potravinárstvo®
Slovak Journal of Food Sciences

Potravinarstvo Slovak Journal of Food Sciences
vol. 15, 2021, p. 460-466
https://doi.org/10.5219/1544
Received: 16 January 2021. Accepted: 15 April 2021.
Available online: 28 May 2021 at www.potravinarstvo.com

ISSN 1337-0960 (online)

POTENTIAL PROBIOTIC YEAST ISOLATED FROM AN INDONESIAN INDIGENOUS FERMENTED FISH (IKAN BUDU)

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ABSTRACT

Budu is a fermented food resulting from the activities of microorganisms like lactic acid bacteria and yeast. Budu, therefore, serves as a source of probiotics that can have beneficial effects on livestock and humans. Nonetheless, their selection has to be done with caution. The current study purposed to find out whether budu has desirable probiotic properties. This was done by determining its pH, bile acid tolerance, hydrophobicity, and inhibition of pathogens such as *Staphylococcus aureus*, *Salmonella enteritidis*, and *Escherichia coli*. An *in vitro* experiment was conducted using three *Saccharomyces cerevisiae* (coded as SC 11, SC 12, and SC 21) in the preparation of budu. The whole experiment was repeated four times. The budus were tested for their probiotic properties (low pH, bile salts, hydrophobicity, and inhibition of pathogenic bacteria). The results showed that the three *Saccharomyces cerevisiae* survived in gastric juice and bile acid, exhibited good hydrophobicity, and could inhibit pathogenic bacteria, both gram-positive and negative pathogens. They were able to survive at pH 2 for 3 h (40.70 to 55.1%), at pH 2 for 5 h (35.25 to 46.88%), in 0.3% bile acid incubated for 3 h (69.69 to 86.56%), in 0.3% bile acid incubated for 5 h (82.22 to 88.18%) and hydrophobicity ability of 97.0 to 98.1%. The inhibition activity against pathogenic bacteria, that is, *Escherichia coli* was 2.50 to 3.81 mm, *Staphylococcus aureus* was 1.66 to 3.71 mm, and *Salmonella enteritidis* was 1.20 to 2.64 mm.

Keywords: probiotic properties; inhibition of pathogenic bacteria; Indonesian fermented fish; Ikan Budu; poultry infection

INTRODUCTION

In recent times, the use of antibiotic growth promoters (AGP) in livestock business or broiler business, in particular, has been banned. This is due to consumer concerns about the presence of AGP residues in products such as meat, milk, and eggs, because of the potential risk of drug resistance they pose to humans. Farmers always try to find a substitute for AGP with organic compounds such as plant extracts, prebiotics in the form of MOS (mannan oligosaccharides) and FOS (fructooligosaccharides), and probiotics such as giving live microorganisms to livestock **(Davari et al., 2019)**. Microorganisms of the lactobacillus genera are mostly used for the commercial production of probiotics, especially in fermented milk worldwide **(Sharif et al., 2017)**. Probiotics promote the growth of healthy microflora in the gastrointestinal tract **(Rajoka et al., 2018)**.

Probiotics can be from bacteria, fungi, and yeast. Bacteria that are widely used as probiotics are lactic acid bacteria, from fungi are *Rhizopus oligosporus*, while from yeast are *Saccharomyces cerevisiae* and *Saccharomyces boulardii*. The source of microorganisms used as probiotics is essential, which is usually obtained from the digestive tract of livestock because they are already adapted to the intestine. There are not many reports showing that probiotics are isolated from fermented foods such as budu.

Traditional fermented fish, also known as budu is produced mainly in West Sumatra, Indonesia. Budu is usually made from leather skin (*Chorinemus* spp.) and Spanish mackerel (*Scomberomorus* spp.) known as Ikan Talangand Ikan Tenggiri, respectively, in the Indonesian language **(Huda, 2012)**. Budu processing starts with the hanging of fresh fish by the tail fin for four hours under room temperature. It is then eviscerated, washed, and covered with a layer of salt in a traditional container. After which, it is stored for one day at room temperature and sun-dried for five days. Garlic and white pepper can be sprinkled on the fish during the drying process to improve the quality of the budu **(Huda and Ahmad, 2006)**.

Anggraini et al. (2019) isolated lactic acid bacteria from budu and found that the LAB produced Gamma-aminobutyric acid (GABA), which served as anti-heat stress for broilers. They also found that yeast undergoes symbiosis with lactic acid bacteria in budu. **Stadie et al. (2013)** reported a symbiotic relationship between yeast and lactic acid bacteria of water kefir origin. Symbiosis

(commensalism or mutualism) widely occurs in fermented foods such as sourdough, milk kefir, and yogurt.

