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Sago Starch as an Innovative Fermentation Aid for Tempeh Fungus (*Rhizopus oligosporus*)

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Abstract: Approximately two percent of starch used as a fermentation aid for steamed soybeans has been used for tempeh production. Three sago starches, extracted using different methods, were sourced from Jayapura, Indonesia (Sample A, wet-extraction), Kuching, Malaysia (Sample B, wet-extraction), and Cebu, Philippines (Sample C, dry-extraction). Sample C demonstrated substantially higher nitrogen, phosphate, potassium, and polyphenol content than Samples A and B. This difference may be attributed to the extraction methods used: wet versus dry-extraction. Inoculating tempeh fungus onto a starch agar medium（SA medium） containing two percent of three sago starches, extracted using different methods, led to the formation of large, rapidly developing colonies, with the largest size observed in Sample C. The dry-extraction method of sago starch, when used as a fermentation aid, was the most important factor for tempeh fungus growth in tempeh production.

Keywords: fermentation aid, fungus, tempeh, wet- and dry-extraction,

Introduction

Tempeh is a food fermented from soybeans using tempeh fungus. It is rich in plant protein (Sjamsuridzal et al., 2021). It is a national dish (Heskamp and Barz, 1997) and is widely produced and consumed in Indonesia, where people effectively utilize protein derived from soybeans. There are an estimated 81,000 tempeh producers in Indonesia, who produce 2.4 million tons of tempeh annually (Romulo and Surya, 2021). Common culinary uses for tempeh include deep frying, using it as an ingredient in vegetable stir-fries and fried noodles (mie goreng), and incorporating it into grilled chicken skewers.

The tempeh fungus, *Rhizopus oligosporus*, is native to tropical and subtropical regions and can be isolated from the inside and underside of plant leaves. Historically, natural tempeh fungus spawn was obtained by covering steamed soybeans and hibiscus leaves (*Hibiscus rosa-sinensis*) with banana leaves (Duniaji et al., 2019). With technological advancements, a mixture of wheat starch and isolated tempeh fungus is now used as a starter. In Japan, the earliest documented mention of tempeh dates back to Nakazawa and Takeda in 1928 (Shurtleff and Aoyagi, 2007). Beginning in 1983, concurrent with the soymilk boom, Japanese food companies began producing tempeh in large quantities. Shurtleff and Aoyagi (2007) reported that by early 1984, the

world's largest tempeh producers were Torigoe Flour Milling (2,623 kg per week) and Marukin Foods (2,100 kg per week). Sjamsuridzal et al. (2021) noted that tempeh production began in Indonesia in the early 1600s, and the term tempeh has been traditionally recognized since at least 1875.

Sago starch holds potential as a beneficial starter for tempeh production. This starch is extracted from the pith of the sago palm (*Metroxylon sagu*) using a wet-extraction method prevalent in most parts of Southeast Asia. In contrast, a dry-extraction method is employed in the Visayas region of the Philippines (Toyota and Okazaki, 2003; Paluga and Ragragio, 2016). The composition of sago starch includes elements such as nitrogen, phosphorus, and a small amount of sodium. This composition varies based on the extraction method. Particularly, organic nitrogen (protein) (Sorenson and Hesseltine, 1966) and organic phosphate (phytin) (Rani and Ghosh, 2011) in the starch agar medium (SA medium) are important growth factors for *Rhizopus*. In this study, we aimed to examine the relationship between the growth of tempeh fungus on SA medium and the concentrations of polyphenols and various elements in sago starch. Furthermore, the performance of tempeh produced from soybeans was assessed based on the percentages of growth areas of tempeh fungus.

Materials and Methods

1. Sago and wheat starch

Sago starch samples were obtained from Jayapura, Indonesia (Sample A, wet-extraction method), Kuching, Malaysia (Sample B, wet-extraction method), and Cebu, Philippines (Sample C, dry-extraction method) (Fig. 1).

