Effect of *Rhizopus azygosporus* UICC 539 growth on the nutrient content of sterile slurry and palm kernel cake mixtures at different temperature

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Abstract. This study reported the effect of *Rhizopus azygosporus* UICC 539 growth on the sterile slurry and palm kernel cake (PKC), and analysis of the nutrient content of the waste mixtures. The fungus showed good growth on Potato Sucrose Agar (PSA) at temperature range of 30 to 50 °C, and could not grow at 55 °C. Inoculum was prepared from fungal culture in Potato Sucrose Broth (PSB) by still fermentation at 30 and 40 °C for 5 days. Sterile slurry and PKC (3:1) in Petri dishes (diameter 9 cm) were prepared and solid-state fermentation was carried out using inoculum (10 %, v/v) at 30 and 40 °C, and incubated for 5 days. *Rhizopus azygosporus* UICC 539 showed good growth and increased cell numbers on sterile waste mixtures. Effect of fungal growth on the nutrient content of the waste mixtures was observed by comparing the treatment and control. There was an increase in the moisture and ash content, and a decrease in protein content, total calorie and carbohydrate content. There was no change of the calorie from fat and total fat content compared to the control.

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

1. Introduction
Palm oil from the oil palm plant (*Elaeis guineensis*) is one of the most important commodities in Indonesia. Sumatra and Kalimantan are the largest oil palm areas in Indonesia [1]. Palm oil mill generates liquid and solid wastes in large quantities. Palm oil mill effluent (POME) is a brown slurry, which composed of 4 to 5 % solids, 0.5 to 1 % residual oil and 95 % water [2]. Palm kernel cake (PKC) is obtained from oil extraction of palm fruit. PKC contains 10 to 17 % crude fibre and 35 % mannan [3]. PKC and POME are acceptable to most ruminants [4].

Treatment of agricultural waste can add value to waste [2]. Solid-state fermentation (SSF) offers numerous opportunities in processing agricultural residues. In SSF the activity of microorganisms is increased since SSF is closer to their natural habitat [5]. The solid matrix becomes the source of nutrients and support the development of the microorganisms. The cultivated microorganisms in SSF can contribute in the disposal of agricultural residues and produce useful value-added products [6].

The microorganisms used in SSF can occur as single pure cultures. Molds are frequently used in SSF for maximizing production of value-added products. *Rhizopus* strains have been used in SSF process using waste material, e.g. tempeh production, since they have abilities to degrade the raw
material [7]. *Rhizopus microsporus* var. *oligosporus* was able to utilize agricultural agro-industrial wastes as substrates [8]. *Rhizopus microsporus* was reported to show good growth on sterile palm oil processing waste [9].

*Rhizopus microsporus* var. *azygosporus* was introduced as a new species, *R. azygosporus* [10]. Universitas Indonesia Culture Collection (UICC) has a collection of *Rhizopus* spp. from tempeh samples from various regions of Indonesia. *Rhizopus azygosporus* UICC 539 was originally isolated from soybean tempeh from Mataram, Lombok Island [data unpublished]. The effect of *Rhizopus azygosporus* UICC 539 growth on sterile slurry and palm kernel cake (PKC) through solid-state fermentation at different temperatures, and analysis of nutrient content after fermentation, was reported in this study.

2. Materials and Methods

2.1 Microorganism and growth temperatures

*Rhizopus azygosporus* UICC 539 was maintained on Potato Dextrose Agar (PDA, Difco) containing 0.02 % (w/v) chloramphenicol (Wako). Fungal culture was inoculated on Potato Sucrose Agar (PSA) plates [11], added with 0.02 % chloramphenicol and incubated at 30 °C for 5 days. Colony disc containing the fungal culture was prepared with modification from Mascarin et al. [12], by cutting the agar from 5 days old culture using sterile plastic straw (diameter 6 mm). Temperature growth of the fungus was observed by transferring the agar blocks onto PSA plates and incubated at 30°, 35°, 40°, 45°, 50°, 51°, 52°, 53°, 54°, 55°, and 60 °C for 5 days. The experiment was performed in five replications.

