

NUTRIENT ENHANCEMENT OF PALM KERNEL CAKE VIA SOLID STATE FERMENTATION BY LOCALLY ISOLATED *Rhizopus oryzae* ME01

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ABSTRACT

Palm kernel cake (PKC) is abundantly generated in palm kernel crushing plant throughout the year. Its use as monogastric animal food source is very limited due to its high fibre and moderate protein content. Therefore, this study is aimed to enhance PKC nutritional value, particularly the protein content through solid state fermentation (SSF), using a locally isolated fungus. Fungal identification was performed using partial 18S ribosomal ribonucleic acid (rRNA) nucleotide sequence. A fungus containing 600 base pairs was sequenced and aligned with GenBank database and named *Rhizopus oryzae* ME01. SSF was carried out using palm kernel expeller (PKE) which is mechanical pressed and PKC which is solvent extracted, as a single carbon source with minimum mineral addition. Results showed that SSF successfully increased crude protein and ash content of fermented PKC and PKE, respectively. Interestingly, this study also found that the size of PKC's particle had impacted protein content of fermented palm kernel cake (fPKC) during SSF. PKC which has a smaller and uniform size than PKE, gave higher crude protein increment than fermented palm kernel expeller (fPKE). However, the protein content of fPKE had increased by 7.64% more than fPKC which was only 3.45%. Thus, fPKE appeared to enhance PKE's nutrient value, especially for monogastric animal feed applications.

Keywords: fungus identification, nutrient enhancement, palm kernel cake, solid state fermentation.

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INTRODUCTION

Raw material for feed formulation in livestock industry, particularly for non-ruminant, such as poultry, depends entirely on the imported raw materials, which resulted in high overall feed cost. The main imported raw materials for non-ruminant feed, such as maize and soybean meal, require approximately RM2.5 billion per year, with maize accounting for more than 50% (Wan Zahari and Wong, 2009). Majority of the livestock gross domestic product (GDP) is contributed mainly by the poultry sub-sector at 62.9%, while the ruminant sub-sector

only contributed 12.1% (Shanmugavelu, 2014). It is therefore crucial to reduce the dependency on imported raw materials by using local raw materials in feed formulation for non-ruminants. Palm kernel cake (PKC) appears to be an alternative protein source in livestock feed formulation to stabilise the feed price.

In 2019, Malaysia produced 19.86 million tonnes of crude palm oil (CPO) and 2.59 million tonnes of PKC after 2.29 million tonnes of crude palm kernel oil (CPKO) was extracted (Parveez *et al.*, 2020; Malaysian Palm Oil Board, 2020). Currently, there are two technologies to process palm kernel in the mills, which are solvent extraction and mechanical press. The product produced via solvent extraction is called PKC while mechanical produces palm kernel expeller (PKE). Due to PKC's high fibre and moderate protein content (Sundu

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and Dingle, 2003), it had not been widely applied for monogastric animal feed particularly, poultry. Moreover, protein quality and presence of non-digestible polysaccharide components such as non-starch polysaccharide (NSP) in PKC also hindered its maximum utilisation by monogastric animals. Protein quality is closely related to amino acid components bioavailability and animal digestive ability (Bryan and Classen, 2020). Protein quality is also determined by the ability of amino acids to be absorbed by the body which is also known as amino acid digestibility (Alshelmani *et al.*, 2017a).

PKC was rarely used in monogastric livestock formulations although it contains up to 18% protein due to the presence of anti-nutritional components besides its high content of mannan (Oluwafemi, 2008). Nevertheless, the potential of PKC can be enhanced through fermentation that serves as an alternative protein source for animal nutrition. Its nutritional quality can be enhanced by the microorganism's biomass and NSP content can be reduced to enable its utilisation as raw material in poultry feed (Alshelmani *et al.*, 2014; 2016; 2017b). Mohd Firdaus (2014) and Noraini *et al.* (2009) had proved that fermented PKC can be given to broiler chicks up to 30% in the feed formulation without adverse effects. Sathitkowitzchai *et al.* (2018) contended that the application of enzyme for β -mannan degradation in PKC will release the sugar and other digestible sugars that can be absorbed and metabolised by monogastric animals (Zamani *et al.*, 2017). Therefore, the solid state fermentation (SSF) method using microorganism is preferred as a treatment for substrates containing high NSP content prior to feed application. The SSF method is also an economically viable method in fermentation biotechnology and its use in the livestock industry which has high operating costs is guaranteed an alternative technology (Lateef *et al.*, 2008; Rodríguez-Jasso *et al.*, 2013). The use of microorganisms in this method is capable of hydrolysing NSP such as lignin, hemicellulose and cellulose, as well as ensuring optimum fermentation factors. This can also assist in lowering the NSP content and increasing substrate utilisation ratio in animal feed, thereby improving animal digestion.