Figures 2 and 3 illustrate the wet- and dry-extraction methods for sago starch. The wet-extraction method uses a drain under running water to obtain the sago starch (Samples A and B). In contrast, the dry-extraction method does not use water. Instead, the local people sliced the pith of the sago palm trunk, struck it, and extracted the sago starch (Sample C)

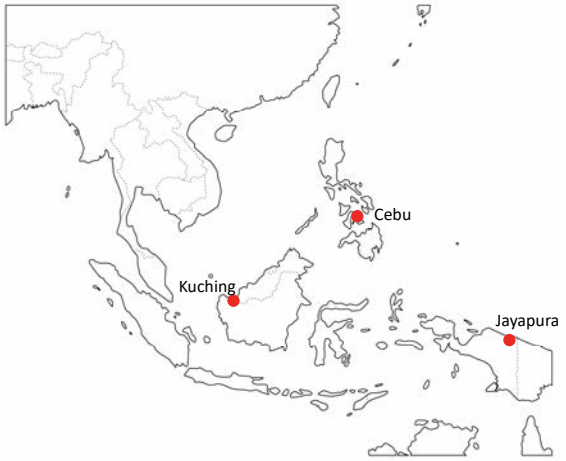

Fig. 1. Sampling sites for sago starch

Fig. 2. Wet-extraction method for sago starch

Fig. 3. Dry-extraction method for sago starch (Toyota and Okazaki, 2003)

(Toyota and Okazaki, 2003). In addition, wheat cake flour from Canada, was used as a reference (Sample D). These samples were stored at a cool temperature (5°C) prior to analysis.

2. Determination of polyphenol in starches

The polyphenol content in starch samples was determined using the colorimetric Folin–Ciocalteu method (Blainski et al., 2013; Ministry of Education, Culture, Sports, Science and Technology, 2015) after extraction with ethanol. A 1000 mg/L gallic acid standard solution was prepared by adding 100 mg of gallic acid (dried at 100°C for 1 hr) to 50% ethanol in a 100 mL volumetric flask. Four grams of the starch sample (W g) was weighed into a 250 mL centrifuge tube, homogenized with 80 mL of 50% ethanol, and centrifuged at 2500 rpm for 5 min. The supernatant was then transferred to a 250 mL volumetric flask. The residue in the centrifuge tube was vibrated for 5 min and then centrifuged again at 2500 rpm for 5 min. This procedure was repeated twice. The volumetric flask was then filled up to the 250 mL mark (V) with 50% ethanol. An aliquot of the sample was filtered using a 0.45 μm membrane filter. A standard solution was prepared from 1 to 50 μg/mL. One mL of the sample solution was mixed with 5 mL of Folin–Ciocalteu reagent, 4 mL of a 0.7 mol/L sodium carbonate solution, and 0.5 mL of ascorbate oxidase (Japan Patent Office, 2008). After thorough mixing and standing for 1 hr, the absorbance at 765 nm was measured using a spectrophotometer (Shimadzu UV-1850). For the blank solution, distilled water was used in place of the phenol reagent. The polyphenol concentration (A μg/mL) in the sample solution was determined from the calibration curve.

$$\text{Polyphenol content (g/100 g)} = (A \times V) \times D \times (100/1{,}000{,}000)/W,$$

where A: polyphenol concentration (μg/mL), V: 250 mL of ethanol solution,

D: Dilution ratio, W: starch sample (g).

Gallic acid and vanillic acid were analyzed using HPLC (Jasco UV-4070) after repeated extraction with 50% ethanol in a 1:40 ratio, followed by sonic vibration (40 W for 4 min), centrifugation (3300 rpm for 10 min), mixing with a mixer, centrifugation, and volume adjustment to 50 mL using distilled water.

3. Determination of elements

For nitrogen determination, starch samples were digested using 2 mL of salicylic sulfate and hydroperoxide. The total nitrogen content in the digested starch samples was determined by employing a 0.005 or 0.025 mol/L sulfuric acid titration method after steam distillation (using Nitrogen and Protein Distillation, MRK Nakayama Rika), followed by collection with a 4% boric acid solution containing bromocresol green and methyl red indicators. The total phosphate content was measured using a colorimeter (Shimadzu UV-1850) employing the molybdenite blue method after digestion with 60% perchloric acid (Suzuki, 1957). The total sodium content in the starch samples was measured using an atomic absorption spectrophotometer (Shimadzu AA-7000). Samples were digested using 60% perchloric acid. Sodium is of significance due to its intermediate hydration energy and substantial effective ion radius in the starch structure (Takahashi et al., 1981).

