genomic DNA kit following the manufacturer's protocol. The quality and quantity of the genomic DNA were monitored by using Thermo Scientific Nanodrop 1000 based on the  $\lambda_{260/280}$  ratio.

Genomic DNA was used for polymerase chain reaction (PCR) amplification using the specific primers of the ITS sequence, namely, ITS1 (5'-TCC GTA GGT GAA CCT TGC GG-3') and ITS4 (5'-TCC TCC GCCTTA TTG ATA TGC-3'), resulting in a ~600 bp product [11]. The PCR mixture (50  $\mu$ L) was prepared as follows: The 25  $\mu$ L GoTaq® Green Master Mix, primer ITS1 (10 pmol), primer ITS 4 (10 pmol), and DNA template (~100 ng/ $\mu$ L) were mixed, and nuclease free water used to adjust the volume of the reaction. The PCR reaction was set as follows: pre-denaturation at 94 °C for 4 min,

1 cycle; denaturation at 94 °C for 30 s; annealing at 49 °C for 30 s; elongation at 68 °C for 1 min; post elongation at 68 °C for 20 s (1 cycle). Denaturation, annealing, and elongation were set for 30 cycles.

The PCR products were visualized by using 1% agarose gel electrophoresis (100 V for 25 min). A total of 1 Kb DNA ladder was used as the marker. Ethidium bromide was used to stain the DNA on the agarose gel. The stained DNA was observed using UV transillumination. PCR sequencing was conducted using the 1st BASE DNA sequencing analysis service. The DNA sequence was then analyzed for the identity and homology by using BLASTN analysis.