The application of SSF using local isolated fungus could enhance the nutritional content of PKC and indirectly enhanced the application of PKC as livestock feed by dissolving its NSP components. The use of new strains in this study also brings novelty and new scientific findings if it can improve the nutrition value of PKC. This article is intended to identify locally isolated fungus strains that infect the palm kernel naturally using the 18S ribosomal ribonucleic acid (rRNA) method. In addition, the effect of SSF on nutrient content of PKE and PKC after fermentation was also studied.

MATERIALS AND METHOD

Chemicals

All chemicals used were of analytical grades. Fungal growth media, namely potato dextrose agar (PDA) and potato dextrose broth (PDB), were obtained from Oxoid, England. The chemicals used for fungus identification (Tris-HCl, Tris-bes and ethidium bromide) were supplied by Sigma Chemical Company, USA. Ammonium sulphate $[(\text{NH}_4)_2\text{SO}_4]$ and potassium dihydrogen phosphate (KH_2PO_4) for fungus fermentation were obtained from BDH AnalaR, USA. The proximate analysis was carried out using sodium hydroxide, ethylenediaminetetraacetic acid (EDTA), sodium borate deacetyl, sodium lauryl sulphate, triethylene glycol, disodium hydrogen phosphate, sulphuric acid, hydrochloric acid, petroleum ether and acetone provided by Hamburg Chemical, Germany and BDH AnalaR, USA.

Enzyme, Kit, DNA Rulers and Vectors

The restriction enzymes were supplied by Vivantis, Malaysia and the DNA 1 kb ruler was supplied by Promega, USA. The polymerase chain reaction (PCR) components namely PCR buffer (10X), magnesium chloride (MgCl_2) (25 mM), deoxynucleoside triphosphate (dNTP) (10 mM), Taq deoxyribonucleic acid (DNA) polymerase, pfu DNA polymerase were supplied by Invitrogen, Brazil and Promega, USA. Promoter DNA (oligo primer) was supplied by First BASE (Malaysia). Vector pGEM[®]T Easy, ligase buffer and T4 DNA ligase were obtained from Promega, USA. The isopropyl β -D-1-thiogalactopyranoside (IPTG) was supplied by Bio Basic Inc., Canada, while X-gal was sourced from 5 Prime, USA. Universal promoters for the T7, SP6 and M13R sequencing processes were obtained from Promega, USA. Fungal DNA extraction was conducted using the DNeasy Plant Mini Kit (Qiagen). For PCR product purification, the Megaquick-spin PCR Kit and Agarose Gel DNA Extraction System obtained from Intron, USA were used. Meanwhile, the plasmid purification was conducted using the Wizard Plus Minipreps DNA Purification System Kit from Promega, USA. ABI PRISM[®] Big Dye[®] Terminator v3.1 Cycle Sequencing provided by Applied Biosystems, USA was used for sequencing.

Fungus Culture

The naturally infected palm kernel samples were taken and cultured on PDA media. New subculture validation test was conducted in triplicates to ensure fungal independence and purity. The locally isolated strain was incubated

for seven days on PDA at 30°C. After that, it was stored in a refrigerator at 4°C until further use. New subcultures were created every four weeks on the PDA to maintain the purity of the fungal species.

Identification of Fungus Strain

Isolation and extraction of fungal genomic DNA. The fungal culture fermented in PDB medium for seven days at 30°C and 180 rpm agitation was harvested for genomic extraction of fungal DNA using the DNeasy Plant Mini Kit (Qiagen).

