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# Potential of *Rhizopus delemar* and *Rhizopus microsporus* as tempeh starters

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**Abstract.** *Hartanti AT, Kurniawan BC, Gunawan AW. 2024. Potential of* Rhizopus delemar *and* Rhizopus microsporus *as tempeh starters. Biodiversitas 25: 4208-4214.* The biodiversity of tempeh-associated *Rhizopus* in Indonesia has decreased due to the widespread use of commercial starters. This study aimed to conserve three pure *Rhizopus* strains (*Rhizopus delemar* ATH 53, *R. microsporus* ATH 1 and ATH 24) of tempeh starters and optimize their use in tempeh production. Using rice flour and *Rhizopus* spores at $10^6$/mL concentration, tempeh starters were made and stored for up to 12 weeks, maintaining spore viability at $10^6$/g with 7-14% water content. Tempeh production experiments utilized three starter doses (0.1, 0.2, 0.3 g per 100 g soybeans) and three different incubation temperatures (27, 30, and 35°C), all of which successfully yielded tempeh. Sensory evaluations revealed that ATH 1 and ATH 53 starters were superior over ATH 24, which produced a distinct yellow tempeh that sporulated easily. It was also noted that 0.1 g of starter per 100 g of soybeans was sufficient for producing high-quality tempeh, offering an efficient use of starter. By successfully conserving these *Rhizopus* strains as viable tempeh starters for at least 12 weeks, results of present study demonstrate a promising approach to maintaining biodiversity in Indonesian tempeh production, paving the way for further exploration of traditional *Rhizopus* strains in tempeh making.

**Keywords:** Conserve, *Rhizopus*, soybean, starters, tempeh, temperature, viability

## INTRODUCTION

Tempeh, a fermented soybean product originating from Indonesia, has garnered significant scientific interest due to its nutritional profile and potential health benefits (Romulo and Surya 2021). The fermentation process in tempeh production is primarily facilitated by the genus *Rhizopus* (Drabo et al. 2023). This process not only enhances the organoleptic properties of the final product but also significantly improves the digestibility and bioavailability of nutrients (Ahnan-Winarno et al. 2021). The biochemical changes occurring during fermentation warrant further investigation to fully elucidate the mechanisms underlying these improvements (Feng et al. 2023).

Various strains of the genus *Rhizopus* play pivotal roles in tempeh fermentation, significantly influencing the nutritional profile, sensory attributes, and textural characteristics of the final product (Wikandari et al. 2021). Among these, *Rhizopus oligosporus* stands out as the predominant strain in tempeh production, valued for its efficient soybean fermentation capabilities and reduced spore formation, which enhances its safety for consumption (Teoh et al. 2024). In contrast, *Rhizopus delemar*, while less commonly employed, contributes to the microbial diversity during the fermentation process. Its presence can potentially introduce nuanced variations in the flavor profile and textural properties of tempeh (Tamang et al. 2022). The inclusion of *R. delemar* in the fermentation ecosystem may offer opportunities for product differentiation and could play a role in preserving the traditional microbial diversity associated with tempeh production (Pouris et al. 2024).

Central to tempeh production is the tempeh starter, an inoculum composed of *Rhizopus* and a substrate. Dewi and Aziz (2011) reported various substrates used in starter preparation, including rice, soybeans, wheat, wheat bran, and *Hibiscus tiliaceus*. The traditional use of hibiscus leaves (*Hibiscus* spp.) as tempeh wrappers is particularly noteworthy (Owens et al. 2015). The fine trichomes on these leaves serve as an effective capture mechanism for ambient *Rhizopus* spp. spores, highlighting the ingenious adaptation of traditional production methods to local environmental conditions. The practice of using dried hibiscus leaves as a tempeh starter, known as *usar*, represents a form of microbial enrichment that likely contributed to the historical diversity of *Rhizopus* strains associated with tempeh. This natural inoculation method contrasts sharply with modern commercial starters, which tend to promote uniformity in the microbial composition of tempeh across Indonesia (Surono 2016).