4. Starch agar medium plate and tempeh fungus inoculation

Tempeh fungus (*Rhizopus*) (Fig. 4) was obtained from tempeh powder sourced from Akita Konno Store (248, Kariwano, Daisen, Akita). The agar medium, consisting of 2% starch (2 g of starch and 1.7 g of agar dissolved in 100 mL of water: SA), was subjected to

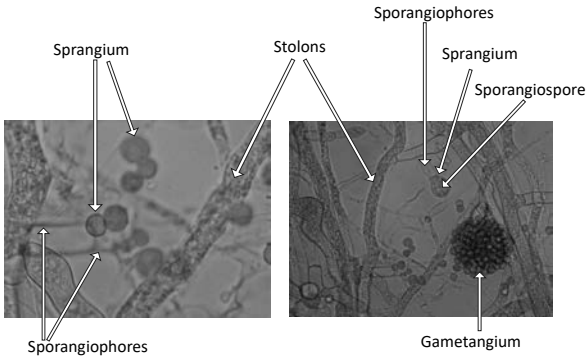

Fig. 4. Microscope images of tempeh fungus (*Rhizopus*)

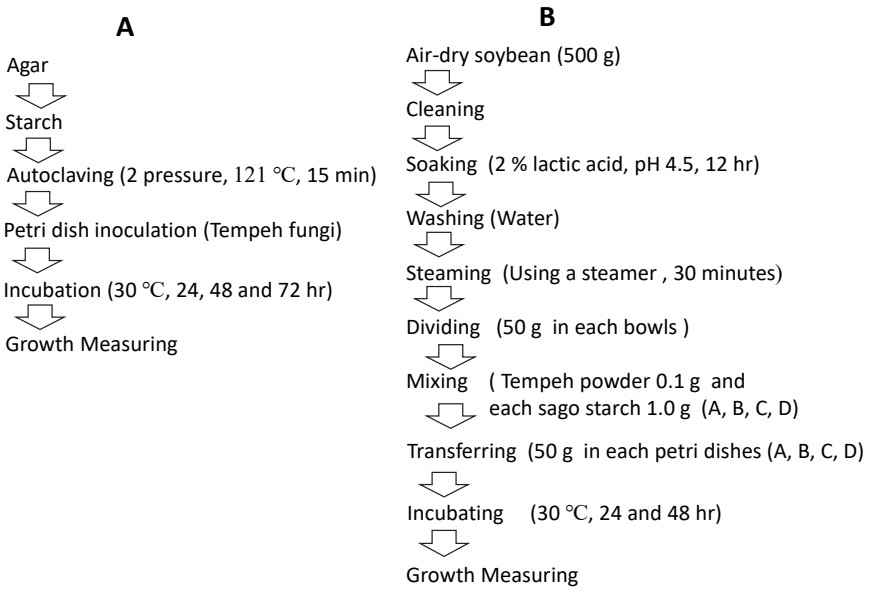

Fig. 5. Flow chart of tempeh production

heat and pressure in an autoclave at 2 atmospheres and 121°C for 15 min. After cooling to 70°C, approximately 15 mL of the medium was poured into each of three petri dishes having a diameter of 9 cm (Fig. 5A). In the same way, the three sago starches and wheat starch were used for the SA medium (n=3). It has been established that *Rhizopus* slightly hydrolyzes soybean protein, leaving mostly medium to high molecular weight compounds. In contrast, *Aspergillus* breaks down protein to low-molecular-weight products (Harayama and Yasuhira, 1989). Inoculation with the tempeh fungus was conducted by placing a sample at the center of the SA medium using an inoculation needle. The three inoculated petri dishes were then incubated at 30°C for intervals of 24, 48, and 72 hr. The growth rate of the tempeh fungus colony was determined by measuring the distance from the inoculation point at the center of the petri dish to the colony edge in four directions, with the result being given in mm (Fig. 6). The differences between Sample A and D, Sample B and D, and Sample C and D were tested by paired-samples t-test using Excel software.