Determination of DNA concentration and quality. Two methods were used to determine the concentration of DNA in this study. The first method was conducted by using the spectrophotometer (Biophotometer, Eppendorf, Germany). One μL DNA was mixed with 49 μL distilled water and the absorption values at 230, 260 and 280 nm were measured, respectively. Distilled water (dH_2O) was used as evaporator. The second method was conducted by directly comparing the intensity level of the DNA band of interest with the known mass ruler band after both samples and rulers were fractionated in the same gel.

Agarose gel electrophoresis of DNA samples. Agarose gel electrophoresis was run at 50-100 V to obtain the desired band separation. The resulting electrophoresis was subsequently absorbed on an ultraviolet (UV) transilluminator and the resulting band images were taken using the AlphaImager™ 2200 gel documentation system (Alpha Innotech, USA). Prior to that, DNA samples were fractionated using 1.0% (w/v) agarose gel. The agarose gel was dissolved in TAE 1X buffer through microwave heating. After the gel was cooled to 50°C, 1 μL ethidium bromide (10 mg mL^{-1}) was added to the agarose solution before being poured into the gel mold. A total of 20 μL of DNA sample was mixed with a 6X load buffer before being filled into frozen gel wells. DNA rulers included in other gel wells served as rulers for DNA samples. The use of DNA rulers depended on the analysis, needs and size of the expected results (Sambrook and Russel, 2001). Common rulers used are DNA 1 kb, 100 bp, DNA mass ruler and λ HindIII.

PCR 18S rRNA amplification. Specific oligo primers were used to generate 18S rRNA fragments through PCR amplification chain reactions. They were known as internal transcribed spacer (ITS), for example ITS1-F: 5'-CTTGGTCATTTTAGAGGAAGT-3' and ITS4-R: 5'-TCCTCCGCTTATTGATATGCC-3' (Kim *et al.*, 2008). The ITS was synthesised by First BASE Laboratories Sdn. Bhd., Malaysia upon designed by Primer-design program software. The PCR reactions were performed using a GeneAmp Thermal Cycler

(Model 2400, USA). *Table 1* summarises the PCR components. The programmed temperature cycles were as follows: A cycle of DNA denaturation at 94°C for 5 min; 25 cycles for denaturation at 94°C for 1 min, followed by annealing at 65°C for 1 min and elongation at 72°C for 1 min. Then, the PCR reaction was terminated with a final elongation process at 72°C for 7 min. The resulting PCR products were subsequently analysed by 1% agarose gel electrophoresis. If the presence of the expected single band is found the PCR product is then cut for further action.

TABLE 1. THE POLYMERASE CHAIN REACTION (PCR) COMPONENTS USED TO AMPLIFY 18s rRNA

Component	Volume (μL)
PCR buffer (10X)	2.0
Magnesium chloride (MgCl_2) (25 mM)	1.0
Deoxynucleoside triphosphate (dNTP) (4 mM)	1.0
Forward primer (12.5 pmol)	1.0
Reverse primer (12.5 pmol)	1.0
DNA genome (250 ng μL^{-1})	3.0
<i>Taq</i> DNA polymerase (5 U μL^{-1})	0.5
Sterile distilled water	10.5
Final volume	20.0

Extraction and purification of DNA from agarosa gel. The PCR products were extracted and purified from agarose gel after the desired band was found. The commercial Megaquick-spin PCR Kit and Agarose Gel DNA Extraction System were used for this purpose.

Sequence analyses. The sequencing process was performed using the protocol of the ABI PRISM® BIG DYE® Terminator Cycle Sequencing Ready Reaction (PE Biosystems, USA). The components of the sequencing reaction were 200-500 ng DNA template, 3.2 pmol of appropriate promoters, 1 μL Big Dye terminator v.3.1, 0.5 μL of 10X sequence buffer and sterile distilled water were added into the PCR tube until the final volume was equal to 10 μL . The promoters used were universal T7 (5 'TAATACGACTCACTATAGGG 3'), SP6 (5 'GATTTAGGTGACACTATAG 3') and M13R (5 'CAGGAAACAGCTATGACC 3'). The tubes were then centrifuged prior to cyclic reaction using TPersonal Thermal Cycler, Biometra, Germany or PTC-100 Thermal Cycler, MJ Research, USA at 25 cycles at 96°C for 30 s, 50°C for 15 s and 60°C for 4 min. An automated DNA sequencing was then performed on that sample.