A comprehensive study by Hartanti et al. (2015) provided crucial insights into the current state of *Rhizopus* diversity in Indonesian tempeh, obtained from 29 locations yielded 36 *Rhizopus* strains, comprising 35 *R. microsporus* strains and one *R. delemar* strain. This finding suggests a predominance of *R. microsporus* in contemporary tempeh production, possibly due to the widespread use of commercial starters (Barus et al. 2019). Three strains were

identified as particularly significant: *R. microsporus* ATH 1, similar to *Rhizopus microsporus* var. *oligosporus*, which appears to be a dominant strain in Indonesian tempeh; *R. microsporus* ATH 24, similar to *R. microsporus* var. *azygosporus*, uniquely found in the Malang area of East Java, Indonesia; and *Rhizopus delemar* ATH 53, the sole *Rhizopus* species identified in the Palu area of Central Sulawesi, Indonesia. The geographical specificity of these strains underscores the potential for regional variations in tempeh characteristics and highlights the importance of preserving microbial diversity in traditional fermented foods.

This research aimed to conserve three pure strains of *Rhizopus* (*R. microsporus* ATH 1 and ATH 24, *Rhizopus delemar* ATH 53) of tempeh starters and optimize their application in tempeh production. This approach offers several potential benefits, including preservation of microbial diversity, standardization with diversity, regional specificity, and the potential for strain-specific benefits.

## MATERIALS AND METHODS

### Materials

*Rhizopus* strains used were strains ATH 53 (*R. delemar*), ATH 1 (*R. microsporus* var. *oligosporus*), and ATH 24 (*R. microsporus* var. *azygosporus*). All three were collected from the collection of the Faculty of Biotechnology, Unika Atma Jaya. Other ingredients were potato dextrose agar (PDA) (Oxoid, England), soybeans (Bola Merah, USA), sterile distilled water, and rice flour (Rose Brand).

### Inoculum preparation of *Rhizopus*

A total of three lines of *Rhizopus* ATH 1, ATH 24, and ATH 53 were cultured on a potato dextrose agar (PDA) slant medium. Cultures were incubated at 30°C until sporulation. The spores were harvested by adding 5 mL of sterile distilled water to the slant culture and gently scraping the surface with a sterile loop to create a spore suspension. The suspension was then transferred to two 2 mL tubes. The concentration of the spore suspension was calculated using a hemacytometer. The spore concentration used to make tempeh starter was $10^6$ spores/mL.

The morphology of three *Rhizopus* strains was observed using the slide culture technique (Rosana et al. 2014). PDA medium was cut using a sterile plastic straw, and then the medium pieces were transferred aseptically to the surface of a sterile glass object. *Rhizopus*, which had been cultured previously, was then inoculated into pieces of the medium. The cover glass was placed on the top of the medium. A total of 2 mL of sterile distilled water was poured onto tissue paper so that the cup became moist. The inoculated medium was incubated at 30°C for three days. The cover glass was carefully removed and placed on a new glass slide prepared with fresh Shear's mounting medium. The original PDA medium was discarded from the glass slide. The specimen was then covered with a new cover glass for observation. The morphological observation included sporangiospore shape, rhizoids, columella, and sporangiophore length. Sporangiophore length was measured using DinoCapture 2.0 software and a Nikon E100 binocular microscope with magnifications of 10×10 and 10×40.

Three *Rhizopus* strains were grown in a PDA medium at different temperatures of 30, 33, 42, and 45°C for two days (Dolatabadi et al. 2014). The variable observed was the growth of mycelium on the PDA medium.

