The viability of *Rhizopus microsporus* APBSMLF19 on different substrates and its application as a starter culture in tempeh fermentation

M K Putri¹, A M Sari¹, M Z Zaman¹, A Nursiwi¹, D Ishartani¹, and Lukitawesa²

¹Department of Food Science Technology, Faculty of Agriculture, Universitas Sebelas Maret, Jl. Ir. Sutami 36 Jebres, 57126, Surakarta, Indonesia

²Swedish Centre for Resource Recovery, University of Borås, Allégatan 1, 50190, Borås, Sweden

Email: m_zukhruf@staff.uns.ac.id

Abstract. In addition to temperature and incubation time, inoculum is one of the key factors contributing to tempeh’s quality. At several regions in Java, *usar*, a traditional fungal inoculum obtained from teak leaves, is used for tempeh fermentation, however, there are some lacks in its application. Rice, cassava root and flour, as well as tapioca flour, were evaluated for their suitability as a substrate to support *Rhizopus microsporus* APBSMLF19 growth (fungi isolated from *usar* in Gunungkidul, Yogyakarta). About 100 ml/kg of 10⁷ CFU/ml fungi suspension were inoculated to each substrate and incubated at 30 °C for 48 hours. After dried at 50 °C for ± 20 hours and mashed, the fungi viability was observed on weeks 0, 1, 2, and 4 during storage. The result showed no significant difference in the fungi’s number based on storage time for each starter culture’s type, but the inoculum exhibited different viabilities. However, there were no differences in texture measurements for all tempeh produced by using experimental and commercial starter culture (RAPRIMA®). Increasing the culture’s storage time for viability test is required in future research.

Keywords: inoculum, rice, cassava root, cassava flour, tapioca flour

1. Introduction

Tempeh is one of the Indonesian traditional fermented foods [1] and its rich variety is often the country’s pride which has gained acceptance and popularity worldwide [2]. The distinctive flavor and texture, high nutritional quality, and mineral source that improves the enzymes’ activities in the body [3] contribute an important role in characterizing a tempeh product. The research on this product is much and diverse to many topics, such as variation in the inoculum, tempeh making, raw material, etc [4]. Soybeans are generally used as one of the raw materials, while others include legumes like jack beans (*Canavalia ensiformis*) [5], kidney beans (*Phaseolus vulgaris* L.) [6], lamtoro seeds (*Leucaena leucocephala*) [7], etc. The fungi or inoculum type such as *Rhizopus microsporus*, *R. delemar* [8], *R. oligosporus* [9], *R. oryzae*, *R. stolonifer* [1], and *N. Intermedia* [10] is also widely researched. Tempeh’s texture, color, and nutritional content are affected by inoculum type [11]. Since there are lots of *Rhizopus* strains, isolation and identification are needed to specify them based on morphology and characteristics.
Therefore, previous research was conducted to isolate and identify seven selected cultures from usar in lamtoro tempeh, among which the one from Gunungkidul, Yogyakarta was Rhizopus microsporus APBSMLF19 [12]. The usar used as an inoculum is very limited, unpractical, and has short shelf life [13]. Hence, further research for developing fungi isolated from usar into a starter culture that can be easily used is needed, but the culture’s diversity is increasing.

Substrates’ nutritional content plays a key role in fungi growth which is one of the key factors for starter culture production. Some elements namely carbon, nitrogen, hydrogen, oxygen, sulfur, phosphorus, magnesium, and iron are fungi’s energy source. Carbon is obtained from carbohydrates which are generally found in cereals and roots such as rice and cassava including their derivative product, such as flour, while nitrogen is obtained from amino acids and protein. The substrate that has a high carbon and nitrogen source is suitable for fungi growth, but the vitamins content are also important [14]. Rice has 77.78 g carbohydrates, 6.67 g protein, 3.33 g fat, and 1.6 mg iron in 100 g. Cassava contains 38.06 g carbohydrates, 1.36 g protein, 0.28 g fat, 16 mg calcium, 0.27 mg iron, 21 mg magnesium, 27 mg phosphorus, 271 mg potassium, 14 mg sodium, 0.34 mg Zn, and 0.1 mg Cu in 100 g. Furthermore, cassava flour contains 87.50 g carbohydrates, 3.12 g protein, 3.1 g fiber, 0.31 iron, and 31 mg sodium in 100 g [15]. Tapioca flour contains 85.00 g carbohydrates, 0.5 g protein, and 0.2 g fat in 100 g [16]. Rice, cassava roots and flour, as well as tapioca flour’s contents, such as carbon, nitrogen, magnesium, and iron provide nutrients and energy for mold (Rhizopus microsporus APBSMLF19) growth in tempeh starter culture production.

