Solid-state fermentation of sterile slurry and palm kernel cake (PKC) mixture using *Rhizopus azygosporus* UICC 539

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**Abstract.** This study described solid-state fermentation of sterile slurry and palm kernel cake (PKC) mixtures by *Rhizopus azygosporus* UICC 539, preparation of the fungus and waste mixture as a formula for animal feed, and analysis of nutrient content of the formula. Preparation of inoculum (v/v) in Potato Sucrose Broth (PSB) was carried out at 30 and 40 °C for 5 days. Wet weight biomass was used as inoculum for solid-state fermentation (SSF) using sterile slurry and PKC (3:1) mixtures and SSF was carried out in flat trays (20×20×5 cm) at 30 and 40 °C for 5 days. The fermented waste mixture was dried at 60 °C for 5 days. Changes in nutrient content of the formula were observed by comparing the treatment and control. Formula prepared at 30 °C showed an increase in carbohydrate, protein and moisture content. A decrease was observed in total fat and ash content, calorie from fat and total calorie. Formula prepared at 40 °C showed an increase in protein and ash content. A decrease was observed in carbohydrate, moisture and total fat content, calorie from fat, and total calorie. *Rhizopus azygosporus* UICC 539 was able to grow and utilize the palm oil processing waste and improved the nutrient content of the formula.

**Keywords:** nutrient content; *Rhizopus azygosporus*; sterile palm kernel cake; sterile slurry

**1. Introduction**

Indonesia produced 37.5 million tons of palm oil from oil palm plantation areas of 11.3 million hectares [1]. Wastes resulted from palm oil processing industry still have economical values as they can be used as cheap raw materials. Since the wastes are rich in carbohydrates and other nutrients, they can serve as substrates for biological treatment [1,2]. The palm oil processing industry also provides by-products feeds (palm press fibre, palm oil mill effluent, palm oil mill effluent (POME), and palm kernel cake, PKC) which can be used by ruminants [3].

POME is a thick brownish yellow colloidal slurry of water (95 %) with average pH about 4.7. POME has high compositions and concentrations of carbohydrate, protein, nitrogenous compounds, lipids and minerals which render it possible to reuse as a possible feedstock for substrate microbial bioconversion. The utilization of POME as animal feed could be enhanced further by addition of other oil palm by-products [4]. PKC has nutritional value and has been accepted as one of the components in...
animal feeds. Combining POME with PKC could create a low-cost and excellent feeding system for ruminants [3,4].

In recent years, solid-state fermentation (SSF) has received more and more interest from researchers for the value-addition of tropical agricultural by-products [2]. SSF is defined as any fermentation process performed on a non-soluble material in which microorganisms are grown on solid substrates in the absence of water [5]. One type of SSF process to perform is using flat trays. The substrate is spread onto each tray forming a thin layer, only a few centimetres deep. The process is kept in a chamber at constant temperature [2]. In this process, the solid substrate not only supplies the nutrient to the culture but also serves as an anchorage for microbial cells [6].

Fungal growth under SSF has been found to be more suitable for low technology applications and there is hardly any waste disposal at the end of the process because the whole product may be used directly in animal feeds [7]. Rhizopus sp. produces mycelium biomass which is a valuable product that tolerant to the lignocellulosic hydrolysates inhibitory [8]. Good growth of Rhizopus microsporus was reported on mixtures of slurry and PKC [9]. The fermented PKC by Rhizopus stolonifer showed reduction in the crude fibre contents and lipid content [5].

A collection of Rhizopus spp. from tempeh samples from various regions of Indonesia was deposited in Universitas Indonesia Culture Collection (UICC). This study described solid-state fermentation of sterile slurry and PKC mixtures by Rhizopus azygosporus UICC 539, preparation of the fungus and waste mixture as a formula, and analysis of nutrient content of the formula.