### Making tempeh starter

Tempeh starter was made using rice flour as a substrate and three *Rhizopus* inoculum. The making of tempeh starter was prepared in triplicate. Put 100 g of rice flour in a heat-resistant plastic bag and 50 mL of distilled water into a 250 mL Erlenmeyer flask, then both were sterilized at 121°C for 15 min. After cooling, the two ingredients were mixed aseptically and inoculated with 1 mL of *Rhizopus* spore suspension at a concentration of $10^6$/mL. The plastic bag was punctured at a distance of 2 cm using a sterile toothpick and placed on the perforated basket. The top of the bag was covered with two layers of sterile cheesecloth and incubated at 30°C. On the second day, the plastic bag was opened so that *Rhizopus* sporulated and incubated until the third day. The variables observed were mycelium growth time, sporulation, and water content. The growth time of mycelium was observed using the following criteria: mycelium covered the entire surface of the substrate (very dense), mycelium grew only in few places (dense), and mycelium grew, but the growth was low (not dense). Observation of sporulation was carried out by observing the formation of black spots dominating on the surface of the substrate (very dense). There were only some black spots on the surface of the substrate (dense), and there were only a few black spots on the surface of the substrate (not dense).

On the third day, substrate and *Rhizopus* colonies were transferred into a new plastic bag and weighed to obtain the wet weight. Both were freeze-dried using a freeze dryer (Eyela FD 551) at -35°C for 24 h and referred to as starter of tempeh (Chutrtong and Bussabun 2014). The dried tempeh was weighed along with the plastic bag as the dry weight. Water content in the fermentation process was calculated according to the formula of AOAC (2023). The starter of tempeh was crushed and stored for 4, 8, and 12 weeks at a temperature of 23°C. Each starter of tempeh was coded ATH 1, ATH 24, and ATH 53.

Tempeh shelf-life time test was carried out using the cup counting (TPC) technique referring to the method of Yunita et al. (2015) with a slight modification. A total of 1 g of starter of tempeh was poured into a test tube containing 9 mL of sterile distilled water and shaken using a vortex. The tempeh starter suspension was diluted to reach $10^{-3}$, and 0.1 mL of the starter suspension was spread aseptically on PDA medium and incubated at 30°C for 18 hours.

$$\% \text{ Moisture content (dry basis)} = \frac{b-(c-a)}{c-a} \times 100\%$$

Where:

B : sample weight (wet) (g)

c-a : dry weight (g)

The moisture content of tempeh powder was calculated using the modified AOAC (2023) method. The aluminium paper was weighed, and 2 g of starter of tempeh was added to the wet weight. Starter of tempeh was heated in the oven at 105°C for 18 h. Melt the dried tempeh, then cool in a desiccator for 15 min. After that, the dried tempeh was weighed as dry weight.

**Making tempeh with three types of tempeh starter**

Tempeh was made according to the method of Koh et al. (2012) with modifications (to tempeh starter doses and incubation temperature). Dried soybeans were washed from impurities and soaked in distilled water for 1 hour. Soybeans were boiled, and the foam was removed. Next, the soybeans were drained, and the epidermis was cleaned. Soak the clean soybeans again in distilled water for 12 h. Soybeans were boiled for 15 min. Drain the soybeans until dry and let stand until warm. A total of 100 g of warm soybeans was put into three plastic bags. Tempeh was made using three types of tempeh starter aged 12 weeks. The treatment doses for tempeh starter were 0.1, 0.2, and 0.3 g for 100 g of cooked soybeans. The plastic bag was pierced with a hole using a sterile toothpick measuring 2×1 cm. Each treatment was incubated at temperatures of 27, 30, and 35°C for three days. This experiment was repeated three times. The variables observed were tempeh color, compactness, and sporulation.

**Sensory evaluation**

The tempeh sensory evaluation was conducted with 30 panelists who underwent a brief training session on tempeh evaluation. These semi-trained panelists were individuals familiar with tempeh consumption but not professional food tasters. Prior to the evaluation, they received a 30-minute instruction on the specific attributes to assess in tempeh (color, aroma, compactness) and how to use the 7-point rating scale. This training aimed to standardize their understanding of the evaluation criteria without reaching the level of expertise of fully trained sensory professionals. The tested samples were tempeh made using three types of starter and two doses of selected starter from the previous treatment. Commercial tempeh was used as a control in this test. The assessment criteria included tempeh color, aroma, compactness, and overall preference, rated on a scale of 1-7, where 1 represented the lowest quality and 7 the highest quality (Meilgard et al. 2016).