The data obtained was displayed on a chromatogram and analysed using ChromasLite201 software. The sequences obtained were compared with nucleotide sequences and amino acids in the National Center for Biotechnology Information

(NCBI) database using BLAST software (Altschul *et al.*, 1990). Through BLAST, the percentage homology of nucleotides and amino acids to sequences from other organisms was determined. Domain analysis was performed using InterProScan software. The sequences then were used to generate phylogenetic tree by using the software (Phylogeny,fr) and the analysis was performed by the software through an automated programme from multiple alignment of the sequence to the construction of phylogenetic tree (Radha and Ashok, 2020).

Solid State Fermentation (SSF)

Preparation of culture media. PKE sample was obtained from Federal Land Development Authority (FELDA) Pasir Gudang, Johor, Malaysia, while PKC sample was produced in-house using a 5 L scale Soxhlet apparatus (Osman *et al.*, 2009). A total of 20 g of untreated PKE and PKC samples were mixed directly with 1% (w/w) $(\text{NH}_4)_2\text{SO}_4$ and 0.3% (w/w) KH_2PO_4 . The mixture was mixed and autoclaved at 121°C for 15 min. The method used was modified from Ilyemi *et al.* (2006). Only two types of minerals were used in this study since PKC already contains several minerals such as magnesium (Mg), iron (Fe), sodium (Na), calcium (Ca), zink (Zn) and copper (Cu) (Osman *et al.*, 2009).

Seed culture production. A total of 1 cm³ of *R. oryzae* ME01 fungal mycelium aged five days was cut from the PDA and cultured in a 500-mL conical flask containing 250 mL PDB to form pellets. The seed culture was carried out for five days at 30°C with 180 rpm agitation.

Solid state culture. Sterilised culture media was mixed aseptically with seed culture at a ratio of 1:1 (volume: medium) to give an initial moisture of 50%.

Fermentation was performed for 10 days according to the study by Swe *et al.* (2003) whereby maximum protein was produced by *Aspergillus niger* on Day 8. To study the effect of fermentation on changes in nutrient content, 20 g samples were harvested from the bioreactor daily, where the growth of fungal biomass was measured.

Bioreactor systems. Figure 1 shows the schematic diagram of the SSF bioreactor system used in this study. This bioreactor system consisted of an air pump, a water temperature probe, a water heater, an air filter and a bioreactor.

Chemical Composition Analysis

Approximate analysis of fermented PKE and PKC were determined according to the standard method described in Association of Official Agricultural Chemists (AOAC) (AOAC, 1998; Gul and Safdar, 2009). Analysis of total moisture content was carried out using AND Machine MX50 (USA) using 5 g of ground samples. Crude protein was determined according to the Kjeldahl method and each sample was calculated by multiplying the nitrogen content by a factor of 6.25. The fat content of the samples was determined using hexane in soxhlet extraction method (Soxtherm machine). The samples were incinerated for 4 hr at 600°C. The percentage of carbohydrate content and the energy value was determined according to Galla *et al.* (2012), using the following Equation (1) and (2):

$$\% \text{ Carbohydrate} = 100 - (\text{sum of moisture, ash, crude protein, crude fibre and crude fat contents}) \quad (1)$$

$$\% \text{ Energy (kcal/100 g)} = 9 \times \% \text{ fat} + 4 (\% \text{ protein} + \% \text{ carbohydrate}) \quad (2)$$