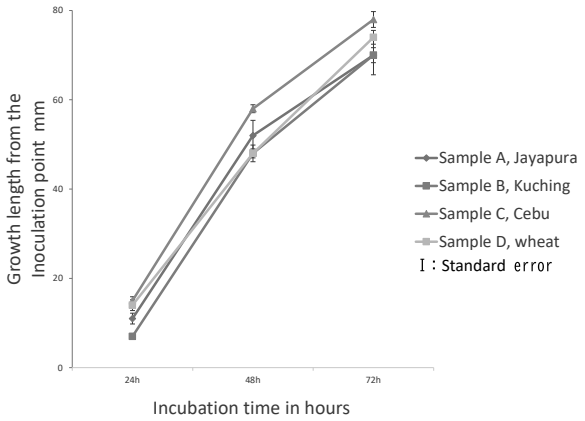

Fig. 6. Growth rate of the tempeh fungus colony on starch agar medium

5. Determining the growth area of tempeh fungus on the boiled soybean medium

Dry soybeans weighing 500 g were washed with water and soaked in a 2% lactic acid solution (pH 4.5) for 12 hr (see Fig. 5B). After soaking, the soybeans were steamed in a steamer for 30 min. After steaming, the soybeans were divided into four bowls containing 50 g each. Tempeh powder weighing 0.1 g and 1.0 g of each starch (A, B, C, D) were incorporated into the soybeans and mixed. This soybean mixture was transferred into a petri dish 9 cm in diameter, and these operations were repeated three times (n=3). After that, these petri dishes were incubated at 30°C for both 24 and 48 hr intervals (n=3).

The growth of tempeh fungus was quantified by evaluating the proportion of the 9 cm petri dish surface

covered by tempeh fungus. The coverage areas were assessed using Canopeo, an automatic color threshold (ACT) image analysis tool, developed using MATLAB programming language (MathWorks, Inc., Natick, MA, USA). This tool employs color values from the red–green–blue (RGB) spectrum to make assessments. The analysis is based on pixel selection within the image (Patrignani and Ochsner, 2015; Kumar et al., 2023). Android mobile devices can access Canopeo via the links provided on its website. The growth areas of tempeh fungus and the boiled soybean medium were distinctly discernible due to their contrasting colorations within the petri dish.

Results

1. Polyphenol and phenolic compounds in starch samples

Interactions between polyphenol and starch are prevalent in food systems and can bestow foods with unique properties and functional effects (Ngo et al., 2022). The polyphenol content of the three analyzed starches, determined by the Folin–Ciocalteu method, varied from 55.0 to 3410 mg GAE/kg (GAE: gallic acid equivalent) (Table 1). Sample D (wheat flour) contained 804 mg GAE/kg. Sample C (Cebu, Philippines), extracted using the dry method, showed an exceptionally high polyphenol content as compared to starches extracted using the wet method. When quantified by the HPLC method, gallic acid levels were 197 mg/kg for the wet extraction and 304 mg/kg for the dry extraction.

On the other hand, vanillic acid was found in Sample A (Jayapura, Indonesia, extracted with the wet-extraction method) at 4.05 mg/kg, and in Sample D (wheat) at 4.37 mg/kg.

2. Element content in starch samples

Table 1 displays the total nitrogen (N), phosphate (P_2O_5), and sodium (Na) content in the sago starch samples. The total nitrogen content in the sago starch samples ranged from 112 to 620 mg/kg. Sample C (Cebu) registered a total nitrogen content that was five times greater than the other two sago starch samples. However, Sample D (wheat) had a higher total nitrogen content than all three sago starch samples. The total phosphate content in Sample C (Cebu, extracted with the dry-extraction method) was 134 mg P_2O_5/kg, triple the content found in the other two sago samples, which were 45.1 and 46.9 mg P_2O_5/kg. However, Sample D (wheat) demonstrated a total phosphate content of 391 mg P_2O_5/kg that was significantly higher than those of all sago starches. The total sodium content in the sago starch samples spanned from 58.0 to 310 mg Na/kg, notably higher than that of Sample D (wheat), which contained 27.3 mg Na/kg.

Table 1. Polyphenols and elements in sago and wheat starches

Sample	Product Region	Exeraction Methods	Total Polyphenol* Mean (SD)	Gallic Acid*2 Mean (SD)	Vanillc Acid*2 Mean (SD)	Total N*3 Mean (SD)	Total P_2O_5*4 Mean (SD)	Total Na*5 Mean (SD)
			mgGAE*6/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
A	Jayapura Indonesia	Wet	55.0 (8.2)	197 (23)	4.05 (2.02)	115 (5)	45.1 (5.8)	58.0 (28)
B	Kuching Malaysia	Wet	143 (0.7)	132 (2)	N.D.	112 (4)	46.9 (2.9)	110 (29)
C	Cebu Philippines	Dry	341×10 (11×10)	304 (6)	N.D.	620 (2)	134 (12)	310 (13)
D	Wheat Canada	Dry	804 (129)	141 (5)	4.37 (0.16)	150×10^2 (4×10^2)	391 (42)	27.3 (6.9)

Abbreviation: SD, Standard deviation ; N.D. Not detected ; * Folin-Ciocalteu method ; *2 HPLC ; *3 Kjeldahl method
*4 Colormetry ; *5 Atomic absorption spectrophotometry ;
Averages based of three replications.