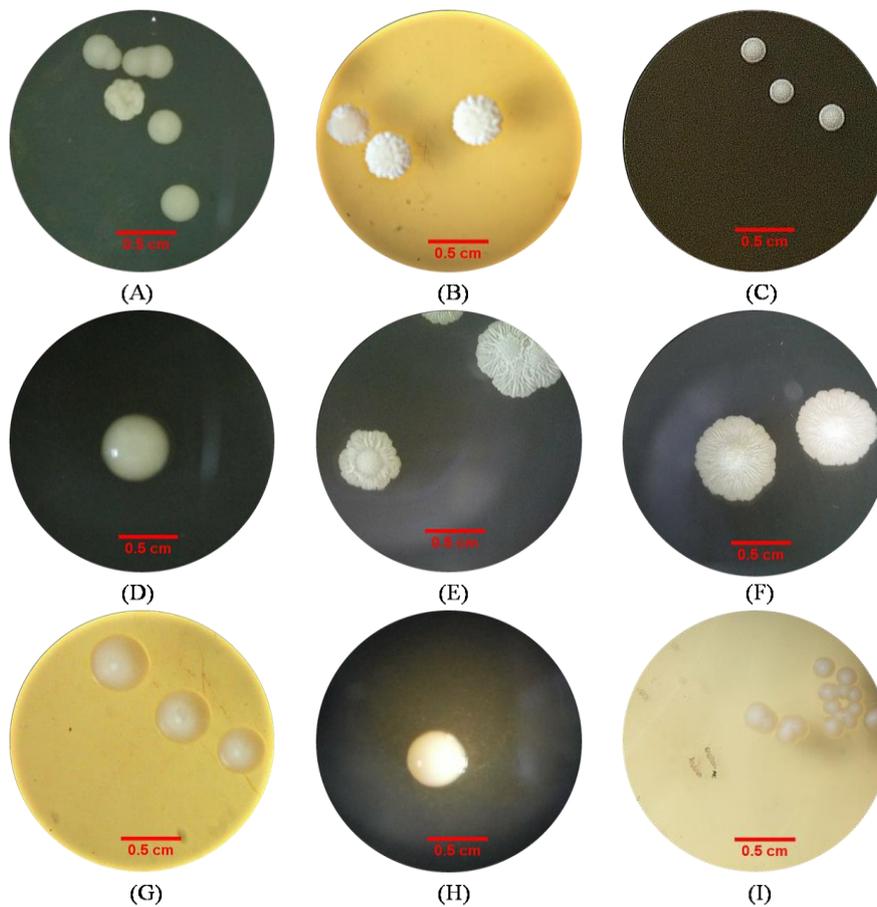
## Results and Discussion

### Isolation and Morphological Characters of Yeasts.

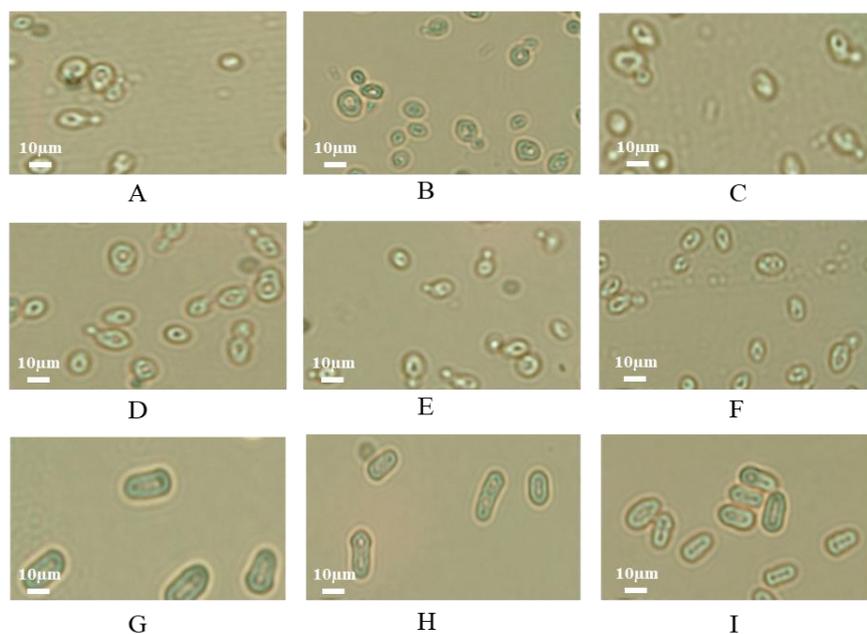
We have isolated 9 yeast isolates from two products of traditional Brem Bali. Five isolates were isolated from the first product and labeled as BT1, BT2, BT3, BT4, and BT5. The other four isolates originated from the second Brem Bali product and labeled as DS1, DS2, and DS3. Each isolate was then analyzed for its morphological characters, including the colony, and microscopic appearance. Several yeast colonies showed markedly distinct characters. Three isolates, namely, BT2, BT5, and BT6, showed irregular colony forms, whereas the others were mostly circular (Figure 1).

For additional insights into the yeast morphological characters, we further observed the cellular microscopy analysis of each yeast isolate. We observed that all isolates found in the first product of Brem Bali were budding yeasts, whereas those that originated from the second products were possibly fission yeasts (Figure 2). In addition, we identified several monopolar/multilateral types of bud in each budding yeast isolate (Figure 2).