2.2 Preparation of inoculum and liquid-state fermentation

The fungus was grown on PSA slants at 30 °C and 40 °C for 5 days. Cell suspension as inoculum was prepared according to Prameswari et al. [9]. The surface of 5 days-old culture was scraped to dislodge mycelia and spores. The cells were added with 5 mL sterile distilled water to obtain inoculum of 5.6×10⁵ CFU/mL from 30 °C culture, and 2.6×10⁴ CFU/mL from 40°C culture. 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. Inoculum (25 % v/v) was prepared from 2 days-old cultures in PSB with cell concentrations of 3.9×10⁵ CFU/mL from 30 °C, and 1.3×10⁵ CFU/mL from 40 °C, 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)

Slurry and palm kernel cake (PKC) were obtained from P.T. Agricinal in Seblat-Putri Hijau, North Bengkulu, Sumatra. The process of SSF was followed as described by Prameswari et al. [9]. Mixtures of slurry and PKC (3:1) were weighted to 18 g and autoclaved for 15 min at 15 psi pressure, 121 °C. After cooling, the sterile waste mixtures were placed in petri dishes (diameter 90 mm) and inoculated with 2 g of inoculum (10 % v/w) from 3 days-old cultures in PSB at 30 °C and 40 °C to give a final weight (20 g). The mixtures were mixed using spatulas. Sterile distilled water without fungal cultures served as controls. The petri dishes were incubated at 30 °C and 40 °C for 5 days. Characteristics of the fermented waste mixtures were determined on pH, fungus growth, mycelial (biomass) coverage, and biomass colour according to Faber-Castell colour chart. Mycelial coverage (%) was determined according to the formula [9]: (colony area/tray area)×100 %. The experiment was performed in five replications.

2.4 Analysis of nutrient composition of SSF

The treatment and control were analysed for nutrient composition (carbohydrate, protein, water content, total fat content, ash content, fat calorie and total calorie) according to Standar Nasional
3. Results and Discussion

*Rhizopus azygosporus* UICC 539 showed good growth at 30 to 40 °C as shown by blackish grey colonies in full sporulation on the PSA plates after day-5 of incubation (Figure 1). Colony growth was restricted at 45 to 50 °C which indicated that *R. azygosporus* has thermo-tolerant nature. At 51 to 55 °C and 60 °C there was no growth, which indicated that the fungus was inhibited at higher temperatures above 50 °C. Good growth was observed at 30 °C and 40 °C, these temperatures were selected for inoculum preparation in liquid-state fermentation in PSB. According to Zheng *et al*. [13] the maximum growth temperature range of *Rhizopus microsporus* and its allies on Potato Dextrose Agar (PDA) are 40 to 51 °C.

![Figure 1. Growth of *R. azygosporus* UICC 539 in Potato Sucrose Agar (PSA) plates, after 5 days of incubation at various temperatures.](image)

*Rhizopus azygosporus* UICC 539 showed good growth in liquid-state fermentation in PSB at 30 °C and 40 °C (Figure 2). Fungal culture in PSB at 40 °C showed luxuriant mycelial growth compared to 30 °C. Dolatabadi *et al*. [14] reported that the optimum temperature for growth of *Rhizopus microsporus* was in the range 36 to 40 °C. Nout and Kiers [15] reported that the optimum condition for biomass production of *R. microsporus* var. *microsporus* and *R. microsporus* var. *oligosporus* on a model medium were 40 °C.

A decrease in the pH of medium from the initial pH 6-7 to 6 was observed during *R. azygosporus* growth in PSB, which indicated that the fungus produced organic acid(s) during growth. According to Abe *et al*. [16] *Rhizopus* spp. could be divided into fumaric acid producers, lactic acid producers, and producers of both fumaric and lactic acids. Sparringa *et al*. [17] reported that the pH optimum for *R. oligosporus* was from 5.5 to 5.8.

