Solid-state fermentation and formulation of non-sterile palm oil processing waste using *Rhizopus azygosporus* UICC 539

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Abstract. This study reported the ability of *Rhizopus azygosporus* UICC 539 to utilize non-sterile slurry and palm kernel cake (PKC) through solid-state fermentation, preparation of the fungus and non-sterile waste mixture as a formula for animal feed, and analysis of nutrient content of the formula. Fungal culture in PSB at 30 and 40 °C for 5 days was prepared and wet weight biomass was used as inoculum for animal feed formula. Solid-state fermentation (SSF) was carried out on the mixtures of non-sterile slurry and PKC (3:1) at 30 and 40 °C for 5 days and the fermented waste mixtures were dried at 60°C for 5 days. The results showed that during SSF there were presence of colonies of other fungi and bacteria from the waste mixture besides *R. azygosporus*. The total cell number of *R. azygosporus* and other fungi were decreased after SSF. Changes in nutrient content in the formula were observed by comparing the treatment and control. Formula prepared at 40 °C showed an increase of carbohydrate content and total calorie, while formula prepared at 30 °C showed an increase only at carbohydrate content. A decrease of protein, water content, ash content, total fat, and energy from fat, was observed in formulas prepared at both temperatures.

Keywords: formulation; palm oil waste; *Rhizopus azygosporus*; solid-state fermentation

1. Introduction

The palm oil industry in Indonesia is the third highest contributor to the entire export in the country. The export is expected to reach 40 million tonnes of production by 2020 [1]. The potential national production of oil palm wastes in 2020 is estimated to be 91,224,865 tons palm oil mills effluent (POME), and 10,389,975 tons of palm kernel shell (PKS). In 2030, there would be production of 130,035,387 tons of POME, and 14,810,264 tons of PKS [2].

Palm kernel cake (PKC) is obtained from extraction process of the oil palm fruit. PKC has a protein content of 16 to 18 %, and ~22 % crude fibre [3]. Raw palm oil mill effluent (POME) is a colloidal suspension containing 95 to 96 % water, 0.6 to 0.7 % oil and 4 to 5 % total solids. Included in the total solids are 2 to 4 % suspended solids, which are mainly debris from palm fruit mesocarp. POME contains water soluble carbohydrates [4]. PKC and POME are considered suitable as possible feedstock particularly for ruminant [3, 4].

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Solid-state fermentation (SSF) has been credited to be responsible for fermentation in ancient time [5], e.g. the traditional tempeh fermentation [6]. SSF stimulates the growth of microorganisms in nature on moist solids [5]. The substrate must possess enough moisture to support growth and metabolism of the organisms. SSF resembles the natural habitat of microorganisms, and therefore, the preferred choice for microorganisms to grow and produce useful value-added by-products [7].

There is challenge of converting POME into environmentally friendly waste. The biological treatment of POME depends enormously on consortium of microorganism activities [8]. The high fibre in PKS can be overcome by the use of fungi, or improvement in nutritive quality, using SSF process [9]. 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 [10]. The fungal biomass obtained after fermentation of PKS could be exploited as fodder enrichment [9]. Members of *Rhizopus* have great potential for bioconversion of various byproducts from the food and agricultural industry to value-added bioproducts [11]. Good growth of *Rhizopus microsporus* was reported on non-sterile palm oil processing waste [12], while *R. microsporus* gave significant reduction of hemicellulose and lignin of PKS, and decreased the crude protein of PKS [9].

*Rhizopus azygosporus* was previously assigned as *R. microsporus* var. *azygosporus*, but was later introduced as a new species, *R. azygosporus* [13]. A collection of *Rhizopus* spp. from tempeh samples from various regions of Indonesia was deposited in Universitas Indonesia Culture Collection (UICC). *Rhizopus azygosporus* UICC 539 was originally isolated from soybean tempeh from Mataram, Lombok Island [data unpublished]. This study reported the ability of *Rhizopus azygosporus* UICC 539 to utilize non-sterile slurry and palm kernel cake (PKC) through solid-state fermentation, preparation of the fungus and non-sterile waste mixture as a formula for animal feed, and analysis of nutrient content of the formula.