2. Materials and Methods

2.1 Microorganism and growth conditions

Rhizopus azygosporus UICC 539 was originally isolated from soybean tempeh from Mataram, Lombok Island by Universitas Indonesia Culture Collection (UICC), Department of Biology, FMIPA Universitas Indonesia. Potato Dextrose Agar (PDA, Difco) containing 0.02 % (w/v) chloramphenicol (Wako) was used for maintenance of the strain. Five days old-culture in Potato Sucrose Agar (PSA) plates [10] added with 0.02 % chloramphenicol at 30 °C was used for colony disc as a inoculum. Colony disc was prepared by cutting the colony using sterile plastic straw (diameter 6 mm) [11]. Growth temperatures of the fungus was observed by transferring the agar discs onto PSA plates and incubated at 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, and 60 °C for 5 days. The experiment was performed in triplicate.

2.2 Preparation of inoculum and liquid-state fermentation

The culture was prepared from fungal growth on PSA slants at 30 °C and 40 °C for 5 days. Cell suspension as inoculum was prepared according to Prameswari et al. [9] by adding 5 mL sterile distilled water into the PSA slants and scraping the colony surface to obtain the mycelia and spores. The cell inoculum (5.68×10^5 CFU/mL) and (2.67×10^4 CFU/mL) were obtained from 30°C and 40°C cultures, respectively. The inoculum (15 % v/v) was transferred aseptically to a final volume of 25 mL Potato Sucrose Broth (PSB) in 200 mL Erlenmeyer flasks and incubated at 30 °C and 40 °C for 2 days without shaking. The 2 days-old cultures in PSB which contained 3.9×10^5 CFU/mL from 30 °C, and 2×10^5 CFU/mL from 40 °C, were used as inoculum (25 % v/v) and transferred to 75 mL PSB in 250 mL Erlenmeyer flasks to give a final volume of 100 ml, and incubated at 30 °C and 40 °C for 3 days without shaking. The experiments were performed in triplicate for each temperature.

2.3 Solid-state fermentation (SSF) and preparation of formula

Slurry and palm kernel cake (PKC) were obtained from P.T. Agricinal in Seblat-Putri Hijau, North Bengkulu, Sumatra. Mixtures of slurry and PKC (3:1) were weighted to final weight of 400 g and autoclaved for 15 min at 15 psi, 121 °C. The culture broth in PSB from 30 °C and 40 °C were filtered to separate the medium using filter paper Toyo Advantec number 5C (diameter 90 mm). The wet biomass obtained from 30 °C and 40 °C were transferred to the sterile waste mixtures in flat trays
(20×20×5 cm) and mixed using spatulas. Sterile waste mixtures added with sterile distilled water without the fungal cells served as controls. The trays were incubated at 30 °C and 40 °C for 5 days. Characteristics of the waste mixtures during SSF were determined on pH, fungal growth, mycelia (biomass) coverage, and biomass colour according to Faber-Castell colour chart. Mycelial coverage (%) was determined as follows [9]: (colony area/tray area) ×100%. The dried formula was obtained by drying the waste mixtures from SSF in the incubator at 60 °C to constant weight, then cooled and weighed. The dried formula was ground into smaller particle sizes using table blender (BL-151 PF-AP, Miyako Indonesia); particles size of 0.2 to 1.5 mm. Enumeration of cell concentration in the formula was carried out using total plate count (TPC).

2.4 Analysis of nutrient composition of dried formula
Analysis of carbohydrate, protein, water content, total fat content, ash content, fat calorie and total calorie in the formula and control were carried out at P.T. Saraswanti Indo Genetech, Bogor, according to Standar Nasional Indonesia (SNI 01-2891-1992, Cara uji makanan dan minuman).

3. Results and Discussions

Rhizopus azygosporus UICC 539 colonies in PSA plates showed good growth at 30 °C, 35 °C, and 40 °C by covering the whole surface of the plates after 5 days of incubation, while colony growth at 45 to 50 °C was restricted. Growth was not observed at 55 °C and 60 °C. Higher temperatures above 50 °C seemed to cause inhibition of the fungus growth. Full sporulation was observed in blackish grey at 30 °C and 40 °C after 5 days of incubation. These temperatures were then selected for preparation of the inoculum for liquid-state fermentation in PSB. Zheng et al. [12] reported that colonies of R. microsporus var. azygosporus on potato dextrose agar (PDA) reached 9 cm diameter in 3 to 4 days at 30 °C. The colony at first was white, then grey, and blackish grey when mature. This species has a maximum growth temperature at 49 to 51 °C.