*Rhizopus azygosporus* UICC 539 showed good growth in solid-state fermentation on sterile waste mixture plates at 30 °C and 40 °C, after 5-days of incubation (Figure 3). Growth of *R. azygosporus* was supported by the nutrients in the slurry and PKC. The fungus colonies were greyish black at 30 °C which indicated aerial mycelia had reached full sporulation, compared to greyish white colonies at 40 °C which indicated the aerial mycelial had not reached full sporulation yet. However, more abundant aerial mycelia were shown by *Rhizopus azygosporus* at 40 °C which indicated accelerated aerial mycelial growth at higher temperature, but slower sporulation. This result indicated that although *R. azygosporus* has a thermo-tolerant nature, however, 30 °C was a favourable temperature for the fungus.
to utilize the nutrients in the palm waste mixture leading to faster sporulation and producing greyish black colonies. According to Biesebeke et al. [18] during SSF the fungal hyphae effectively colonise and penetrate the solid substrate. During the process the fungus respiration has a temperature optimum between 30 and 35 °C. When abundant aerial mycelium is formed in *Rhizopus oligosporus*, a diffusion of oxygen is limited in SSF in the layer of densely packed fungal hyphae leading to limited fungus growth in the substrate particle.

**Figure 2.** Growth of *R. azygosporus* UICC 539 in liquid-state fermentation in PSB at 30 °C and 40 °C. (a) Culture in 25 mL PSB, 30 °C, after 2 days, (b) Culture in 100 mL PSB, 30 °C, after 3 days, (c) Culture in 25 mL PSB, 40 °C, after 2 days, and (d) Culture in 100 mL PSB, 40 °C, after 3 days.

**Figure 3.** Growth of *R. azygosporus* UICC 539 in solid-state fermentation on sterile waste mixture plates at 30 °C and 40 °C, after 5 days of incubation. (a) Fungal cultures on sterile waste mixture plates at 30 °C, (b) Fungal cultures on sterile waste mixture plates at 40 °C.

The sterile waste mixtures (slurry: PKC = 3:1) had acidic pH 5 and brownish colour (Table 1 and Table 2). No change of pH (5) was shown by the control at 30 °C, while there was a slight change of pH from 5 to 4-5 at 40 °C. Fungus growth on the waste mixture caused a decrease of pH from 5 to 4-5 during SSF. The acidic character of POME has been reported. POME becomes acidic due to the corrosion of iron used during processing [19]. The culture medium pH may change as a result of the metabolic activities of microorganisms [8]. Mycelium during fungal metabolism in SSF secreted some metabolites, including organic acids [18]. Organic acids secretion may cause a decrease in the culture medium of *R. oligosporus* [8].

*Rhizopus azygosporus* UICC 539 caused changes of nutrient content of the waste mixtures before and after growth (Table 3 and Table 4). Growth of the fungus at 30 °C and 40 °C caused an increase in the ash and moisture content, and a decrease in protein content, total calorie and carbohydrate content. Meanwhile, there was no change of the calorie from fat and total fat content compared to the control.
Slurry and PKC were complex substrates and provide carbon source and energy for *Rhizopus azygosporus* UICC 539 during SSF. There were reports that members of *Rhizopus* showed amylolytic activity [20], proteolytic activity [21], lipolytic activity [22], and cellulolytic activity [23]. These abilities to degrade different substrates may lead to the decrease of carbohydrate and protein contents of the waste mixtures due to the metabolic activities of the fungus. This was also an indication that the fungus could improve the nutritive contents of the fermented waste mixtures. Increase in ash content was in line with report from Falaye *et al.* [24] when *Rhizopus microsporus* was grown on PKC. The fibre fractions and nutritive content of fermented PKS was improved by *R. microsporus* by degrading the lignin and hemicellulose in PKS.