### 2. Materials and Methods

#### 2.1 Microorganism and growth conditions

Potato Dextrose Agar (PDA, Difco) containing 0.02 % (w/v) chloramphenicol (Wako) was used for maintenance of *Rhizopus azygosporus* UICC 539. The fungus in slants of Potato Sucrose Agar (PSA) [14] added with 0.02 % chloramphenicol was incubated at 30 °C for 5 days. Fungal colony was cut using sterile plastic straw (diameter 6 mm) for preparation of colony disc as an inoculum according to Mascarin *et al.* [10] with some modification. The agar blocks were transferred onto PSA plates and fungal growth was observed 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

Cell suspension as inoculum was prepared according to Prameswari *et al.* [12]. Inoculum was prepared by scraping the mycelia and spores from five-days old fungal colonies on PSA slants, and added with 5 ml sterile distilled water to obtain $5.6 \times 10^7$ CFU/mL, and $2.6 \times 10^4$ CFU/mL, from 30 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 without shaking. After incubation for 2 days, the inoculum (25 % v/v) in PSB was obtained with cell concentrations of $3.9 \times 10^5$ CFU/mL and $1.3 \times 10^4$ CFU/mL from 30 and 40 °C, respectively. The inoculum was 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. Raw mixtures of slurry and PKC (3:1) were weighted to final weight of 400 g.
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 non-sterile waste mixtures in flat trays (20×20×5 cm) and mixed using sterile spatulas. Non-sterile waste mixtures without the fungal cells served as controls. The trays were incubated at 30 °C and 40 °C for 5 days. Characteristics of the non-sterile waste mixtures during SSF were determined on pH, growth of *R. azygosporus* and naturally occurring fungi, mycelial (biomass) coverage, and biomass colour according to Faber-Castell colour chart. Mycelial coverage (%) was determined according to Prameswari *et al.* [12] as follows: (colony area/tray area) x100 %. The dried formula was obtained by drying the non-sterile 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

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

### 3. Results and Discussions

After 5 days of incubation, *R. azygosporus* grew well and colonies of covered the culture plates and showed full sporulation in blackish grey at 30 to 40 °C, but growth was restricted at 45 to 50 °C. This was an indication that the fungus was thermo-tolerant. Growth was not observed at 51 to 55 °C and 60 °C, indicating that higher temperatures above 50 °C caused inhibition of the fungus growth. At 30 °C and 40 °C the colony diameter, 78.93 cm and 83.3 cm, were observed to be nearly similar compared to colony diameter at other temperatures, respectively. These temperatures were then selected for preparation of the inoculum for liquid-state fermentation in PSB. Dolatabadi *et al.* [15] reported that *R. microsporus* on Malt Extract Agar (MEA) grew well at 30 °C and 45 °C, showing thermo-tolerant. At almost 55 °C no growth was observed.

Good growth was shown by *Rhizopus azygosporus* UICC 539 in liquid-state fermentation in PSB at 30 °C and 40 °C (Figure 1). Mycelial biomass was formed on the liquid medium surface at both temperatures within 2-3 days. Inoculum for 400 g final weight of non-sterile waste mixture was obtained as wet biomass from PSB at 30 °C (11.67 g, 2.83 % w/v) and from PSB at 40 °C (13.06 g, 3.10 % w/v). Higher amount of wet biomass was obtained from culture grown at 40 °C compared to 30 °C, which indicated that more mycelial biomass was achieved at 40 °C than at lower temperature. However, Nout and Kiers [16] noted that spore germination and luxuriant growth of mycelium was observed during fermentation of *Rhizopus* spp. at ambient temperature (25 to 30 °C).

Cultivation of *R. azygosporus* UICC 539 in PSB at 30 °C and 40 °C showed that there was a decrease in the pH of medium from the initial pH 6-7 to 6. This was an indication that the fungus produced organic acid(s) during growth. A report from Plassard and Fransson [17] stated that fungi are well known for their natural capability to produce high amounts of various organic acids. Members of the genus *Rhizopus* produce lactic and fumaric acids. Amiri *et al.* [18] reported that mycelial growth of *Rhizopus stolonifer* was optimal at pH 7 and was reduced at 4≤ pH ≥9 in a liquid medium.

The non-sterile waste mixtures (slurry:PKC = 3:1) had acidic pH 5 and brownish colour. The control showed an increase of pH from 5 to 7 at 30 °C and 40 °C, while the treatment showed slightly change of pH 5 to 6 during SSF (Table 1 and Table 2). Observation on the non-sterile waste mixtures in control and treatment during SSF showed that there was growth of naturally occurring fungi and bacteria (Figure 2). The occurrence of naturally occurring fungal isolates and bacteria in this study was an indication that slurry and PKC contained nutrients that support growth of these microorganisms. The presence of naturally occurring microorganisms in the non-sterile waste mixture control might contribute to change of waste mixture acidity towards neutral, while the slightly changed of pH in the...
waste mixture treatment was probably due to the presence of \( R. \) azygosporus UICC 539. Amiri et al. [18] stated that fungi are known to grow well at acidic conditions similar to those found in their natural habitats. According to Plassard and Fransson [17] fungal natural production of organic acids has many key roles in nature. These roles either due to the pH decrease consecutive to organic acid secretion or to direct interaction of the organic acid with the environment. The acidic character of POME has been reported. The corrosion of iron used during processing might have influenced POME to become acidic [8].

