Exploration of bacteria and analysis of alcohol concentration in pongasi a tolaki’s typical alcoholic beverage

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Abstract. Pongasi is a typical traditional drink of Tolakinese made from fermented cooked rice with yeast. The present study explores the types of bacteria and analyzes the alcohol concentration of Pongasi drinks. It is an experimental research that explore the sample of pongasi from one of home-industry factory, isolation and bacterial inoculation on NA (Nutrient Agar) media, Gram staining, biochemical testing on the Vitek 2 Compact Systems, measuring the alcohol concentration of the distillation method, and beverage sensitive test Pongasi with bacteria that have been isolated using MHA (Mueller Hinton Agar) media. The results suggest in the sample of Pongasi were white, round, and Gram positive bacteria colonies. The results of identification and biochemical tests showed that 91% were Staphylococcus carnosus bacteria. The results of alcohol analysis indicate that fermentation 2 has a percentage of alcohol concentration at 0.8%, fermentation 3 at 8.2%, and fermentation 4 at 21%. The three treatment groups were continued with a sensitivity test with three repetitions, and the average area of inhibition obtained in each treatment group was fermentation 2 at 9.05 mm, fermentation 3 at 12.12 mm, fermentation 4 at 12.14 mm.

1. Introduction
Southeast Sulawesi is a province known for producing a Pongasi drink, especially in the area of Konawe Regency. Pongasi is a fermented beverage with alcohol content. The people of Konawe Regency, especially the Tolakinese, carry out the tradition of drinking Pongasi during weddings or special moments. Pongasi is a fermented beverage product made from rice added by yeast. The yeast used is a type of Saccharomyces cerevisiae [1]. The Tolakinese people make Pongasi for four or five days of fermentation. After that, it will be consumed by the people by adding mineral water first.

Microbes have been used to help the fermentation process for centuries. The most widely used fermentation organism to produce alcoholic drinks is Saccharomyces cerevisiae[2][3][4]. In the process of making Pongasi, there is a stage of silence after mixing the yeast inoculums in that process, bacteria can participate in helping the fermentation process. It is the focus of this study in the context of conducting exploration with the isolation and of bacteria contained in the Pongasi as a traditional drink of Tolakinese.

Alcohol fermentation is a complex biochemical process that involves interactions between yeast, some fungi, and bacteria. During the alcohol fermentation process, yeast converts sugars into ethanol and carbon dioxide [5][6]. Therefore, bacteria can participate in helping the fermentation process of traditional Pongasi drink. This study aims to explore the type of bacteria and analyze the alcohol concentration of pongasi drinks.
2. Materials and Methods

2.1. Materials
The present study used sample of Pongasi drink obtained from one of the home-industry factories. The samples used were fermentation in day 2, 3, and 4 are known as Wulele Pongasi. Fermentation day 1 is not used because there is no fermentation product of drink produced. Other material used are Nutrient Agar (Oxoid) media, Mueller Hinton Agar (Himedia) media, Aquades (OneLab Waterone), Gram staining Reagent (ST-Reagents), NaCl (Merck), Alcohol (onemed). Equipment used in this activity are Autoclave (Hirayama), Incubator (Yenaco), Microscope (Boeco), Hot plate, Laminar Air Flow (Pharmeqlab), Analytical Scales (Durascale), Vitek 2 Compact Systems (bioMérieux), Pycnometers (Pyrex), petri dish (Pyrex), and micropipette (JoanLab).

2.2. Procedure

2.2.1. Bacteria Isolation and Characterization
Prepare the samples of pongasi on fermentation day 2, 3, and 4. Sample of pongasi were isolated on Nutrient Agar media to identify bacteria that participate in the fermentation process [7]. Furthermore, it is incubated for 48 hours at 37°C. Bacterial colonies were observed macroscopically (shape, color, surface) [8].

2.2.2. Gram Staining
Gram staining were used by microscopic observation. Prepare slide that have been cleaned with 70% alcohol. Make a slide of cell bacteria to be stained. Heat fix the cell bacteria to the slide by carefully passing in the slide with a drop of cell bacteria on it through a Bunsen Burner three times. Add the crystal violet to the slide and incubated for 1 minute. Rinse slide with a gentle stream of water to remove unbound crystal violet. Add lugol solution for 1 minute then rinse slide with a gentle water. Add 95% alcohol solution for 30 seconds then rinse slide with a gentle stream of water. Add safranin to the slide and incubated for 1 minute, rinse slide with a gentle stream of water then dry air. Observing using a microscope [9,10,11].

2.2.3. Identification and biochemical testing using Vitek2CompactSystems
Gram positive bacterial identification was carried out using pure bacterial isolate grown in Nutrient agar media. Then, from the bacterial isolate a suspension was made into 3 mL of sterile NaCl solution. After that, the suspension was measured for turbidity using DensiChekTM. Then, the identification card with the BCL code (Gram positive spore-forming bacteria) is inoculated with the bacterial suspension using a vacuum device. Then, the card is sealed and incubated at 35.5 ± 1.0°C for 15 minutes. The results of the test reactions appear on Vitek 2 Compact Systems are as +, -, (+), (-). Reaction appears in parentheses indicates that the reaction is weak. Then, bacterial identification results that appear in the form of species names and scale or level of qualitative identification based on numerical probability calculations [12, 13].