**Statistical analysis**

Tempeh-making data (sensory evaluation) was tested using IBM SPSS 25 statistical software. The test used was the ANOVA test, which has a confidence level of 95%. Data that are significantly different will be tested further using the Duncan test.

## RESULTS AND DISCUSSION

***Rhizopus* inoculum**

The color of colonies of three *Rhizopus* strains was white on the first day. Colonies of three *Rhizopus* strains experienced color changes on the third day. *Rhizopus* ATH 1 and *Rhizopus* ATH 53 had brownish grey colonies, while *R. microsporus* ATH 24 was blackish grey (Figures 1-3).

Each *Rhizopus* strain has different morphological characteristics. *Rhizopus microsporus* strain ATH 1, and *R. delemar* ATH 53 were similar in their sporangiospores, columella and rhizoid shape. *Rhizopus* strain ATH 53 has longer sporangiophores compared to strain ATH 1. *R. microsporus* strain ATH 24 had uniform sporangiospores, avocado columella shape, branched rhizoids, and formed azygospores (Table 1). *Rhizopus* morphology can affect the quality of tempeh. Each strain has different morphological characteristics. Rhizoids are morphological structures of *Rhizopus* which are useful for binding soybean pieces when making tempeh. The rhizoid shape must develop sufficiently so that the soybean pieces can bond and the tempeh becomes compact. These three strains have good rhizoid development, resulting in compact tempeh. Apart from rhizoids, mycelium growth also affects the compactness of tempeh (Kustyawati et al. 2017).

Tempeh starter is the inoculum used to make tempeh. The composition of the tempeh starter consists of *Rhizopus* mold and substrate. Some other substrates used are rice, soybeans, wheat, wheat bran, and *Hibiscus tiliaceus* L. leaves. In ancient times, tempeh artisans used *H. tiliaceus* leaves as tempeh wrappers. The fine hairs on hibiscus leaves are helpful for capturing *Rhizopus* spp. spores existing in the environment. Tempeh makers usually dry the *H. tiliaceus* leaves left over from wrapping tempeh to make tempeh starter. Tempeh starter made from waru leaves is called usar (Dewi and Aziz 2011). Usar is used because in ancient times, no starter was commercialized like it is now. It allows for the diversity of *Rhizopus* associated with tempeh.

The commercial use of starter causes the *Rhizopus* associated with tempeh in Indonesia to become uniform. Hartanti et al. (2015) obtained 36 *Rhizopus* strains consisting of 35 *R. microsporus* strains and one *R. delemar* strain in fresh tempeh samples at 29 locations in Indonesia. *R. microsporus* ATH 24 is a strain that is only found in the Malang area, East Java, Indonesia. *Rhizopus delemar* is also the only *Rhizopus* species found in the Palu area, Central Sulawesi (Hartanti et al. 2015).

**Table 1.** Observation of morphology and growth temperature of three *Rhizopus* strains

| Strains | Shape and size of spores | Columella | Rhizoids | Length sporangiophore (µm) | Structure reproduction | Temperature (45°C) |
|---|---|---|---|---|---|---|
| ATH 1 | Vary 2.42-6.42 µm | Vary | Simple, fingering | 60-357 | Sporangiospore | Growing |
| ATH 24 | Uniform 2.51-3.54 µm | *Pyriform* | Simple, branching | 135-368 | Sporangiospore and azygospore | Growing |
| ATH 53 | Vary 2.92-8.08 µm | Vary | Simple, fingering | 325-1084 | Sporangiospore | Not growing |