Rhizopus azygosporus UICC 539 showed good growth in liquid-state fermentation in PSB at 30 °C and 40 °C (Figure 1). The fungus formed mycelial biomass and covered the liquid medium surface at both temperatures within 2-3 days. Inoculum for 400 g final weight of sterile waste mixture was obtained as wet biomass from PSB at 30 °C (13.44 g, 3.25 % w/v) and from PSB at 40 °C (11.43 g, 2.77% w/v). Higher amount of wet biomass was obtained from culture grown at 30 °C compared to 40°C, which indicated that more mycelial biomass was achieved at 30 °C than at higher temperature. Nout and Kiers [13] reported that incubation at ambient temperature (25 to 30 °C) allows spore germination and luxuriant growth of mycelium. During fermentation, the temperature will increase to 40 to 50 °C since fungal metabolic activity releases considerable heat.

Growth of R. azygosporus UICC 539 in liquid-state fermentation in PSB at 30 °C and 40 °C after 5 days of incubation was accompanied by a decrease in the pH of the medium from the initial pH 6-7 to 6. There were reports that species of Rhizopus have more acidic optimum pH during growth [14,15]. In this study, a decrease of pH of the growth medium indicated that R. azygosporus UICC 539 secreted organic acid(s) into the medium. According to Abe et al. [16] members of the genus Rhizopus could be divided into fumaric acid producers, lactic acid producers and producers of both fumaric and lactic acids. According to Liaud et al. [17] the consecutive decrease in pH upon organic acid secretion may give a competitive advantage to the acid-tolerant filamentous fungi. Organic acids produced by fungi may have many roles such as in nutrient acquisition, and/or participation in metal detoxification by metal complexion.

The sterile waste mixtures (slurry:PKC = 3:1) had acidic pH 4.5 and brownish colour. Growth of R. azygosporus UICC 539 in SSF in the sterile waste mixtures at 30 °C and 40 °C was accompanied by an increase in the pH of the waste mixtures from 4-5 to 6 (Table 1 and Table 2). Good growth of R. azygosporus UICC 539 was shown on the waste mixtures at 30 °C and 40 °C. However, mycelium coverage on the waste mixtures reached 100 % and full sporulation at 30 °C after 3 days of incubation, which was higher than mycelium coverage (97.5 %) and full sporulation at 40 °C after 5 days of
incubation. This result showed that the waste mixtures with acidic pH was a good substrate for the growth of *R. azygosporus* UICC 539. Wu *et al.* [4] reported that POME includes various liquids, dirt, residual oil and suspended solids, mainly cellulosic material from the palm fruits. Suyala *et al.* [18] reported that POME contained high C/N ratio, and high organic matter (36,000 g/L COD, 10 g/L oil) but was low in nitrogen content (1.04 g/L). Prasertsan and Binmaeil [15] reported that POME had acidic pH (4.5). Cultivation of *Rhizopus oryzae* in POME at the optimum temperature of 45 °C for 5 days showed slightly increased pH from 4.5 to 5.2-5.5. FazeliNejad *et al.* [8] reported that treatment of lignocellulosic material with the *Rhizopus* sp. at low pH and high temperature allows for sufficient enzymatic decomposition of the cellulose into monomers.

During SSF of the waste mixtures by *Rhizopus azygosporus* UICC 539, it was indicated that the fungus was able to degrade cellulosic wastes and oil contained in the slurry and PKC, and protein contained in PKC, as shown by good growth and observation on mycelial biomass (Figure 2). *Rhizopus oryzae* have been reported to produce enzymes such as cellulase, amylase, lipase, and protease, respectively [19]. *Rhizopus oligosporus* was reported to liberate a large range of water-soluble high molecular weight oligosaccharides by enzymic degradation of polysaccharides by major carbohydrases (endocellulase and xylanase) during tempeh fermentation [13]. During solid-state fermentation of waste agricultural residues of grain crops by *Rhizopus oligosporus*, the fungus produced various enzymes that hydrolysed the raw materials and changed their textures, taste and aroma [20].