As a probiotic, yeast must be able to withstand gastric pH, bile acids, and pathogenic bacteria. **Brandão et al. (2014)** found that acidic pH was not affected by the fatty acid composition of *S. boulardii*. Yeast is capable of maintaining its internal pH by consuming H+ through a metabolic pathway and by using cell buffer systems. **Ogunremi et al. (2015)** added that *Pichia kudriavzevii* ROM 11, that is, yeast from Ogi, which is a cereal pudding fermented food from Nigeria usually made from corn, sorghum, or millet, had a resistance of 86.36% against bile acids with a concentration of 0.3%.

The purpose of the research was to find yeast present in budu that can serve as a probiotic for potential application for poultry production.

Scientific hypothesis

As the yeast-derived from fermented foods from West Sumatra such as fermented fish (budu) has not been exploited, especially as a candidate for probiotics, the study hypothesizes that probiotic yeast exists in fermented fish Ikan Budu.

MATERIAL AND METHODOLOGY

Samples

A sample of budu was purchased from a traditional producer at Pariaman Regency, West Sumatra, Indonesia. Budu was made from coral reef fish such as red Kakap (*L. campechanus*) and Tenggiri fish (*Scomberomorini*) as shown in Figure 1.

Chemicals

Chemicals used in this study were NaCl (Merck, Germany), glycerol (Merck, Germany), HCl (Merck, Germany), oxgall (synthetic bile salts) (Merck, Germany), phosphate buffer (Merck, Germany), and lactic acid (Merck, Germany). The media used in this study were yeast universal agar, nutrient broth, and nutrient agar. All media used were also purchased from Merk, Germany.

Biological Material

Biological material involved in this study was isolated of *Salmonella enteritidis*, *Staphylococcus aureus, Saccharomyces cerevisiae,* and *Escherichia coli.*

Equipment

The equipment used in this study were microscope (Merck, Germany) spectrophotometer (Mettler Toledo UV Vis, Inggris), incubator (Thomas Scientific, USA), autoclaved (Systec autoclaved company, Japan), and caliper (Misumi, Indonesia).

Description of Experiments

Isolation of yeast was conducted according to the method of **Bajwa and Sharma (2018)**. The budu (1 g) was added to 9 mL of 0.9% NaCl (saline) solution and mixed thoroughly for 60 s. Serial dilution was then carried out in saline solution and spread plated onto yeast universal agar. The yeast universal agar was composed of 3.0 $g.L^{-1}$ malt extract, 3.0 $g.L^{-1}$ yeast extract, 10.0 $g.L^{-1}$ glucose, 5.0 $g.L^{-1}$ peptone, and 15.0 $g.L^{-1}$ agar. The spread plated yeast universal agar was incubated for 72 h at 28 °C. Presumptive yeast showed white-to-yellow colonies under the microscope. Such isolates were randomly selected and further purified on yeast universal agar. Yeasts showing the typical appearance of *Saccharomyces* (white-to-yellow colonies) were selected. The selected yeast strains were further purified by successive streaking on yeast universal media. Three isolates were maintained at -80 °C in 20% (v/v) glycerol (Hi-Media).

pH resistivity test was done using a modified nutrient broth in which 0.1 N HCl was added to achieve a pH of 2 which corresponds with gastric pH as described by **Ogunremi et al. (2015)**. The yeast extract (1 mL) was inoculated in modified HCl nutrient broth and incubated at 37 °C for 3 and 5 h. After which, the absorbance was read at a wavelength of 600 nm. This research was conducted with three replications. Isolates resistance was also expressed as a percentage, according to **Ogunremi et al. (2015)**.

Bile salt resistivity test was conducted using mixed modified HCl nutrient broth with 0.3% oxygall (synthetic bile salts) and incubated for 3 and 5 h **(Ogunremi et al., 2015)**. The culture was streaked onto a modified nutrient broth and incubated at 37 °C. 1 mL of yeast isolate was streaked onto the bile salt modified nutrient broth medium. Incubation was done at 37 °C **(Ogunremi et al., 2015)**. The results were obtained qualitatively by comparison of the yeast absorbancy of the control (not streaked with yeast) with the streaked modified nutrient broth (0.3% bile salts).

Hydrophobicity test or attachment was carried out by the method of **Vinderola et al. (2004)** using stainless steel plates. Clean and dried stainless-steel plates were marked on one side. One hundred (100) mL of distilled water was used to dissolve 0.8 g of nutrient broth. Growth media and stainless steel were autoclaved at 121 °C for 15 min.

Figure 1 Fermented fish (budu) made from Tenggiri fish (*Scomberomorini*).