3. Sago starch as a fermentation aid for tempeh fungus

The growth of tempeh fungus on the SA medium, measured in millimeters from the inoculation point at intervals of 24, 48, and 72 hr, is presented in Fig. 6. The growth rate of tempeh fungus on Sample C (Cebu) was faster than those in Sample A (Jayapura) and Sample B (Kuching). The differences between Samples A and D (wheat), Samples B and D, and Samples C and D after 72 hr incubation, tested by the paired-samples t-test using Excel software, were significant at the level of $P<0.01$.

For Sample C, the color of the petri dish changed to a brownish hue, attributed to the pigment produced by the fluffy tempeh fungus and its sporangia, a hallmark of *Rhizopus*. While the growth curves of the tempeh fungus on both wet- and dry-extraction starch agar mediums were relatively consistent, Sample C demonstrated the most pronounced growth curve.

4. Tempeh fungus development on the boiled soybean medium

Figure 7A illustrates the progression of tempeh fungus colonies on the boiled soybean medium after 48 hr. The tempeh fungus manifested as a white, glittering (including the glint of water) mycelium, spreading uniformly across the gaps in the boiled soybeans. The areas covered by the tempeh fungus in various boiled soybean samples were as follows: 67% for Sample A (Jayapura), 45% for Sample B (Kuching), 96% for Sample C (Cebu), and 98% for Sample D (wheat) (Fig. 7B). The sago starches (Samples A, B, and C) employed as fermentation aids significantly stimulated the growth of the tempeh fungus on the boiled soybeans, as compared to Sample D. Among the sago starches, Sample C (Cebu) fostered the largest area of the three.

Discussion

1. Polyphenols and other elements in sago starch derived from the different extraction methods

The extraction methods for sago starch greatly influence its compositional elements. Many water-soluble polyphenols tend to dissolve and be lost during the starch extraction process. Polyphenols are the most abundant antioxidants in our diet (Scalbert et al., 2005). While the interaction of starch with polyphenols can enhance the functional attributes of phenolic compounds, this interaction also invariably alters the properties of starch (Ngo et al., 2022). For various growth stages of sago palm folk varieties, the

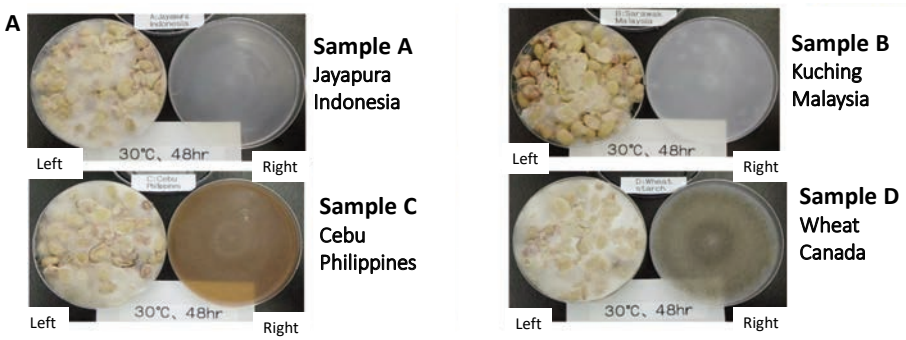

Fig. 7A. Growth areas of the tempeh fungus colony on boiled soybean plate (left) and on starch medium plate (right)

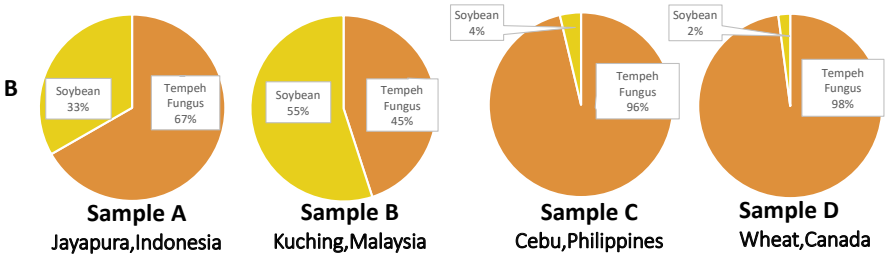

Fig. 7B. Growth rate (%) of the tempeh fungus colony on boiled soybean plate (Fig. 7A, left)