The dried formula of *R. azygosporus* UICC showed that the fungus was still viable after the drying process at 60 °C for 5 days (Figure 2). However, the formula showed a decrease of cell number after drying (3.3×10⁴ CFU/g) compared to the cell number before drying (1.5×10⁷ CFU/g) for formula prepared at 30 °C. Similar observation was shown for formula prepared at 40 °C, which showed a decrease of cell number after drying (3.3×10⁴ CFU/g) compared to before drying (6.67×10⁶ CFU/g). This result indicated that drying temperature influenced the viability of the fungal cells. It was noteworthy that formula prepared from cells which was incubated at higher temperature (40 °C) showed higher cell viability compared to formula prepared at lower temperature. Cell viability at higher temperature indicated that it could tolerate high temperature due to adaption to higher temperature during growth. Nout and Kiers [13] reported that the viability of *Rhizopus oligosporus* as starter for tempeh, in dried pulverized powder (aw 0.48) remained high (ca 10⁷ CFU/g) up to 30 weeks at 5 °C, and 25 °C. The viable spores only represented 5 % of the total spores present in rice-grown starter. The remaining spores were not dead, but were present in a dormant state.

**Figure 1.** Growth of *R. azygosporus* UICC 539 in liquid-state fermentation in PSB.
Table 1. Characteristics of the sterile waste mixtures during solid-state fermentation by *R. azygosporus* UICC 539 at 30 °C after 5 days of incubation.

| Day | pH control | pH treatment | Colour of waste mixture (control) | Colour of waste mixture (treatment) | Mycelial coverage (%) |
|-----|------------|--------------|-----------------------------------|------------------------------------|-----------------------|
| 0   | 4.5        | 4.5          | Sepia light                       | Waste mixture: van dyck brown      | 0                     |
|     |            |              |                                   | *R. azygosporus* colony: no growth |                       |
| 2   | 4.5        | 4.5          | Sepia light                       | Waste mixture: van dyck brown      | 32                    |
|     |            |              |                                   | *R. azygosporus* colony: white     |                       |
| 3   | 4.5        | 4.5          | Sepia light                       | Waste mixture: van dyck brown      | 100                   |
|     |            |              |                                   | *R. azygosporus* colony: cold grey IV |                     |
| 4   | 5.5        | 5.5          | Sepia light                       | Waste mixture: van dyck brown      | 100                   |
|     |            |              |                                   | *R. azygosporus* colony: cold grey IV |                     |
| 5   | 6          | 6            | Sepia light                       | Waste mixture: van dyck brown      | 100                   |
|     |            |              |                                   | *R. azygosporus* colony: cold grey IV |                     |

Table 2. Characteristics of the sterile waste mixtures during solid-state fermentation by *R. azygosporus* UICC 539 at 40 °C after 5 days of incubation.

| Day | pH control | pH treatment | Colour of waste mixture (control) | Colour of waste mixture (treatment) | Mycelial coverage (%) |
|-----|------------|--------------|-----------------------------------|------------------------------------|-----------------------|
| 0   | 4-5        | 4-5          | van dyck brown                    | Waste mixture: van dyck brown      | 0                     |
|     |            |              |                                   | *R. azygosporus* colony: no growth |                       |
| 1   | 4-5        | 4-5          | van dyck brown                    | Waste mixture: van dyck brown      | 3                     |
|     |            |              |                                   | *R. azygosporus* colony: no growth |                       |
| 3   | 4-5        | 4-5          | van dyck brown                    | Waste mixture: van dyck brown      | 78                    |
|     |            |              |                                   | *R. azygosporus* colony: white     |                       |
| 4   | 4-5        | 5-6          | van dyck brown                    | Waste mixture: van dyck brown      | 91.5                  |
|     |            |              |                                   | *R. azygosporus* colony: warm grey I |                     |
| 5   | 6          | 6            | van dyck brown                    | Waste mixture: van dyck brown      | 97.5                  |
|     |            |              |                                   | *R. azygosporus* colony: warm grey III |                     |