This research aims to determine the effect of using several substrate types and storage time on Rhizopus microsporus APBSMLF19 viability in the starter culture because such has never been conducted. Its objective is to also determine substrate types and starter culture concentration’s effect on fermented tempeh’s physical characteristics in order to increase the culture’s diversity and shelf life.

2. Material and Methods

2.1 Culture preparation

Rhizopus microsporus APBSMLF19 [13] was used in starter culture preparation. Its frozen samples in microtube were thawed within 10 – 15 minutes at room temperature. The culture was inoculated (0.5 ml) on Acidified Potato Dextrose Agar (APDA), incubated at 30°C for 3-7 days to induce sporulation and then stored at 4°C until further utilization [12].

2.2 Inoculum preparation

Rhizopus microsporus APBSMLF19 was inoculated on APDA at 30°C for 7 days. The spores were harvested by adding 10 ml of 0.5% Tween 80 on the aforementioned culture’s surface and gently rubbing with sterile ose. Afterwards, 10 ml of the culture suspension was centrifuged at 5000 rpm for 15 minutes and the supernatant was removed. Sterile water was added to the spores until 10 ml, then the fungal amount was counted with Hemocytometer, and cultures that had 7 log CFU/ml were used for starter’s preparation [10].

2.3 Substrates preparation

Rice (0.5 kg) was boiled on the stove for about 30 minutes using medium heat, with rice: water ratio as 1:2. The half-cooked rice was then steamed for ±1 hour until it cooked evenly and was ready to be used as a substrate (1 kg). The next was cassava roots (2 kg), of which the outer surfaces were removed and the inner part was grated roughly and (1 kg) steamed for ±30 minutes until became cooked. Finally, cooked rice and cassava were sterilized in an autoclave at 121°C for 15 minutes [17]. For cassava and tapioca flours (1 kg), they were sieved to remove impurities and then sterilized.
2.4 Starter cultures preparation
Each substrate (1 kg) was inoculated and thoroughly mixed with 100 ml/kg fungi suspension (10 ml of 7 log CFU/ml Rhizopus microsporus APBSMLF19 and 90 ml of sterilized aquades). Inoculated substrates were placed in sterile trays, covered with sterile perforated aluminum foil, and incubated at 30°C for 48 hours. The fermented substrates were dried in a cabinet dryer at 50°C for ±20 hours, and mashed up with a middle-speed blender for 1 minute then sieved to remove impurities. Subsequently, the powders were sealed in plastic bags and stored at room temperature (27°C) [10].

2.5 Determination of Rhizopus microsporus APBSMLF19 viability
The fungi’s viability was checked after 0, 1, 2, and 4 weeks during storage. Each substrate’s starter culture (10 g) was poured in 90 ml of Potato Dextrose Broth (PDB) and incubated for 24 hours. Then, 1 ml samples from this were serially diluted in Buffered Pepton Water and spread (0.1 ml) in duplicates on APDA plates. These were subsequently incubated at 30°C and colonies formed were counted after 24 hours [10].

2.6 Preparation and textural analysis of tempeh
Soybeans were washed with tap water to remove the impurities, then cooked for 30 minutes with medium heat on the stove until boiled, where soybeans:water ratio was 1:2. The cooked soybeans were cooled with tap water and then removed from the husk. They were soaked in tap water for 18 hours following the aforementioned ratio. Afterwards, they became slimy because there was microbial growth including coliforms while soaking [18], which was then removed with water. The cleaned ones were cooked again for 30 minutes until boiled and drained, then placed in wide trays to cool. According to previous trials, 0.2% experimental starter culture’s concentration has not produced a compact tempeh product as it should be. Therefore in this research, 3 variations (0.4%, 0.8% and 1.2%) of experimental and commercial (RAPRIMA®) (0.2%) starter culture were added to the soybeans. These were thoroughly mixed, deposited in layers (1.5 to 1.8 cm thick) in greaseproof paper, and incubated at 27-30°C (room conditions) for 2 days.