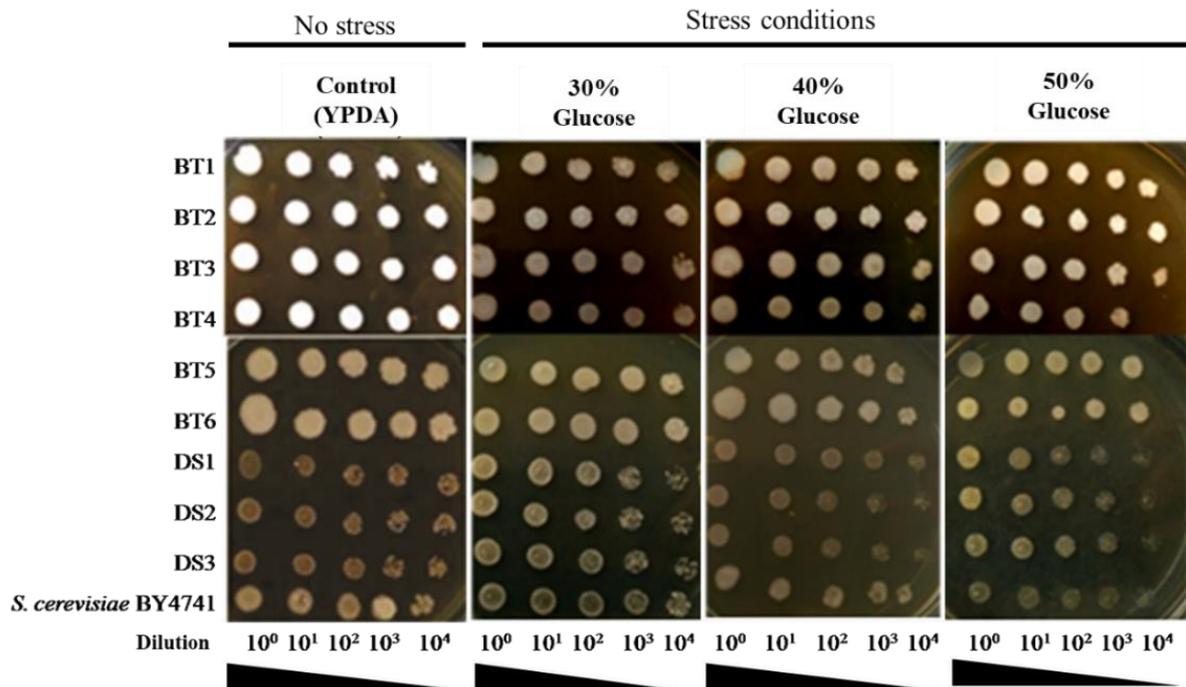
**Stress-tolerant Yeast Phenotypes.** By using the spot assay, we can determine the ability of yeast isolates to deal with stress conditions. All isolates grew at high osmotic stress conditions caused by high glucose supplementation (40%) on the YPDA medium (Figure 3). This finding was revealed by the fully grown colony of each designated spot at each dilution series. However, most of the BT isolates exhibited higher tolerance against high osmotic pressure (50% glucose) compared with the DS isolates and *S. cerevisiae* BY4741 (Figure 3). However, although each isolate can substantially deal with glucose-induced osmotic stress, only BT5 was tolerant against (12.5%) ethanol-induced stress conditions (Figure 4). The industrial yeast, *S. cerevisiae* BY4741, was poorly grown under particular stress conditions. Meanwhile, all isolates were tolerant against 5% ethanol stress conditions. All yeasts cannot grow under 15% ethanol conditions (data not shown).



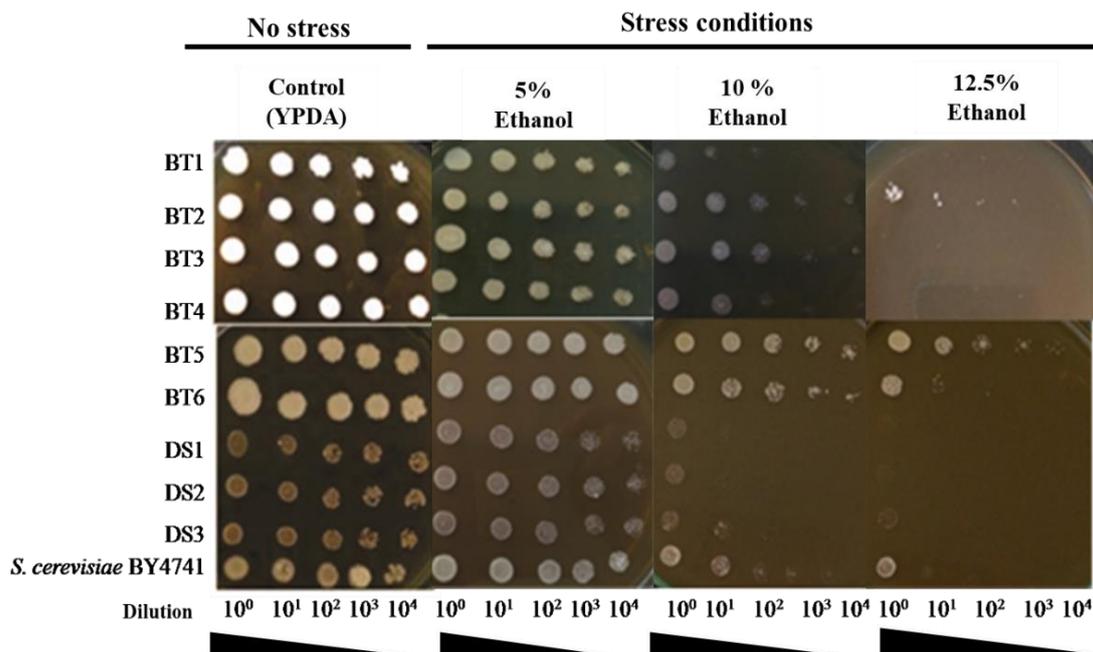
**Figure 1.** Colony Appearance of Yeast Isolates Grown on Yeast Peptone Dextrose Agar Medium for 3 Days at Room Temperature. A) BT1, (B) BT2, (C) BT3, (D) BT4, (E) BT5, (F) BT6, (G) DS1, (H) DS2, and (I) DS3. Yeast Isolates were observed under Stereo Microscopic Observation with 10X Magnification)