**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 fungus (treatment) | Mycelial coverage (%) |
|-----|------------|--------------|-----------------------------------|-------------------------------|-----------------------|
| 0   | 5          | 5            | Van dyck brown                    | No growth                     | 0                     |
| 2   | 5          | 5            | Van dyck brown                    | No growth                     | 0                     |
| 3   | 5          | 4-5          | Van dyck brown                    | Cold grey III                 | 72.98                 |
| 4   | 5          | 4-5          | Van dyck brown                    | Cold grey IV                  | 95.30                 |
| 5   | 5          | 4-5          | Van dyck brown                    | Cold grey IV                  | 100                   |

**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 fungus (treatment) | Mycelial coverage (%) |
|-----|------------|--------------|-----------------------------------|-------------------------------|-----------------------|
| 0   | 5          | 5            | Van dyck brown                    | No growth                     | 0                     |
| 1   | 5          | 5            | Van dyck brown                    | No growth                     | 0                     |
| 3   | 4-5        | 4-5          | Van dyck brown                    | White                         | 80.90                 |
| 4   | 4-5        | 4-5          | Van dyck brown                    | Warm grey I                   | 86.62                 |
| 5   | 4-5        | 4-5          | Van dyck brown                    | Warm grey II                  | 94.06                 |

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

| Parameter           | Original sterile waste mixture (Control) | Treated sterile waste mixture | Value differences | % Increase or decrease |
|---------------------|------------------------------------------|------------------------------|-------------------|------------------------|
| Carbohydrate        | 20.28 %                                  | 18.30 %                      | -1.98             | 9.76 decrease          |
| Protein content     | 5.02 %                                   | 4.88 %                       | -0.14             | 2.78 decrease          |
| Moisture content    | 73.42 %                                  | 75.40 %                      | +1.98             | 2.69 increase          |
| Ash content         | 1.28 %                                   | 1.42 %                       | + 0.14            | 10.93 increase         |
| Total fat content   | <0.02 %                                  | <0.02 %                      | <0.02             | constant               |
| Calorie from fat    | 0 Kcal/100 g                             | 0 Kcal/100 g                 | 0                 | constant               |
| Total calorie       | 101.2 Kcal/100 g                         | 92.72 Kcal/100 g             | -8.48             | 8.37 decrease          |
Table 4. The composition of sterile waste mixtures before and after growth of *Rhizopus azygosporus* UICC 539 at 40°C for 5 days incubation.

| Parameter                | Original sterile waste mixture (Control) | Treated sterile waste mixture | Value differences | % Increase or decrease |
|--------------------------|------------------------------------------|------------------------------|-------------------|------------------------|
| Carbohydrate             | 21.48 %                                  | 19.10 %                      | -2.38             | 11.08 decrease         |
| Protein content          | 4.58 %                                   | 4.22 %                       | -0.36             | 7.86 decrease          |
| Moisture content         | 72.60 %                                  | 75.26 %                      | +2.66             | 3.66 increase          |
| Ash content              | 1.34 %                                   | 1.42 %                       | +0.08             | 5.97 increase          |
| Total fat content        | <0.02 %                                  | <0.02 %                      | <0.02             | constant               |
| Calorie from fat         | 0 Kcal/100 g                             | 0 Kcal/100 g                 | 0                 | constant               |
| Total calorie            | 104.24 Kcal/100 g                        | 93.28 Kcal/100 g             | -10.96            | 10.51 decrease         |

4. Conclusion

*Rhizopus azygosporus* UICC 539 was able to grow and utilize the sterile mixtures of slurry and PKC during solid-state fermentation. Growth at 30 and 40 °C was favourable for *R. azygosporus* UICC 539 in the waste mixtures; however, better growth was achieved at 30 °C as shown by faster sporulation and greyish black colonies. The fungus was potential in improving the nutrient content of the agricultural by-product, i.e. palm oil processing waste. Further study is needed explore the use of this fungus for formulation of animal feed.

Acknowledgement

This research was funded by Hibah Publikasi International Terindeks 9 (PIT 9) 2019 contract number NKB-0019/UN2.R3.1/HKP.05.00/2019 awarded to A.O. The authors thank P.T. Agricinal in Seblat-Putri Hijau, North Bengkulu, Sumatra for the palm oil wastes, Dhian C.A.F. Sari for technical assistance, and Universitas Indonesia Culture Collection (UICC) for the fungus culture.

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