2.2.4. Alcohol Concentration Analysis
Prepare the Pongasi sample for fermentation of day 2, 3, and 4. The alcohol contained in the pongasi is by the distillation method. Enter the sample of pongasi in the distillation flask and set the distillation temperature not more than 78°C. Determine the specific gravity of alcohol by using a pycnometer [9,14].

2.2.5. Sensitivity test
Sensitivity test using the well diffusion method. Bacteria test were bacteria isolated from the traditional Pongasi drink is S. carnosus. This test was carried out to determine the ability and beverage of pongasi to inhibit the growth of S. carnosus. Sensitivity test using Mueller Hinton Agar
media. the pongasi sampel used fermentation day 2, 3, and 4 [15, 16]. Cell mass in 0.9% NaCL solution compared to turbidity equivalent to solution the Mc-farland standard 0.5. The Mc Farland 0.5 standard coat is assumed to be equivalent to a 1.5x10^8 CFU/mL culture [17, 18, 19, 20]. Samples were put 80 µL into the well repetition is done three times. Incubated for 48 hours at 37°C. After that, observe and measure the inhibition zone.

3. Results and Discussion

3.1. Morphology of Bacterial

Bacteria are prokaryotic microorganisms, the most abundant. Because most prokaryotes are less than ten micrometers (μm) in size, microscopes are used to study bacteria [21]. Therefore, it is very important to explore and identify bacteria so that information about the bacteria contained in the pongasi drink can be obtained. Based on observations of bacterial morphology on the isolation and identification of traditional drinks, suggest the presence of bacteria in fermentation day 2 and 3. While on the day 4 fermentation, there was no bacterial growth found in Nutrient Agar (NA) media. The characteristics of the colony suggest in macroscopic observation are round colony, white colony color and smooth surface. While microscopic observation using Gram staining suggests Coccus and Gram-Positive bacteria (Figure 1). The morphology of bacteria is very diverse. The specific form is a consequence of adaptive pressure that optimizes the ability of bacteria. Forms affect biological functions, including nutrient acquisition, motility, dispersion, resistance to stress and interactions with other organisms [22].

| Sample         | Shape of colony | Colour of colony | Surface    | Gram Staining         |
|----------------|-----------------|------------------|------------|-----------------------|
| Fermentation 2 | round           | White            | Smooth     | Coccus, Gram Positive |
| Fermentation 3 | round           | White            | Smooth     | Coccus, Gram Positive |
| Fermentation 4 | -               | -                | -          | -                     |

Note: - (no bacteria founded)

Figure 1. a) The sample of pongasi; b) macroscopic of bacteria colonies; c) Microscopic of bacteria with Gram staining used magnification of 1000 x
3.2. Identification and Biochemical Test With the Vitek 2 Compact System.

The results of identification and biochemical tests on samples of bacterial isolation using the Vitek 2 Compact System suggest that the bacteria were 91% likely to be a *Staphylococcus carnosus* species (Table 2). *S. carnosus* is a Gram positive cocci, negative coagulase, positive catalase, 0.5 to 1.5 pm in diameter, nonmotile, occurs mainly in pairs and alone. The colonies are slightly raised, round, smooth, slightly sparkling, and are usually white-gray and have a diameter of about 1 to 3 mm [6][23]. These bacteria are used as starter cultures for sausage fermentation, where they contribute to food safety, flavor, and controlled fermentation processes [24]. *S. carosus* and *S. xylosus* are species worldwide that are used as starter cultures in food fermentation, either alone or in combination with prescribed lactobacilli or other microorganisms [24, 25].