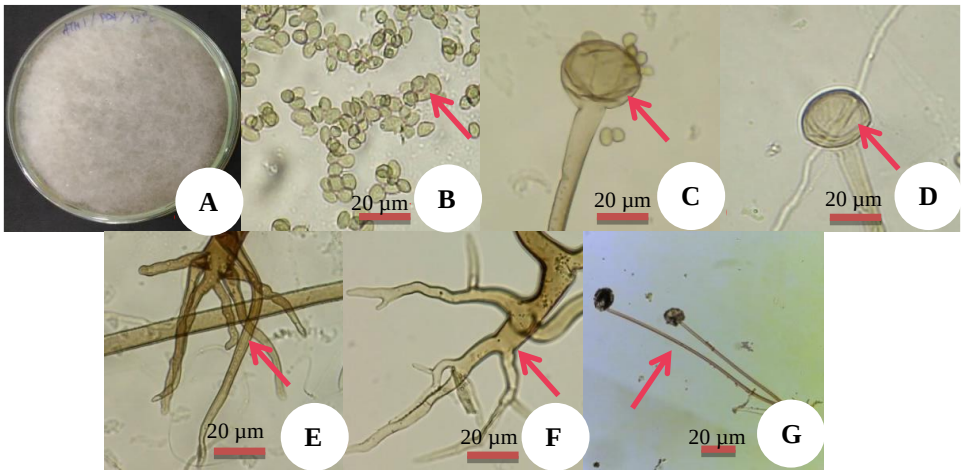


**Figure 1.** Morphology of *Rhizopus microsporus* ATH 1. A. Colony on a medium plate with a diameter of 9 cm; B. Various shapes and sizes of sporangiospores; C-D. Columella; E-F. Fingered rhizoids; and G. Sporangiophores. (B-G) magnification 400×

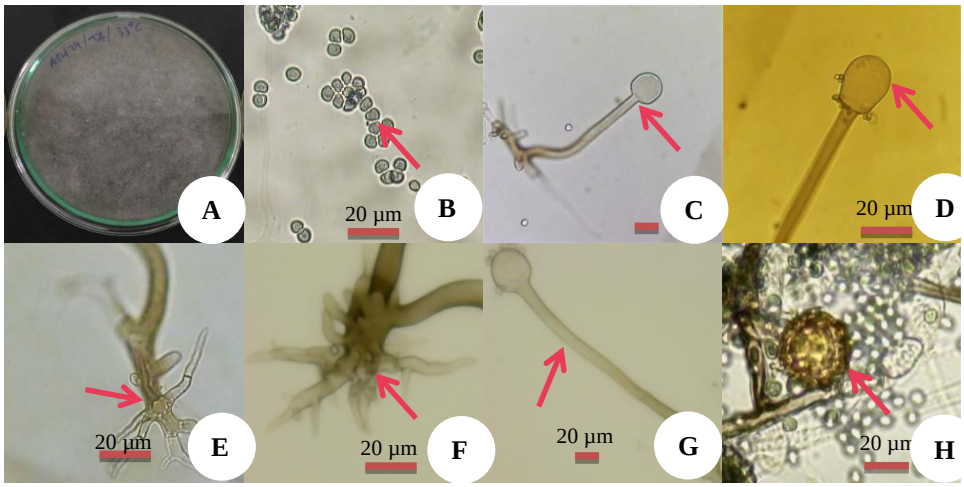


**Figure 2.** Morphology of *Rhizopus microsporus* ATH 24. A. *Rhizopus* colony on a medium plate with a diameter of 9 cm; B. Uniform shape and size of sporangiospores; C-D. Round and pyriform columella; E-F. Root-like rhizoids; and G. Sporangiophores; H. Azygospores. B-H. Magnification 400×