The stainless steel plate was placed in 25 mL nutrient broth inoculated with 1 mL of LAB in an Erlenmeyer and incubated for 24 h at 29 °C. Furthermore, the surface of the stainless steel was evenly wiped with a swab. The swab was put in a tube containing 10 mL of phosphate buffer and homogenized. It was then measured by looking at the absorbance at a wavelength of 600 nm (A). To measure the growth in the liquid phase, 1 mL of liquid was taken from nutrient broth media and diluted in 9 mL of phosphate buffer solution. After which, it was measured at a wavelength of 600 nm (Ao). The percentage hydrophobicity was calculated using the formula of **Fadda et al. (2017)**.

The antimicrobial activity test of 3 yeast isolates against *Salmonella enteritidis*, *Staphylococcus aureus,* and *Escherichia coli* was carried out based on a modification from **Diosma et al. (2013)**. Nutrient agar (10 grams) was added to 500 mL of distilled water, homogenized, heated in a water bath, and autoclaved. The media was allowed to cool (± 45 °C), and 0.2% of test bacteria was added into ±10 mL Petri dishes and allowed to solidify. Meanwhile, a blank antibiotic disk was soaked in the lactic acid bacteria solution for approximately 10 min and was placed on the surface of the nutrient agar medium containing the pathogenic bacterial isolates. It was then incubated aerobically at 37 °C for 24 h. After incubation, the diameter of the inhibition zones was measured using a caliper.

Statistical Analysis

Data were subjected to a one-way analysis of variance (ANOVA), and Tukey's test was used for comparison of means using SPSS version 20.0 Software (SPSS Inc., Chicago, IL, USA). A significant difference was defined at $p < 0.05$.

RESULTS AND DISCUSSION

Resistance test of yeast isolates to gastric pH

The resistance of yeast to gastric pH was tested at pH 2 because the pH in the proventriculus and gizzard is 2.0 – 3.5 **(Rougie`re and Carre, 2010)**. The gastric pH was tested for 3 h and 5 h, the results for which can be seen in Table 1. The results of the study showed that all yeast isolates could survive at pH 2 with resistance >30%. The resistance of the yeast isolates to pH did not differ significantly ($p > 0.05$) from each other. The three yeast isolates of budu origin grew at pH 2 with the viability of 55.1% for isolate SC 11, 43.70% for isolate SC 12, and 40.70% for isolate SC 21, which were incubated for 3 h. When the time incubation time was increased to 5 h, the percentage viability decreased.

The pH of 2.0 – 3.5 is pH in the proventriculus, where HCl is produced. The probiotic yeast work in the gastrointestinal tract (GIT) by providing nutrients that aid in the digestion of food and inhibition of harmful bacteria. Probiotics are also mixed or added to feed to increase the rate of feed and nutrient utilization **(Markowiak and Śliżewska, 2018)**.

The results in Table 1 show that the yeast isolate that had the highest resistance to gastric juice was SC 11, with resistance >50%. **Nurnaafi, Setyaningsih and Desniar (2015)** explained that good probiotic isolates are those with a survival rate of more than 50% under low pH conditions and are resistant to bile salts. The resistance of isolate SC 11 at 3 h incubation time was 55.10% and decreased at 5 h incubation time to 46.88%, a difference of 8.22%. The results of this study were similar to those of **Kumura et al. (2004),** who found that yeast (*Kluyveromyces lactis* S25) isolated from commercial blue cheese and kefir had a resistance of 54.7%. The results of this study for SC 11 were higher than those of **Diosma et al. (2013)**. They examined yeast isolates from kefir (tested at pH 2.5 with an incubation time of 3 hours) and reported that *Kluyveromyces marxianus* 8116 had 45.5% resistance, *Saccharomyces cerevisiae* 8115 had 40.5% resistance, and *Saccharomyces boulardii* had 45.5% resistance.

Tovar et al. (2002) reported that when yeast isolates enter the digestive tract of poultry, they must be able to survive at low pH because the proventriculus and gizzard have a pH of 2.0 – 4.5. **Zubaidy and Khanda (2014)** added that *Saccharomyces cerevisiae* var *boulardii* (S.b32) was able to survive at low pH. Glucomannan, chitin, mannoprotein, and beta-glucan make up the cell component of *Candida* sp. **Drabikova et al. (2009)**. However, beta-glucan forms the largest (50 – 60%) component of the inner layer of the cell wall, while chitin forms 1 – 10%. Mannoproteins form mainly 30 – 40% of the outer layer of the cell wall. They play a major role in interactions with the host, determine the nature of the cell surface and cell-to-cell recognition **(Vickova et al., 2004)**.