The textural quality of 20 g (2.6 x 6.0 x 1.6) tempeh samples from each substrate was determined after 2 days of fermentation using a Kramer shear-compression test cell attached to a Universal Testing Machine. The energy required to break and completely shear compress samples was calculated by integrating the area under the force deformation curve up to the compression motion’s completion [10].

2.7 Statistical analysis
The data presented in this report represented the means of values obtained from 3 replicate trials. The significant difference was determined by using analysis of variance (ANOVA). Furthermore, the Duncan test was used to compare the means and p-values <0.05 indicated a significant deviation.

3. Result and Discussion
3.1 Viability of Rhizopus microsporus APBSMLF19
Four substrates were used for Rhizopus microsporus APBSMLF 19 starter culture production, namely rice, cassava roots and flour, as well as tapioca flour. The viability test result after drying and storage for 0, 1, 2, and 4 weeks is illustrated in Figure 1. The graph showed that all the substrate populations during storage were 3.4 - 5.9 log CFU/g. This means they were perfect for starter culture production because a minimum of 3 - 4 log CFU/g spores was needed for initial tempeh fermentation [19]. The fungi population in Figure 1 was subjected to One Way ANOVA with a significance level of 0.05 and no difference was found in their number based on each starter culture type’s storage time. The highest populations were noted in cassava flour’s tempeh starter culture as 5.8 – 5.9 log CFU/ml during 4 weeks of storage, rice and tapioca substrates had 4.5 – 4.6 log CFU/ml, while cassava roots had the
lowest which was 3.4-3.5 log CFU/ml. The *Rhizopus microsporus* APBSMLF19 percentage population decline in experimental starter culture indicated rice had the lowest value (1.37%), followed by cassava flour (1.57%), and cassava roots (2.73%), while tapioca flour (3.25%) had the highest. This proved that the fungi’s viability was most stable in the starter culture with rice substrate.

**Figure 1.** Changes in the population of *Rhizopus microsporus* APBSMLF19 in tempeh starter culture from each substrate type during storage.

| Substrate       | Population (Log CFU/g) |
|-----------------|------------------------|
| Rice            | 5.800 - 5.900          |
| Cassava roots   | 4.800 - 4.900          |
| Cassava flour   | 4.400 - 4.500          |
| Tapioca flour   | 3.800 - 3.900          |

*Rhizopus microsporus* APBSMLF19 population in cassava flour starter culture was the highest compared to tapioca flour, rice, and cassava. This is due to carbohydrates’ number in cassava flour, which is 87.50 g per 100 g [15] as well as the highest. Cassava flour also contains 3.12 g protein, 3.1 g fiber, 0.31 g iron, 84 mg calcium, and 125 mg phosphorus [15]. Carbohydrates and protein amounts in tapioca flour are lesser, namely 85 g and 0.5 g per 100 g of ingredients [16]. The population in tempeh starter culture with rice and cassava substrate was also below that of cassava flour. Moreover, rice contains 77.78 g carbohydrates, 6.67 g protein, 3.33 g fat, and 1.6 mg iron per 100 g [15], while cassava contains 38.06 g carbohydrates, 1.36 g protein, 0.28 g fat, 16 mg calcium, 0.27 mg iron, 21 mg magnesium, and 27 mg phosphorus per 100 g [15]. A high carbohydrates amount in the substrate was needed to support *Rhizopus microsporus* growth [20]. Therefore, the fungi converted the carbohydrates (starch) into simple sugars to facilitate their utilization as carbon and energy sources [21]. Later the cassava flour’s carbohydrates dissolved easily than how the energy was metabolized by *Rhizopus microsporus* APBSMLF19 for growth support [22]. Furthermore, the protein content served as a nitrogen source to meet the nutritional needs, while others such as iron and phosphorus also support cassava flour’s high population size [14].