**Figure 2.** Cellular Morphology of Yeast Isolates from Traditional Brem Bali. A) BT1, (B) *Wickerhamomyces anomalus* BT2, (C) BT3, (D) BT4, (E) *Wickerhamomyces anomalus* BT5, (F) *Wickerhamomyces anomalus* BT6, (G) DS1, (H) DS2, and (I) DS3. Yeast Isolates were grown on Yeast Peptone Dextrose Agar Medium and Incubated for 2 Days at 30 °C. The Yeast Cells were observed under Binocular Microscopy Analysis with 1000X Magnification



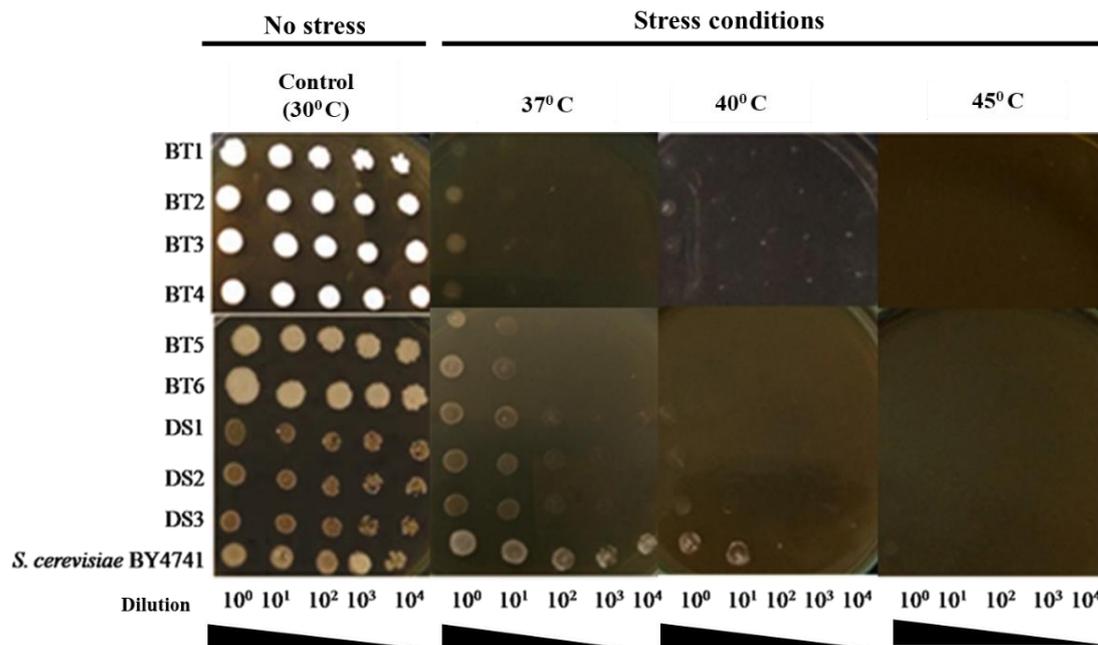
**Figure 3.** Tolerant Phenotypes of Yeast Isolates against Glucose-induced Osmotic Stress Conditions. Each Yeast Isolate was Grown on the YPD Broth Medium as the Main Culture and Serially Diluted as Indicated. Each Suspension was Spotted on top of the YPDA Medium Supplemented with Various Glucose Concentrations to Induce Osmotic Pressure. The Yeast Grown on YPDA (2% Glucose) was Designated as the Control (No Stress Conditions). The Plates were Incubated for 3 Days at 30 °C