composition of associated components (extracted via the wet method) revealed that the amount of phenolic compounds was below 1% (10 mg/g) (Pei-Lang et al., 2006). Konuma et al. (2013) also reported that the total phenolic content (TPC) of sago starch ranged from 0.063 to 0.119 mg CE/g (CE: catechin equivalent) with a mean value of 0.089 mg CE/g (25.8 mg/g) for Ronpiboon and 0.61 to 0.183 mg CE/g with a mean value of 0.115 mg CE/g (33.4 mg/g) for Kreng. One of the factors for the fastest *Rhizopus* growth, as compared to Samples A and B, might be shown by the high polyphenol content in the sago starch of Sample C (Cebu).

In fermentation processes with *Rhizopus*, organic and inorganic nitrogen are the most crucial elements. *Rhizopus* fermentation often results in a noticeable reduction in protein nitrogen and an increase in ammoniacal-nitrogen and amino-nitrogen levels in soybeans (Nowak and Szebiotko, 1992). Harayama and Yasuhira (1989) reported that *Rhizopus* produces primarily neutral and acidic proteases, which are influenced by the C/N ratios in the substrates, and that soybeans fermented by *Rhizopus* tended to exhibit higher soluble protein content than those fermented by *Aspergillus*.

Rhizopus may have a remarkable ability to utilize total phosphate, based on the growth results on Sample C (Cebu) and D (wheat) media. The growth areas on boiled soybean plates were almost covered by the tempeh fungus colony (Fig. 7). According to Table 1, Sample C (Cebu) was 134 mg P_2O_5/kg, and Sample D (wheat) was 391 mg P_2O_5/kg. On the other hand, Sample A (Jayapura) was 45.1 mg P_2O_5/kg, and Sample B (Kuching) was only 46.9 mg P_2O_5/kg.

Regarding 0.5~4% sodium content, Uyar and Uyar (2016) noted that increased sodium concentrations may hinder *Rhizopus* growth. However, in our study, the *Rhizopus* growth on the SA medium of Sample C (Cebu) might not have been suppressed by slightly higher medium concentrations than those of Samples A and B because the concentration of sodium was less than sufficient to influence *Rhizopus* growth.

2. Tempeh fungus growth

The growth of *Rhizopus* on SA medium and boiled soybeans is influenced by multiple factors, with particular emphasis on nitrogen and total phenol levels. Nowak and Szebiotko (1992) highlighted that fermentation resulted in a marked decrease in protein levels while augmenting ammonium–nitrogen and amino–nitrogen concentrations in soybeans. Aoki et al. (2020) analyzed isoflavone aglycone (IS), total polyphenols, and low-molecular-weight soluble dietary fiber (LMWSDF) across *Rhizopus* strains; *R. oligosporus*, *R. oryzae*, and *R. stolonifer*. Their findings indicated that tempeh produced by *R. stolonifer* showcased a composition of 0.065 g IS/100g, 2.5 g LMWSDF/100 g, and 0.35 g polyphenol/100 g. Rani and Ghosh (2011) demonstrated that phytase production occurred during solid-state fermentation using *R. oryzae*. Uyar and Uyar (2016) suggested that the growth trajectory of *R. oryzae* and its lactic-acid production in a liquid medium was contingent on NaCl and KCl concentrations and decreased as salt levels increased. Vincent et al. (2020) cultivated *R. oligosporus* in sago effluent across several initial pH levels (namely pH 4, 5, and 6) and posited that an initial pH of 4 was most conducive for protein production in fungal biomass.

R. oligosporus has a wide range of assimilation abilities and holds the vital activity for many kinds of media (such as sago and wheat starch).

Conclusion

Extraction methods play a pivotal role in determining the compositional components of sago starch. Samples obtained with the dry-extraction method (Sample C, Cebu) retained a greater quantity of beneficial components as compared to samples obtained by the wet-extraction method (Samples A and B, Jayapura and Kuching), which brought a faster initial growth of *R. oligosporus* on the agar medium as a starter. *R. oligosporus*, derived from dry extraction (Sample C, Cebu), developed optimally on soybeans, leading to a vibrant and lively tempeh.

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