\[\text{Figure 1. Growth of} \ R. \ azygosporus \ \text{UICC 539 in liquid-state fermentation in PSB}\]

| Day | pH control | pH treatment | Colour of non-sterile waste mixture (control) | Colour of non-sterile waste mixture (treatment) | Mycelial coverage (%) |
|-----|------------|--------------|-----------------------------------------------|------------------------------------------------|-----------------------|
| 0   | 5          | 5            | WM: van dyck brown No fungi colony             | WM: van dyck brown \( R. \) azygosporus colony: no growth | 0                     |
| 2   | 5-6        | 5            | WM: van dyck brown FC A: warm grey II          | WM: van dyck brown \( R. \) azygosporus colony: warm grey II FC A: warm grey II | 100                   |
| 3   | 7          | 6            | WM: Sepia light FC A: warm grey II             | WM: van dyck brown \( R. \) azygosporus colony: warm grey II FC A: warm grey II | 100                   |
| 4   | 7          | 5-6          | WM: Sepia light FC A: warm grey II FC B (mature): light ochre FC B: white | WM: sepi light \( R. \) azygosporus colony: warm grey II FC A: warm grey II FC B (mature): light ochre FC B: white | 100                   |
| 5   | 7          | 5            | WM: Sepia light FC A: warm grey III FC B (mature): light ochre | WM: van dyck brown \( R. \) azygosporus colony: cold grey II FC A: cold grey II FC B (mature): light ochre FC B: white | 100                   |

Note: WM = waste mixture; FC = fungi colony
Figure 2. Growth of naturally occurring fungi and bacteria, and *R. azygosporus* UICC 529 during SSF on non-sterile mixtures at 30 °C and 40 °C.

Table 2. Characteristics of the non-sterile 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 non-sterile waste mixture (control) | Colour of non-sterile waste mixture (treatment) | Mycelial coverage (%) |
|-----|------------|--------------|-----------------------------------------------|-------------------------------------------------|-----------------------|
| 0   | 5          | 5            | Waste mixture: van dyck brown                  | Waste mixture: van dyck brown *R. azygosporus* colony: no growth | 0                     |
| 1   | 5          | 5            | Waste mixture: van dyck brown                  | Waste mixture: van dyck brown *R. azygosporus* colony: no growth | 0                     |
| 2   | 7          | 5            | Waste mixture: van dyck brown Fungi colony A: white | Waste mixture: van dyck brown *R. azygosporus* colony: white Fungi colony A: white | 58.5                   |
| 4   | 7          | 5            | Waste mixture: van dyck brown Fungi colony A: white | Waste mixture: van dyck brown *R. azygosporus* colony: warm grey I Fungi colony A: warm grey I Fungi colony C: hooker’s green | 100                    |
| 5   | 7          | 6            | Waste mixture: van dyck brown                  | Waste mixture: sepia light *R. azygosporus* colony: warm grey I Fungi colony A: warm grey I Fungi colony C: hooker’s green | 100                    |

Before the drying process of the formula, the cell number were enumerated on the control and treatment. The result showed that *R. azygosporus* UICC 539 was able to grow in the non-sterile waste mixture treatment at 30 °C and 40 °C although there were naturally occurring fungi and bacteria in the waste. The enumeration result showed that the cell number of microorganisms from the formula...
prepared at 30 °C before drying contained naturally occurring mould isolate (control: 5.5×10⁸ CFU/mL, treatment: 1.7×10⁷ CFU/mL), yeast isolate (control: 2.3×10⁹ CFU/mL, treatment: 4.3×10⁷ CFU/mL), and \textit{R. azygosporus} UICC 529 (treatment: 8.6×10⁶ CFU/mL).

The bacterial colonies were isolated but not enumerated. The cell number of microorganisms from the formula prepared at 40 °C before drying contained naturally occurring mould isolate (control: 3.1×10⁶ CFU/mL, treatment: 2.8×10⁶ CFU/mL), no growth was observed on the yeast isolate, and \textit{R. azygosporus} UICC 529 (treatment: 1.5×10⁷ CFU/mL).

After the drying process of the formula at 60 °C for 5 days, the enumeration result showed that there was a decrease of cell number from the formula prepared at 30 °C after drying as follows: naturally occurring mould isolate (control: 5×10³ CFU/mL, treatment 6.5×10² CFU/mL), no growth of the yeast isolate, and \textit{R. azygosporus} UICC 529 (treatment: 1.5×10² CFU/mL). The cell number from the formula prepared at 40 °C after drying as follows: naturally occurring mould isolate (control: 1×10⁵ CFU/mL, treatment 8.6×10⁵ CFU/mL), no growth of the yeast isolate, and \textit{R. azygosporus} UICC 529 (treatment: 6.7×10⁴ CFU/mL).