**Figure 4.** Tolerant Phenotype of Yeast Isolates under Ethanol Stress Conditions. Each Yeast Isolate was Grown on the YPD Broth Medium as the Main Culture and Serially Diluted as Indicated. Each Suspension was Spotted on top of the YPDA Medium Supplemented with Various Ethanol Concentrations to Induce Stress Conditions. The Yeast Grown on YPDA (2% glucose) was Designated as the Control (No Stress Conditions). The Plates were Incubated for 3 Days at 30 °C

Based on stress tolerance assay against high-temperature stress, BT1, BT2, BT3, BT4, and BT5 were sensitive against high-temperature conditions, with the isolates exhibiting poor growth at 37 °C (Figure 5). A relatively better tolerance phenotype was exhibited by isolates DS1, DS2, and DS3 at the particular 37 °C stress conditions. However these isolates were likely sensitive against high-temperature stress compared with *S. cerevisiae* isolates (at 37 °C). All yeast isolates were not viable at temperatures 40 °C and above.

**Identification of Potential Yeasts.** Among the yeast isolates, BT2, BT5, and BT6 markedly showed potential characters based on the stress tolerance assay. These isolates were primarily capable of dealing with ethanol stresses (Figure 4) compared with the other isolates, including industrial yeast *S. cerevisiae*. Based on the ITS sequence homology analysis, these three isolates shared a strong identity toward *Wickerhamomyces anomalus* isolates (Table 1).



**Figure 5.** Tolerant Phenotype of Yeast Isolates against High-temperature Stress Conditions. Each Yeast Isolate was Grown on the YPD Broth Medium as the main Culture and Serially Diluted as Indicated. Each Suspension was Spotted on top of the YPDA Medium and Incubated for 3 Days at Various Temperature Conditions (37 °C, 40 °C, and 45 °C). The Yeast Grown at 30 °C was Designated as the Control

**Table 1.** Homology Analysis of the ITS Sequence from Selected Yeast Isolates using BLASTN Approach Oil

Isolate		Species Homology	Query cover (%)	Identity (%)	Total score score/max score	E- value	Accession Number
Code	Accession Number						
BT2	MN720560.1	<i>Wickerhamomyces anomalus</i> culture CBS:1978	98	99	1114 /1114	0.0	KY105887
BT5	MN720561.1	<i>Wickerhamomyces anomalus</i> strain M297B	98	99	1107 /1107	0.0	KP675493
BT6	MN720562.1	<i>Wickerhamomyces anomalus</i> strain UFLA ARC235	98	99	1105 /1092	0.0	KY363460

## Discussions

Fermented food and beverages are sources of potential microbes, including probiotics, fermentative microbes, or other unique microbes with distinct physiological and genetic properties [12–14]. In this study, traditionally made Brem Bali was used as the source of yeast isolates. Brem Bali provides distinctive environmental conditions that support the growth of yeasts that can deal with severe stress conditions. Brem Bali contained more than 11% alcohol, which can be increased to 25% following prolonged storage [7, 15].

We isolated nine yeast isolates from Brem Bali [16]. A previous study discovered more than 50 yeast strains from five different Brem Bali products as revealed by pulsed field-gel electrophoresis analysis [7]. We supposed that the different substrates used for Brem Bali production may contribute to the number of isolates obtained in this study. Black and white glutinous rice can be used as substrates to produce Brem Bali. In addition, the isolation medium may be a critical factor of the isolation result. Malt Extract Agar is often applied for yeast isolation, in addition to the rich PDA medium that was used in this study [7, 17].