*Rhizopus microsporus* APBSMLF19 population number in each substrate’s tempeh starter culture decreased after drying during the preparation process, namely from $10^8$ - $10^9$ CFU of the amount of fungus inoculated and incubated at 30°C for 48 hours to $10^5$ - $10^6$ CFU. This population decline occurred because *Rhizopus* spores entered the dormancy stage during drying and storage which reduced viability and germinability [23]. There are 4 stages of fungi spores’ natural life pathway during storage, namely active, dormant, sub-lethally damaged, and dead. It is suspected that the declined population in this research was also due to a transition in the life path. This transition is thought to occur due to changes in the fungi’s environmental conditions during incubation to drying at 50°C for 20 hours, and then to room temperature storage. These changes have led to several active *Rhizopus microsporus* APBSMLF19 spores entering the dormant stage, and some may become sublethally damaged. When starter culture was tested for viability, the spores counted were only active because they formed colonies on solid media. Meanwhile, dormant and sub-lethally damaged spores were present and alive but were not counted due to their inability to form colonies on solid media [24].
Each experimental starter culture was packed with plastic (Polypropylene) and sealed with an impulse sealer [25]. Then, they were stored at 27°C (room temperature) and 61-72% RH (room humidity). Tempeh starter cultures stored at high temperature and/or high humidity had a rapid population decrease during the first few weeks. The decline was from each substrate because the room condition during storage had a high RH which caused the culture (powdered) to absorb moisture and clump, thereby inhibiting the oxygen supply to the fungi growth [25].

Table 1. Texture analysis result of tempeh produced using each substrate type’s experimental and commercial starter culture.

| Type of starter culture | Concentration (%) | Fmax a (N) |
|-------------------------|-------------------|------------|
| RAPRIMA®                | 0.200             | 0.618 ± 0.115ab |
|                         | 0.400             | 0.495 ± 0.208ab |
| Rice                    | 0.800             | 0.647 ± 0.143b |
|                         | 1.200             | 0.607 ± 0.115ab |
| Cassava roots           | 0.400             | 0.514 ± 0.207ab |
|                         | 0.800             | 0.431 ± 0.172ab |
|                         | 1.200             | 0.342 ± 0.195a |
| Cassava flour           | 0.400             | 0.419 ± 0.076ab |
|                         | 0.800             | 0.566 ± 0.048ab |
|                         | 1.200             | 0.408 ± 0.120ab |
| Tapioca Flour           | 0.400             | 0.533 ± 0.098a |
|                         | 0.800             | 0.492 ± 0.142ab |
|                         | 1.200             | 0.466 ± 0.216ab |

*Values in the same column which are not followed by the same letter are significantly different (p ≤ 0.005)
*a is the average result of two analysis replications and three sample replications.

3.2 Texture analysis of tempeh
Tempeh from each substrates type’s (0.4%, 0.8%, and 1.2%) experimental and commercial starter culture called RAPRIMA® (0.2%) was tested to determine type and concentration’s effects on its fermented form. The results of textural measurement of tempeh produced using RAPRIMA® are summarized in Table 1. Also, the One-Way ANOVA test indicated that the effects were uniform (p >0.05). Therefore, tempeh made with both starter cultures had the same texture. The one produced by experimental yeast had a white color with the entire soybean’s surface covered by fungal mycelia, and it was compact. The characteristics of tempeh have been stated by previous research [1].

4. Conclusion
It was concluded that rice, cassava roots and flour, as well as tapioca flour were suitable substrates for tempeh starter culture based on Rhizopus microsporus APBSMLF19 viability during 0, 1, 2, and 4 weeks storage. All tempeh fermented with experimental and commercial starter cultures had the same texture. Increasing the starter culture’s storage time is needed to determine the stability of the fungi’s viability in future research.

Acknowledgements
The authors are grateful to the research project from Universitas Sebelas Maret, Surakarta, Indonesia for the provision of financial support.