Changes of nutrient content of the formula before and after growth of *R. azygosporus* UICC 539 is shown in Table 3. Growth of the fungus on the waste mixture at 30 °C changed the nutrient content of the formula as shown by an increase in carbohydrate, protein and moisture content, compared to the control. A decrease was observed in total fat and ash content, calorie from fat and total calorie compared to the control. Growth of the fungus on the waste mixtures at 40 °C showed an increase in protein and ash content, compared to the control. A decrease was observed in carbohydrate, moisture and total fat content, calorie from fat, and total calorie compared to the control. The waste mixture as a substrate was rich in carbohydrate, lipid and protein. This substrate was fermented and utilized by *R. azygosporus* UICC 539 for the fungus metabolism and growth. The increased protein content of the formula prepared at 30 °C and 40 °C was probably due to the consequence of the mycelial growth. According to Laconi and Jayanegara [21] treatment of agricultural by-product by white rot fungi may increase digestability of a feed, however, there was a loss of yield or biomass since the fungi require and utilize part of the feed nutrients for their metabolism and activity. Belewu and Babalola [20] reported that solid-state fermentation of waste agricultural residues of grain crops by *Rhizopus oligosporus* increased the limiting nutrients, e.g. proteins and minerals for ruminant animals. The process also reduced or eliminated the anti-nutritive components in the fermented products.
Figure 2. Solid-state fermentation of the waste mixture by *Rhizopus azygosporus* UICC 539, and the formula.

**Table 3.** The composition of sterile waste mixtures before and after growth of *Rhizopus azygosporus* UICC 539 at 30 °C and 40 °C for 5 days incubation.

| Parameter                | Original non-sterile waste mixture (control) | Treated non-sterile waste mixture |
|--------------------------|---------------------------------------------|-----------------------------------|
|                          | 30 °C           | 40 °C           | 30 °C           | 40 °C           |
| Carbohydrate             | 66.37 %         | 68.13 %         | 66.67 %         | 67.67 %         |
| Protein content          | 14.70 %         | 14.37 %         | 16.60 %         | 16.27 %         |
| Moisture content         | 6.36 %          | 5.62 %          | 6.43 %          | 5.42 %          |
| Ash content              | 5.22 %          | 5.32 %          | 5.20 %          | 5.72 %          |
| Total fat content        | 7.35 %          | 6.56 %          | 5.10 %          | 5.42 %          |
| Calorie from fat         | 66.15 Kcal/100g | 59.04 Kcal/100g | 45.90 Kcal/100g | 44.28 Kcal/100g |
| Total calorie            | 390.43 Kcal/100g | 389.04 Kcal/100g | 378.98 Kcal/100g | 380.04 Kcal/100g |

| Parameter    | Value differences % Increase or decrease |
|--------------|-----------------------------------------|
|              | 30 °C         | 40 °C         | 30 °C         | 40 °C         |
| Carbohydrate | + 0.30        | - 0.46        | 0.45 increase | 0.68 reduction |
| Protein content | + 1.90      | + 1.90        | 12.93 increase | 13.22 increase |
| Moisture content | + 0.07      | - 0.20        | 1.10 increase | 3.56 reduction |
| Ash content     | - 0.02        | + 1.14        | 0.38 reduction | 17.38 increase |
| Total fat content | - 2.25      | - 0.40        | 30.61 reduction | 7.52 reduction |
| Calorie from fat | - 20.25      | - 14.76       | 30.61 reduction | 25 reduction |
| Total calorie    | - 11.45       | - 9           | 2.93 reduction | 2.31 reduction |
4. Conclusion

In conclusion, *R. azygosporus* UICC 539 was able to grow and utilize the sterile waste mixtures during solid-state fermentation. The fungus was potential in improving the nutrient content of the agricultural by-product from palm oil processing waste. Future studies will be required to optimize bioprotein production using this fungus.

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