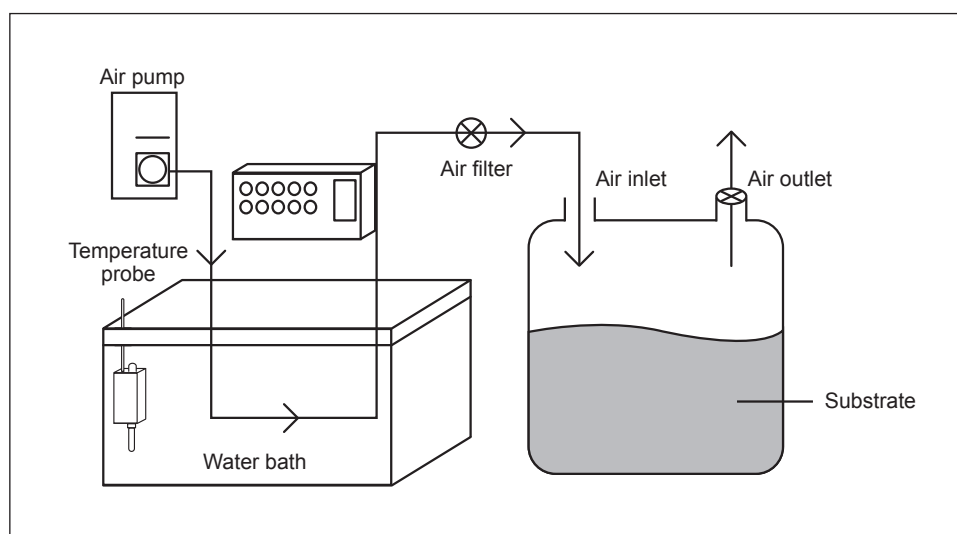


Figure 1. Bioreactor system designed for solid state fermentation of palm kernel cake.

Statistical Analysis

Data were analysed using the general linear models procedure of the Statistical Analysis System (SAS). Differences among treatments were by the least significant difference at $p < 0.05$.

RESULTS AND DISCUSSION

Identification of Fungus Strain

The quality and quantity of locally isolated fungal obtained after DNA extraction needs to be determined to ensure high levels of DNA purity and to produce accurate results in strain identification. Thus, spectrophotometric measurements were taken and agarose gel electrophoresis analysis was performed. Table 2 shows the average spectrophotometer measurements indicating the concentration and purity of the fungal DNA. According to Sambrook and Russell (2001), the measurement of $A_{260/280}$ and $A_{260/230}$ referred to protein and carbohydrate contamination.

Based on Table 2, the measurements of $A_{260/280}$ and $A_{260/230}$ samples were 1.86 and 0.41, respectively. This result signified that the $A_{260/280}$ sample was free of protein contamination as the value was more than 1.80 but with low carbohydrate contamination. To examine the DNA quality, agarose gel electrophoresis analysis was performed. Single strand of DNA produced (Figure 2a) proved that the DNA sample was not degraded and has a satisfactory concentration for further study. PCR amplification of fungal DNA samples was promoted by internal transcribed spacers (ITS) 1 and ITS 4. It was found that good primers combination and annealing temperature above 53°C as suggested by Cappa and Coconcelli (2001), produced good

results and specific amplitude (Figure 2b), as well as reducing products from non-specific reactions.

TABLE 2. CONCENTRATION AND PURITY OF DNA FROM LOCALLY ISOLATED FUNGUS

Fungus	DNA concentration (ng μL^{-1})	Spectrophotometer measurement	
		$A_{260/280}$	$A_{260/230}$
Unknown	5.03 ± 0.57	1.857 ± 0.22	0.407 ± 0.03

Nucleotide sequence analysis was performed using 18S rRNA to determine the unknown strain. 18S rRNA is a small nuclear subunit of ribosomes that evolves very slowly and is therefore suitable to be used to identify and to locate organisms (Dresler-Nurmi *et al.*, 1999). After replication by 18S rRNA, a comparison of sequence data (Figure 3) with the European Molecular Biology Laboratory (EMBL) data from GenBank via BLAST was able to identify the unknown strain as *Rhizopus oryzae* as it has a homology sequence and highest score of 100% E value with some *R. oryzae* strain.

To strengthen the finding, DNA of the unknown strain was aligned with the *R. oryzae* strain TY.GF1 sequence (GenBank accession number: JN003654.1) and the *R. oryzae* strain CS1217 sequence (GenBank accession number: GU126375.1). This multiple alignment was performed to seek the relationships and associations between the genus *Rhizopus* and to look for the distinct differences between the base pairs. The JN and GU sequences were chosen because they had the closest homology and highest score to the unknown strain. According to Schabereiter-Gurtner *et al.* (2001), sequence comparison analysis revealed similarity of the strain studied with the reference strain. Phylogenetic analysis was performed to determine the relationship of strains studied with genus and other fungal strains.