Resistance test of yeast isolates to bile salts

Resistance of the yeast isolates to bile salt was not significantly different ($p > 0.05$). The results in Table 1 shows that isolate SC 11 had a resistance ability of 69.69% at an incubation time of 3 hours and increased to 82.02% at an incubation time of 5 hours, a difference of 12.33% increase. The results of this study are comparable to those of **Chen et al. (2010)**, who examined yeast isolated from fresh milk on Beijing and Heilongjiang farms against 0.3% bile salts and reported that *Pichia fermentans* HJ15 isolate had 79% resistance, *Pichia kudriavzevii* BY10 isolate had a resistance of 25.9%, and *Yarrowia lipolytica* HJ6 isolate had a resistance of 62.9%.

Yeast can survive in bile salt solutions because of its extreme environmental resistance **(Chen et al., 2010)**. **Chen et al. (2010)** explained that yeast develops resistance properties in stressful environments (salt, acids, and sugars), and in competition with other microbial yeasts, they can live a normal life. The difference in the results of this study can be due to the differences in the type of yeast tested against 0.3% bile salts. Yeast cell walls are mostly composed of beta-glucan **(Lee et al., 2001)**. **Ooi and Liu (2000)** reported that beta-glucan is a linear polysaccharide that contains monomers of glucose that are linked by glycosidic bonds. Beta-glucan is water-soluble, and a small concentration will produce high viscosity **(Suzuki et al., 2001)** and will form a gel in the digestive tract to increase the excretion of bile acids. By this, the fat is not emulsified and absorbed in the stomach.

Inhibition test against pathogenic bacteria

The ability of yeast to act antagonistically is due to changes in medium pH, competition for nutrients, secretion of antimicrobial agents, and production of ethanol in high concentrations. *Staphylococcus aureus*, *Escherichia coli,* and *Salmonella enteritidis* were chosen because they are among the pathogenic bacteria associated with poultry and other animals. The results of the study (Table 2)showed that the inhibitory zone produced by isolate SC 11 against *Escherichia coli* was 3.81 mm, and greater than the inhibition zone of 3.71 mm produced against *Staphylococcus aureus*. However, the inhibition of the yeast isolates to *Staphylococcus aureus,* and *Escherichia coli* did not differ significantly (p >0.05). The differences in the bacterial cell walls could not cause significant differences in their resistance to yeast. *Staphylococcus aureus* is gram-positive bacteria, while *Escherichia coli* is gram-negative bacteria. **Saidi et al. (2019)** reported that gram-negative bacteria have a thinner layer of peptidoglycan (5 – 10 cm), while gram-positive bacteria have a thicker layer of peptidoglycan (20 – 80 cm). Therefore, it was more difficult for yeast isolates to penetrate the cell wall of *Staphylococcus aureus* bacteria than the cell wall of *Escherichia coli* bacteria.

Table 2 shows that the inhibition of *Salmonella enteritidis* by yeast was numerically lower than that of *Staphylococcus aureus* and *Escherichia coli.* This might be influenced by antigens present in *Salmonella*. According to **Wang et al. (2020)**, *Salmonella* has three main antigens, namely: somatic antigens or O antigens, flagellate antigens or H antigens, and capsule antigens or Vi antigens; which produce enterotoxins and cytotoxins, making it difficult for yeast to inhibit their growth.

The inhibitory strength possessed by yeast varies; therefore, different yeast species will produce different inhibition and metabolite activities during fermentation. **Freimoser et al. (2019)** studied the antagonistic activity of *Kloeckera* and *Kluyveromyces* species against bacteria and found that they produce intracellular and extracellular antimicrobial compounds that inhibit the growth of gram-negative and gram-positive bacteria. Research by **Suzuki et al. (2001)** and **Marquina et al. (2002)** found yeast to produce antagonistic activity due to the production of killer toxins or mycotoxins. These toxins are extracellular proteins or glycoproteins that can damage the cell membrane. The antimicrobial activity of yeast through the secretion of organic acids and antimicrobial peptides has been reported **(Boirivant and Strober, 2007; Vanderpool, Yan and Polk, 2008; Ciorba, 2012)**. *S. boulardii* secretes mainly capric acid, a medium-chain fatty acid which showed bioactivity against *Candida albicans* and formation of biofilms **(Krasowska et al., 2009; Murzyn et al., 2010)**. *S. cerevisiae* secretes antimicrobial peptides (saccharomycin), which inhibits the growth of pathogenic bacteria **(Hammami et al., 2013)**. Antimicrobial peptides inhibit bacteria growth by absorbing the cell membrane receptors, destructing cell membrane permeability and alteration of intracellular pH **(Rizk et al., 2018)**.