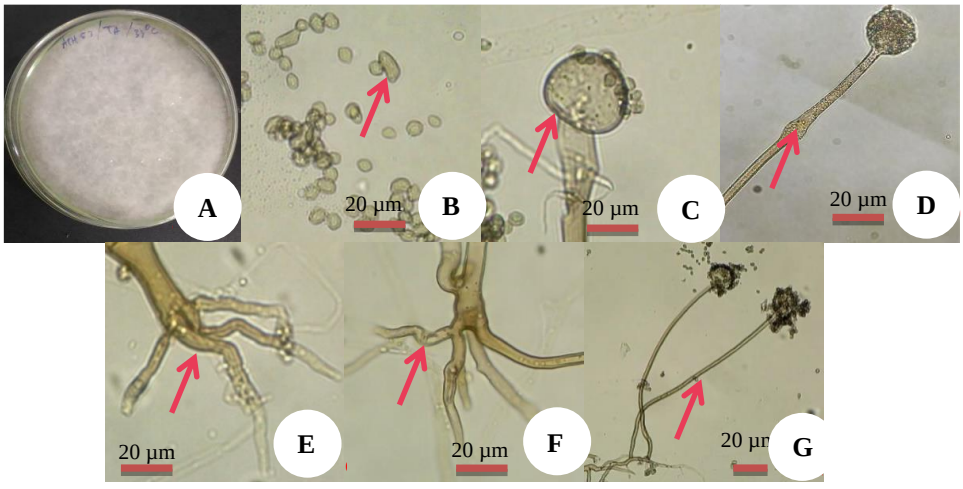


**Figure 3.** Morphology of *Rhizopus delemar* ATH 53. A. *Rhizopus* colony on a medium plate with a diameter of 9 cm; B. Various shapes and sizes of sporangiospores; C. Columella; D. Swollen cells in the sporangiophores; E-F. Fingered rhizoids; and G. Sporangiophore. B-F. Magnification 400×; and G. Magnification 100×

**Tempeh starter**

Tempeh starter was successfully produced using rice flour as a substrate. Rice flour offers an advantage over whole rice grains due to its larger surface area, which promotes better aeration during fermentation. The use of perforated containers covered with thin, sterile cloth facilitated proper aeration while preventing contamination. These aerobic conditions supported both the growth and sporulation of *Rhizopus* mold. The addition of 50 mL of sterile distilled water (resulting in 41% water content in the substrate) created a moist environment conducive to *Rhizopus* growth. Table 2 shows the progress of mycelium growth and sporulation over time, with both parameters increasing as fermentation proceeded. Interestingly, the water content in the substrate decreased during fermentation process (Table 2). *Rhizopus* utilize water for growth and respiration processes (Manan and Webb 2017). The observed mycelium growth and sporulation, coupled with the changes in substrate moisture, demonstrate the successful development of the tempeh starter under the provided conditions.

The present study successfully demonstrated the conservation of three pure *Rhizopus* strains (*R. microsporus* ATH 1, *R. microsporus* ATH 24, and *R. delemar* ATH 53) in tempeh starters. The tempeh starters produced using these strains maintained spore viability at $10^6$/g for up to 12 weeks of storage, with water content ranging from 7-14% (Table 3). This stability is crucial for preserving the microbial diversity and ensuring consistent tempeh production over time. The low water content likely contributed to the absence of bacterial contamination during storage, as bacteria are unable to grow under such conditions (Manan and Webb 2017).

Differences in sporulation rates were observed between strains. *Rhizopus microsporus* ATH 24 exhibited faster sporulation, beginning on the second day of fermentation, compared to ATH 1 and ATH 53 which sporulated on the third day. This variation in sporulation rates could have implications for tempeh production and starter preparation protocols.

In tempeh production trials, all three strains successfully produced tempeh across various incubation temperatures (27, 30, and 35°C) and starter doses (0.1, 0.2, and 0.3 g per 100 g soybeans). Notably, tempeh made with ATH 24 had a distinct yellow color and sporulated more easily, setting it apart from the white tempeh produced by ATH 1 and ATH 53. This color difference and sporulation tendency of ATH 24 may offer opportunities for product differentiation in the tempeh market.

Sensory evaluations revealed that tempeh made with ATH 1 and ATH 53 starters were comparable to commercial tempeh in terms of color, aroma, compactness, and overall preference. The results suggest that a starter dose of 0.1 g per 100 g of soybeans was sufficient for producing high-quality tempeh, offering an efficient use of starter material. This optimization could have significant implications for commercial tempeh production, potentially reducing costs while maintaining product quality.