Based on morphological analysis, each isolate showed a relatively grouped morphological character. The BT yeast isolates were characterized as budding yeasts, whereas the DS isolates were likely fission yeasts. However, further physiological, biochemical, and molecular analysis are needed to clarify these results. Although BT5 and BT6 showed similar morphological characters (Figures 1 and 2), each of these isolates exhibited different responses against ethanol stress (Figure 4). BT5 was markedly tolerant against ethanol stress than other isolates, including *S. cerevisiae* BY4741. DNA barcoding analysis based on the ITS sequence showed that BT2 and BT5 shared high identities with *Wickerhamomyces anomalus*. Clarification of the identity of the particular yeast may be necessary to gain insights into the yeast isolates, possibly by using other gene markers of yeast DNA barcoding, such as the region of D1/D2 of the nuclear large subunit (28S) [18] or the largest subunit of RNA polymerase II (RPB1) [19].

The mechanism of yeast tolerance against stresses has been widely studied. Yeasts may confer stress conditions by activating various stress response pathways. Exogenous or indigenous accumulation of proline is one of the stress tolerance mechanisms of *S. cerevisiae* in combating fermentation-related stresses, such as air drying and high sucrose content [20, 21]. Proline-accumulating *S. cerevisiae* and *Pichia kudriavzevii*-mutant cells show stress tolerance phenotypes against ethanol [22, 23] and freezing stress [24]. In yeasts, proline is involved in the proline–

arginine metabolism pathway [25]. The particular metabolism may lead to the synthesis of signaling molecule nitric oxide, thus inducing the gene clusters involved in the cell survival against stresses [26, 27].

All isolates exhibited resistance against high glucose content of up to 40%. However, most of the BT isolates showed better viability under high glucose stress (50%) compared with the other isolates, including *S. cerevisiae*. Although BT5 was tolerant against 12.5% ethanol stress, this particular yeast isolate was high-temperature stress sensitive. The acquisition of thermotolerance phenotype is strongly correlated to the activity of heat shock protein (Hsp) [28, 29]. *S. cerevisiae* Hsp104 plays a critical role in particular phenotypes [30]. In addition, genes encoding Hsp (*ssq1* and *hsp90*) in *Pichia kudriavzevii* RZ8-1 are upregulated during the fermentation process at 42 °C [31].

*W. anomalus* (formerly *Pichia anomala*) that was isolated from Brem Bali in this study belongs to the group of non-conventional yeasts. *W. anomalus* was possibly derived from the rice used as substrate in making the Brem Bali. As reported previously, this species has been frequently associated with food and feed products [32]. *W. anomalus* also produces alcohol (ethanol) in oxygen-limited conditions and other aromatic compounds, such as ethyl acetate [33,34], that bring out certain aromatic flavor of alcoholic beverages. In addition, *W. anomalus* has been used as a killer yeast in the production of alcoholic beverages such as wine [35]. The toxin of *W. anomalus* is potentially applied to eliminate contamination and wine deterioration caused by spoilage yeast [36]. The cocultivation of *S. cerevisiae* and *W. anomalus* positively influences the chemical composition and sensory features of alcoholic beverages [37]. A previous study reported that *W. anomalus* predominates the middle stage of alcoholic beverage production when ethanol levels reach 3–4%. Based on our study, *W. anomalus* BT2, BT5, and BT6 remained viable at high ethanol concentrations (10%–12.5%). The data indicate that particular yeast isolates likely develop stress tolerance mechanism. Further studies on the ability of this yeast strain in producing ethanol is needed to give valuable insights into the application of yeast isolates for industrial ethanol production.

## Conclusion

Based on our study, alcoholic beverages such as Brem Bali may act as source for potential yeasts with stress-tolerant phenotypes. Such stress-tolerant yeasts may be applied in alcohol and other fermentation-based industries. In this study, a non-conventional yeast belonging to *Wickerhamomyces* was isolated and showed better tolerance against high ethanol stress

(12.5%) than the industrial baker yeast *S. cerevisiae* BY4741. The ethanol stress-tolerant phenotype was unlikely correlated to the ability of isolates in combating other stresses.

## Acknowledgements

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