Table 1 The resistance of yeast isolates towards acid and bile salt conditions.

Isolates yeast	Time (3 h)(%)	Time (5 h)(%)
Acid condition		
Saccharomyces cerevisiae (SC) 11	55.10 ±2.19	46.88 ±1.82
Saccharomyces cerevisiae (SC) 12	43.70 ±1.35	39.36 ±0.80
Saccharomyces cerevisiae(SC) 21	40.70 ±0.87	35.26 ±0.38
Bile salt condition		
Saccharomyces cerevisiae(SC) 11	69.69 ±0.14	82.02 ±0.53
Saccharomyces cerevisiae(SC) 12	84.54 ±1.37	87.43 ±1.91
Saccharomyces cerevisiae(SC) 21	86.56 ±1.71	88.18 ±1.72

Note: values were reported as means ±*SD* of triplicate groups.

Table 2 The resistance of yeast isolates towards pathogenic bacteriaassociated with poultry.

Yeast isolates	Diameter inhibition zone (mm)		
	Escherichia coli	*Staphylococcus aureus*	*Salmonella enteritidis*
Saccharomyces cerevisiae (SC) 11	3.81	3.71	2.64
Saccharomyces cerevisiae (SC) 12	2.50	2.56	2.52
Saccharomyces cerevisiae(SC) 21	1.63	1.66	1.20

Note: values were reported as means ±*SD* of triplicate groups; mean values in the same column with different lowercase were significantly different (p <0.05).

Table 3 The hydrophobicityof yeast on stainless steel plates.

Yeast Isolates	Hdrophobicity (%)
Saccharomyces cerevisiae (SC) 11	97.00 ±0.24
Saccharomyces cerevisiae (SC) 12	97.96 ±0.72
Saccharomyces cerevisiae (SC) 21	98.71 ±0.19

Note: values were reported as means ±*SD* of triplicate groups.

Hydrophobicity using stainless steel plates

Table 3 shows the hydrophobicity of the yeast isolates. From Table 3, all the yeast isolates had a hydrophobicity percentage >90%. The hydrophobicity of yeast isolates was not significantly different (p >0.05).

The results of this study were higher than those of **Sourabh et al. (2011)**, who showed that *Saccharomyces cerevisiae* isolated from traditional West Himalayan fermentation food, Sc01 had a hydrophobicity percentage of 59.65%. **Fadda et al. (2017)** found that *Saccharomyces boulardii* isolated from codex had a hydrophobicity ability of 55.9%, *Kluyveromyces lactis* isolated from kefir had a hydrophobicity ability of 74.1 – 79.4% and *Kluyveromyces marxianus* had a hydrophobicity ability of 75.9%.

The ability of microorganisms to attach to the digestive tract becomes one of the selection criteria for probiotics. The formation of colonies in the digestive tract is influenced by the ability of microorganisms to attach to the digestive tract that is specific to the host **(Suzuki et al., 2001)**. **Tovar et al. (2002)** reported that some yeast species can synthesize and secrete polyamine molecules which can stimulate the development of the digestive tract and the production of digestive enzymes.

CONCLUSIONS

The results indicated that all the isolates identified were *Saccharomyces* sp., (SC 11; SC 12 and SC 21) and showed notable potential probiotic properties. They exhibited a better survival in gastric juice and bile acid, showed high hydrophobicity, and the ability to inhibit pathogenic bacteria (gram-positive and negative pathogens) associated with poultry. They were able to live at pH 2 for 3 h (40.70 to 55.1%), pH 2 at 5 h (35.25 to 46.88%), in bile acid 0.3% incubated for 3 h (69.69 to 86.56%), and at 5 h (82.22 to 88.18%), and hydrophobicity ability of 97.0 to 98.1%. The inhibition zones produced by *Saccharomyces* sp. against *Escherichia coli* was 2.50 to 3.81 mm, *Staphylococcus aureus* was 1.66 to 3.71 mm, and *Salmonella enteritidis* was 1.20 to 2.64 mm.

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Funds:

The authors are grateful to the Ministry of Research, Technology and Higher Education of Indonesia for funding the BOPTN Andalas University Grants through Research Cluster Grant No: 57/ UN.16.17/HGB/LPPM/2019. Grant for publication fee was by Universiti Malaysia Sabah, Jalan UMS, 88400, Kota Kinabalu, Sabah, Malaysia.

Acknowledgments:

We would like to thank the University of Andalas, University for Development Studies and Universiti Malaysia Sabah for the support to conduct research in the area of food science and technology.

Conflict of Interest:

The authors declare no conflict of interest.

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