**Tempeh from three types of tempeh starter**

Tempeh production was successfully achieved across various temperature treatments (27, 30, and 35°C) and starter doses (0.1, 0.2, and 0.3 g per 100 g soybeans). Results showed no significant interaction between starter dose and incubation temperature for tempeh made with ATH 1, ATH 24, and ATH 53 starters (Table 4). While ATH 53 tempeh at 35°C with 0.3 g dose showed incomplete mycelium coverage, likely due to uneven starter distribution, the spore concentration of 106/g was generally sufficient for quality tempeh production. Lower spore concentrations (<106/g) can increase contamination risk due to insufficient inoculum density (Manan and Webb 2017). ATH 1 and ATH 53 produced white tempeh, while ATH 24 yielded yellow tempeh with notably faster mycelium growth at all temperatures, allowing harvest in under 30 hours. This rapid growth may be attributed to high amylase and protease enzyme activities in *Rhizopus* strain ATH 24, though further enzymatic studies are needed.

**Table 2**. Observations of sporulation and water content during fermentation in making starter of tempeh

| Codes | Mycelium growth | Sporulation | Moisture content (%) |
|---|---|---|---|
| ATH 1 | Very dense | Dense | 37.16±1.20 |
| ATH 24 | Very dense | Very dense | 40.42±5.91 |
| ATH 53 | Very dense | Dense | 35.74±2.66 |

**Table 3.** Viability of spores/g of tempeh spores and water content of tempeh starter

| Starters of tempeh | 4 Weeks | | 8 Weeks | | 12 Weeks | |
|---|---|---|---|---|---|---|
| | Viability ($\times 10^6$/g) | Water content (%) | Viability ($\times 10^6$/g) | Water content (%) | Viability ($\times 10^6$/g) | Water content (%) |
| ATH 1 | 1.03±0.01 | 11.33±2.60 | 1.00±0.02 | 7.30 ±1.76 | 1.01±0.00 | 14.67±1.67 |
| ATH 24 | 1.36±0.01 | 8.83±1.58 | 1.42±0.01 | 5.30±0.44 | 1.33±0.01 | 13.00±2.00 |
| ATH 53 | 1.04±0.01 | 7.83±1.24 | 1.05±0.01 | 10.16±1.83 | 1.04±0.02 | 10.67±2.02 |

So far, the cause of the yellow color produced in ATH 24 tempeh was unknown. It is necessary to carry out in-depth study on ATH 24 yellow tempeh. Incubation temperature can influence the production of yellow color in ATH 24 tempeh at 27°C showed pale yellow and became more intense at 35°C (Table 4). The weakness of *Rhizopus* in the ATH 24 starter was that it easily sporulates when exposed to oxygen. This may be related to the ability of *Rhizopus* ATH 24 to produce spores more quickly and in more significant numbers compared to the other two strains. Making tempeh at a temperature of 27°C requires an incubation time of three days, but at a temperature of 30-35°C it was enough to incubate for two days. This is because a temperature of 27°C was not the optimum for *Rhizopus* growth, so it required a longer incubation time. Based on the appearance and compactness of tempeh, giving a late dose of 0.2 and 0.3 g has the same quality. Tempeh was made by administering a late dose of 0.1 and 0.2 g was chosen for sensory evaluation.

Tempeh was successfully made by administering two subsequent doses, 0.1 and 0.2 g. Commercial tempeh, often called control tempeh, was known for its high quality. It has a firm, compact texture, a distinct tempeh aroma, and a clean, white appearance. Tempeh ATH 1 and ATH 53 had the same quality as control tempeh in color, aroma, compactness and liking. Tempeh ATH 24 was less preferred because it had a different appearance, and there were black spots on the surface of the tempeh due to sporulation (Table 5).

**Table 4.** Qualitative observations of tempeh treated with three incubation temperatures of 27, 30, and 35°C and three doses of 12-week-old tempeh