References
[1] Yang Y, Kameda T, Aoki H, Nirmagustina D E, Iwamoto A, Kato N, Yanaka N, Okazaki Y, and Kumrungsee 2018 Journal of Functional Foods 49 162-167
[2] Senapati A K, Ann A, Raj A, Gupta A, Sharma A, Neopany B, Pannei C, Diwedi D H, Raj D R, Anghoch D, Bakar F A, Chye F Y, Rapsang G F, Vyas G, Devi G A S, Prajapati J P, Sim K Y, Targais K, Reddy L V A, Swain M N, Reza M S, Zaman M Z, Garg N, Singh N S, Sharma N, Ray R C, Thorat S S, Pinto S V, Gautam S, Thokchom S, Joshi S R, Khomdram S, and Stobdan T 2016 CRC Press 69-106 V K Joshi (Ed) (ISBN 978-1-4398-8790-5).

[3] Nout M J R and Kiers J L 2005 Journal of Applied Microbiology 98 789 – 805

[4] Ogawa Y, Tokumasu S, and Tubaki K 2004 Mycoscience 45 271 – 276

[5] Erkan S B, Gurler H N, Bilgin D G, Germec M, and Turhan I 2020 Food Science an Technology 119 108880

[6] Ferial M, Abu S, Rasha K, Mohamed, Ahmed Y, Gibriel, Nagwa M, and Rasmy H 2014 International Journal of Biological, Agricultural, Biosystems, Lide Science and Engineering 8 280 – 285

[7] Nursiwi A, Ishartani D, Sari A M, and Nisyah K 2017 International Symposium on Food and Agro-biodiversity 102 012093

[8] Hartanti A T, Rahayu G, and Hidayat I 2015 Hayati Journal of Biosciences 22 136 – 142

[9] Jennessen J, Schnurer J, Olsson J, Samson R A, and Dijksterhuis J 2008 Mycological Research 112 547 – 563

[10] Shambuyi M, Larry R, Yen C, and Nakayama T 1992 Journal of Microbiology 15 77 – 85

[11] Karsono Y, Tunggal A, Wiratama A, and Adimulyo P 2009 Research Report from Food and Technology of Institut Pertanian Bogor, Indonesia (In Bahasa Indonesia)

[12] Rachmawati F 2019 Isolasi, Identifikasi, dan Karakterisasi Kapang Proteolitik dari Usar Tempe Mlanding yang Berasal dari Gunungkidul, Wonogiri, dan Pacitan (in Bahasa Indonesia) Skripsi, Ilmu dan Teknologi Pangan, Fakultas Pertanian, Universitas Sebelas Maret Surakarta

[13] Dewi R S, and Aziz S 2011 Molekul 6 94 – 101

[14] Buckle K, Edward R, Fleet G, and Wootton M 2010 Ilmu Pangan (Bahasa Indonesia) UI-Press Jakarta

[15] USDA 2020 Agricultural Research Service United States Department of Agriculture National Nutrient Database for Standard Reference https://ndb.nal.usda.gov/ndb/foods/show/16114?fgcd=&manu=&format=&count=&max=25&offset=&sort=default&order=asc&qlookup=Tempeh&ds=&qt=&qp=&qa=&qn=&q=&i=url

[16] Mulyana, Susanto W, and Purwantiningrum 2014 Jurnal Pangan dan Agroindustri 2 113 -120

[17] Sukardi 2008 Jurnal Teknologi Pertanian 9 207 – 215

[18] Ashenaft M, and Busse M 1991 International Journal of Food Science and Technology 26 501 - 506

[19] Asmoro N W 2016 Jurnal Ilmiah Teknosains 2 66 – 72

[20] Crueger W and Crueger A 1984 Biotechnology: A Textbook of Industrial Microbiology Sinauer Tech, Inc Madison

[21] Trakarnpaiboon S, Srisuk N, Piyachomkwan K, Yang S, and Kitpreechavanich V 2017 Process Biochemistry 63 26 – 34

[22] Destina D, Widodo Y, and Tantalo S 2015 Jurnal Ilmiah Peternakan Terpadu 3 140 - 144

[23] Hutkins R 2019 Microbiology and Technology of Fermented Foods (USA: John Wiley & Sons,Inc) p 538

[24] Thanh N, Frans M, and Nout M 2007 Antonie van Leeuwenhoek 91 35 - 44

[25] Rusmin S and Ko S D 1974 Applied Microbiology 28 347 – 350