**Table 2. The Identification Results Using Vitek 2 Compact Systems**

| No | Parameters | Result | No | Parameters | Result | No | Parameters | Result |
|----|------------|--------|----|------------|--------|----|------------|--------|
| 1  | AMY (D-Amygdalin) | -16 | 16 | dXYL (xylose) | - | 31 | AMAN (Alpha-mannosidase) | - |
| 2  | APPA (Ala-Phe-Pro Arylamidase) | -17 | 17 | AspA (L-Aspartate Arylamidase) | - | 32 | PyrA (L-Pyrrolodonyl- Arylamidase) | + |
| 3  | LenA (Leucine Arylamidase) | -18 | 18 | BGUR (beta-Glucorinidase) | - | 33 | POLYB (Polymixin B Resistance) | + |
| 4  | AlaA (alanine arylamidase) | -19 | 19 | dSOR (Sorbitol) | - | 34 | dMAL (D-Maltose) | - |
| 5  | dRIB (D-Ribosa) | +20 | 20 | LAC (Lactose) | - | 35 | MBdG (Methyl b-glucopyranidose) | - |
| 6  | NOVO (Novobiocin resistance) | +21 | 21 | dMAN (D-Mannitol) | - | 36 | dTRE (D-Trehalose) | - |
| 7  | dRAF (D-rafinose) | -22 | 22 | SAL (Salicin) | - | 37 | AGLU (Alpha-Glucosidase) | - |
| 8  | OPTO (Optochin resistance) | +23 | 23 | ADH1 (Arginine dihydrolase) | + | 38 | PHOS (Phosphatase) | - |
| 9  | PIPLC (Phosphatidyl inositol) | -24 | 24 | BGAR (b-galactopurinosidase) | - | 39 | BGUR (beta-Glucorinidase) | - |
| 10 | CDEX (Cyclodextrin) | -25 | 25 | AGAL (Alpha-Galactosidase) | - | 40 | dGAL (D-Galactosa) | - |
| 11 | ProA (Pro arylamidase) | -26 | 26 | URE (Urease) | - | 41 | BAC1 (Bacitracin resistance) | + |
| 12 | TyrA (tyrosine arylamidase) | (-27 | 27 | NAG (N-Acetyl-Glucosamine) | + | 42 | PUL (Pullulan) | - |
| 13 | ILATk (lactate) | -28 | 28 | dMNE (D-Mannose) | + | 43 | ADH2s (arginine dihydrolase) | + |
| 14 | NC6.5 (growth in 6.5) | +29 | 29 | SAC (Sucrose) | - | | | |
| 15 | O129R (O/129 resistance) | +30 | 30 | BGAL (Beta-Galactosidase) | - | 91% Probability *Staphylococcus carnosus* | |

3.3. Measurement of Alcohol Concentration in Pongasi Samples

Based on Table 3, the results of alcohol concentration fermented on the day 2 was 0.8%, day 3 was 8.2%, and day 4 was 21%. It suggests that the highest alcohol concentration in day 4 fermentation is 21%, and the result of the lowest alcohol concentration 0.8%. The results indicate bacterial growth were influenced by incubation time. In day 4 fermented beverage, there were no bacteria found due to their high alcohol concentration, which is 21%. The manufacture of alcoholic drinks such as beer with high gravity, causes yeast to face extremely high alcohol concentrations, sometimes reaching up to 20% v/v - 25% v/v. It can quickly become toxic to cells. High alcohol concentration can endanger quality of the product. In addition, excessive alcohol consumption can cause the various health problem [26].
### Table 3. Analysis of Alcohol Concentration Using the Distillation Method

| No  | Sample          | Number of Distillation (mL) | Alcohol Concentration (% v/v) |
|-----|-----------------|-----------------------------|-----------------------------|
| 1   | Fermentation 2  | 0.2                         | 0.8                         |
| 2   | Fermentation 3  | 4.1                         | 8.2                         |
| 3   | Fermentation 4  | 10.5                        | 21                          |

### 3.4. Sensitivity Test for Pongasi Drink

The sensitivity test of traditional Pongasi drink against isolated bacteria (*S. carnosus*) was carried out with three repetitions using the media Mueller Hinton Agar (MHA). The measurement results suggest the average inhibition on day 2 fermentation was 9.05 mm, day 3 fermentation was 12.12 mm, and day 4 fermentation was 12.14 mm (Table 4). These results indicate that *S. carnosus* bacteria are susceptible to pongasi (Figure 2). Based on the results of the study it was found that during the fermentation process, yeast and Lactic Acid Bacteria must respond to several adverse conditions, exceptionally low pH, increased ethanol concentration, nutritional limits, fluctuations in oxygen concentration, and the presence of various compounds with antimicrobial effects [27][28][29]. It causes *S. carnosus* bacteria to be inhibited by fermentation drinks of day 2, 3, and 4. Even bacterial growth can no longer survive day 4 fermentation marked by the absence of these bacteria in isolation using the Nutrient Agar media (Table 1). Bacteria are strongly influenced by changes in environmental conditions. Some species undergo morphological changes under certain conditions. These changes may be related to increasing nutritional requirements, changes in temperature or the presence of threats [22]. Threats can occur because they are caused by secondary metabolites that are produced during the fermentation process. Secondary metabolites that are produced in the fermentation process of pongasi drinks are alcohol which is antibacterial.

### Table 4. Inhibitory Zones in Test Bacteria Against Pongasi as Traditional Drink

| Treatment   | Inhibition Zone Results Against *Staphylococcus carnosus* Bacteria | Amount (mm) | Average (mm) |
|-------------|-------------------------------------------------------------------|-------------|--------------|
|             | I (mm)                II (mm)            III (mm)            |             |              |
| Fermentation 2 | 8,15                  8,93               10,08             | 27,16       | 9,05         |
| Fermentation 3 | 14,75                 11,85              9,77              | 36,37       | 12,12        |
| Fermentation 4 | 11,95                 11,98              12,5              | 36,43       | 12,14        |

**Figure 2.** Inhibitory zones for three repetitions (a, b, c); II= Fermentation day 2; III = Fermentation day 3; IV= Fermentation day 4
4. Conclusions
The results of identification of bacteria in Pongasi drink were 91% Probability Staphylococcus carnosus bacteria and alcoholic concentration results in fermentation day 2 at 0.8%, fermentation day 3 at 8.2%, and fermentation day 4 at 21% which indicated the more extended the fermentation of Pongasi, the higher of the alcohol concentration.

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