| Tempeh | Temperature (°C) | Doses (g)[a] | Color of tempeh | Compactness * | Sporulation** |
|---|---|---|---|---|---|
| ATH 1 | 27 | 0.1 | White | ++++ | - |
| | | 0.2 | White | ++++ | - |
| | | 0.3 | White | ++++ | - |
| | 30 | 0.1 | White | ++++ | - |
| | | 0.2 | White | ++++ | - |
| | | 0.3 | White | ++++ | - |
| | 35 | 0.1 | White | ++++ | - |
| | | 0.2 | White | ++++ | - |
| | | 0.3 | White | ++++ | - |
| ATH 24 | 27 | 0.1 | Pale yellow | ++++ | ++ |
| | | 0.2 | Pale yellow | ++++ | ++ |
| | | 0.3 | Pale yellow | ++++ | ++ |
| | 30 | 0.1 | Yellow | ++++ | +++ |
| | | 0.2 | Yellow | ++++ | ++ |
| | | 0.3 | Yellow | ++++ | ++ |
| | 35 | 0.1 | Yellow | ++++ | - |
| | | 0.2 | Yellow | ++++ | - |
| | | 0.3 | Yellow | ++++ | - |
| ATH 53 | 27 | 0.1 | White | ++++ | - |
| | | 0.2 | White | ++++ | - |
| | | 0.3 | White | ++++ | - |
| | 30 | 0.1 | White | ++++ | - |
| | | 0.2 | White | ++++ | - |
| | | 0.3 | White | ++++ | - |
| | 35 | 0.1 | White | ++++ | - |
| | | 0.2 | White | ++++ | - |
| | | 0.3 | White | ++++ | - |

Note: *++++ : compact; +++ : rather compact; ++ : less compact; + : barely grows; - : no growth; **++++ : dense; +++ : quite dense; ++ : few; + : very few, - : no sporulation

**Table 5.** Sensory evaluation of tempeh using selected starter dosages

| Tempeh | Doses of starter (g) | Color of Tempeh | Aroma | Compactness | Overall |
|---|---|---|---|---|---|
| ATH 1 | 0.1 | $6.03\pm0.18^{c}$ | $5.90\pm0.17^{d}$ | $5.80\pm0.15^{c}$ | $5.87\pm0.16^{c}$ |
| | 0.2 | $5.97\pm0.23^{c}$ | $5.53\pm0.20^{cd}$ | $5.87\pm0.15^{c}$ | $5.70\pm0.19^{bc}$ |
| ATH 24 | 0.1 | $3.90\pm0.28^{b}$ | $4.13\pm0.27^{a}$ | $5.10\pm0.24^{b}$ | $4.27\pm0.26^{a}$ |
| | 0.2 | $3.10\pm0.25^{a}$ | $4.30\pm0.26^{ab}$ | $4.53\pm0.25^{a}$ | $3.90\pm0.21^{a}$ |
| ATH 53 | 0.1 | $6.13\pm0.17^{c}$ | $5.13\pm0.28^{c}$ | $5.53\pm0.18^{bc}$ | $5.20\pm0.25^{b}$ |
| | 0.2 | $5.73\pm0.18^{c}$ | $4.90\pm0.25^{bc}$ | $5.43\pm0.19^{bc}$ | $5.47\pm0.18^{bc}$ |
| Control | Control | $5.80\pm0.18^{c}$ | $5.33\pm0.28^{cd}$ | $5.90\pm0.17^{c}$ | $5.77\pm0.18^{bc}$ |

Note: Different letters in each column indicate significant differences. Significance value $P\leq0.05$

The appearance of ATH 24 tempeh was less favorable because it was different from tempeh in general, which was yellow and easy to sporulate. The aroma produced by all tempeh was typical tempeh. Tempeh ATH 1 had a more favorable tempeh aroma and is similar in aroma to control tempeh. Other tempeh has a distinctive aroma like tempeh, but not as strong as ATH 1 tempeh and control tempeh. The compactness of ATH 24 tempeh was not good compared to other tempeh. Giving a starter can affect the cohesiveness of tempeh. Based on the results in Tables 4 and 5, giving a late dose of 0.1 g for 100 g of mature soybeans can produce good tempeh. This 0.1 g dose is recommended for efficient starter usage while maintaining tempeh quality.

In conclusion, three strains of *Rhizopus* were used as starter for making tempeh. By preserving these *Rhizopus* strains as viable tempeh starters for at least 12 weeks, results of present study demonstrate a promising approach to maintaining biodiversity in Indonesian tempeh production, paving the way for further exploration of traditional *Rhizopus* strains in tempeh making.

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