The result showed that \textit{R. azygosporus} UICC 539 cells were still viable after undergone the drying process and the obtained cell number of \textit{R. azygosporus} UICC 539 was higher from the formula prepared at 40 °C compared to 30 °C. It was assumed that due to the growth preparation of \textit{R. azygosporus}, UICC 539 has become adapted to the higher temperature during growth, and thus, more viable cells were obtained.

During SSF of the non-sterile waste mixtures, it was indicated that the naturally occurring microorganisms and \textit{R. azygosporus} UICC 539 were able to degrade cellulosic wastes and oil contained in the waste mixtures. The waste mixture as a substrate was rich in carbohydrate, lipid and protein. This substrate was fermented and utilized by the microorganisms for metabolism and growth. There were reports that \textit{Rhizopus} spp. showed degrading abilities on various substrates containing cellulose (carboxymethyl cellulose, CMC) \[19\], carbohydrate (starch) \[20\], lipid (olive oil) \[21\], and protein (casein) \[22\] which proved that \textit{Rhizopus} produced enzymes such as cellulase (CMCase), amylase, lipase, and protease, respectively. Thermophilic lipolytic microorganisms degraded the oil and grease in POME. Fungi from the genera \textit{Aspergillus}, \textit{Penicillium} and \textit{Mucor} were presence in the POME, while bacteria from the genera \textit{Micrococcus}, \textit{Bacillus}, \textit{Pseudomonas} and \textit{Staphylococcus} have been isolated \[8\].

Changes of nutrient content of the formula before and after growth of \textit{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 content compared to the control. A decrease was observed in protein content, moisture content, ash content, total fat, 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 carbohydrate content and total calorie, compared to the control.

At both temperatures, the content of protein, moisture, ash, total fat, and calorie from fat was decreased compared to the control. The increased carbohydrate content of the formula prepared at 30 °C and 40 °C was probably due to the consequence of the growth of naturally occurring microorganisms from the non-sterile waste mixtures and \textit{R. azygosporus} UICC 539. There was a loss of \textit{R. azygosporus} biomass since the fungus utilized the nutrients along with naturally occurring microorganisms for their metabolism and activity. Falaye \textit{et al.} \[9\] reported that increased non-starch polysaccharides (carbohydrate) was observed in non-sterile palm kernel sludge. Crude enzyme of \textit{R. microsporus} degraded the lignin and hemicellulose in palm kernel sludge (PKS) thereby improving the nutritive content of fermented PKS.
Table 3. The composition of non-sterile waste mixtures before and after growth of *Rhizopus azygosporus* UICC 539 at 30 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            | 61.34 %                                     | 64.71 %                          |
|                         | 65.07 %                                     | 67.02 %                          |
| Protein content         | 17.66 %                                     | 17.49 %                          |
|                         | 16.50 %                                     | 16.70 %                          |
| Moisture content        | 7.22 %                                      | 6.80 %                           |
|                         | 7.06 %                                      | 6.23 %                           |
| Ash content             | 6.20 %                                      | 6.37 %                           |
| Total fat content       | 7.58 %                                      | 4.63 %                           |
|                         | 5.72 %                                      | 4.39 %                           |
| Calorie from fat        | 68.22 Kcal/100 g                            | 41.67 Kcal/100 g                 |
|                         | 51.48 Kcal/100 g                            | 39.51 Kcal/100 g                 |
| Total calorie           | 384.22 Kcal/100 g                           | 370.47 Kcal/100 g                |
|                         | 377.76 Kcal/100 g                           | 374.39 Kcal/100 g                |

| Parameter               | Value differences | % Increase or decrease |
|-------------------------|-------------------|------------------------|
|                         | 30 °C | 40 °C | 30 °C | 40 °C |
| Carbohydrate            | + 3.73 | + 2.31 | 6.08 increase | 3.50 increase |
| Protein content         | - 1.16 | - 0.79 | 6.56 decrease | 4.51 decrease |
| Moisture content        | - 0.16 | - 0.57 | 2.21 decrease | 8.30 decrease |
| Ash content             | - 0.55 | - 0.71 | 8.87 decrease | 11.14 decrease |
| Total fat content       | - 1.86 | - 0.24 | 24.50 decrease | 5.18 decrease |
| Calorie from fat        | - 16.74 | - 2.16 | 24.50 decrease | 5.18 decrease |
| Total calorie           | - 6.46 | + 3.92 | 1.68 decrease | 1.05 increase |

4. Conclusion
This study showed that *R. azygosporus* UICC 539 was able to grow and utilize the non-sterile waste mixtures during solid-state fermentation. The fungus was potential in improving the nutrient content of the agricultural by-product from non-sterile palm oil processing waste. This study showed that *R. azygosporus* UICC 539 was potential as a nutritional improvement for animal feed formulation.

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