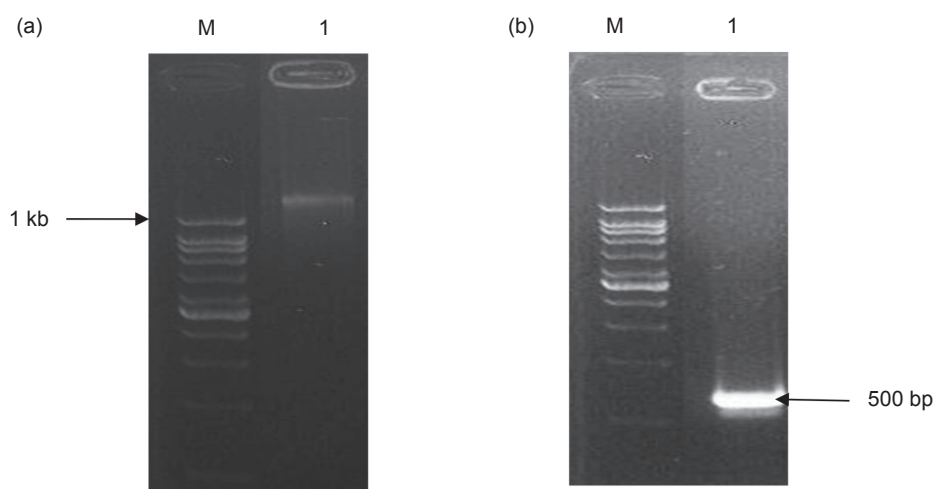


Figure 2. Electrophoresis of agarose gel (1.8% w/v) on extracted DNA; (a) the DNA genome has a single non-degradable band and its size was larger than 1 kb, and (b) the polymerase chain reaction (PCR) 18S rRNA product resulting from DNA amplification where the resulting single band was larger than 500 base pairs (bp) with high concentration.

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1   TCGATTTTCTGCCCTATCATGGTTGAGATTGTAAGATAGAGGCTTACAATGCCCTACAACG
61  GGTAACGGGGAAATTAGGTTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCC
121 AAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCACACGGGGAGGTAGTGACAATACA
181 TAACAATGCAGGSCCTTTAAGGTCTTGCAATTGGAATGAGTACAATTTAAATCCCTTAAC
241 GAGGATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGGTAATCCAGCTCCAATAGC
301 GTATATTAAGTTGTTGCGAGTTAAAACGTCCTAGTCAAACCTTAGTCTACCGCGGTAG
361 TGGCCTGGTCTTATTGACCAAGCTCATTGCTGCCGAGACTCCATGTCATTGACTCCT
421 AGTCCTCGTGGCTAGGGTTTTCTGGACAATTACCATGAGCAAATCAGAGTGTTTAAAGCA
481 GGCTTTTAAGCTTGAATGTGTAGCATGGAATAATGAAATATGACTCGCTCAGCATCTTA
541 GCGAAAGTAAAGTTTTTGGGTTCTGGGGGAGTATGGGACGCAAGGCTGAAACTTAAAGG
601 AATTGACGGAAGGGCACCACAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGG
661 AAACCTCACCAGGTCAGACATAGTAAGGATTGACAGATTGAAAGCTCTTTCTAGATTCTA
721 TGGGTGGTGGTCATGGCCGTTCTAGTTCGTGGAGTGATTGTCTGGTTAATCCGATA
781 ACGAACGAGACCTTATTCTGCTAATTAGACAGGCTAACTCTTTGGGTTGGTTTATATTT
841 AATATTTAACTGGCTTCTTAGAGAGACTATCGGCTTCAAGCCGAAAGGATTTTAGGCAA
901 TAACAGGTCTGTGATGCCCTTAGATGTTCTGGCCGACGCGCTACACTGATGAAGTC
961 AGCGAGTTTATAACCTTGGCCGGAAGGCTGGGTAAACTTTTGAACCTTATCGTGTCTGG
1021 GGATAGAGCATTGTAATTATTGCTCTTCAACGAGGAATTCCTAGTAAGCGCAAGTCATCA
1081 GCTTGCCTGATTACGTCCTGCCCCTTTGTACACACCGCCGCTGCTACTACCGATTGAA
1141 TGGTTATAGTGAGCATATGGGATCAGTAGGA

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Figure 3. Complete nucleotide sequence of fungal strain A containing 1171 base pairs. Through nucleotide searches using BLAST, the organism has the highest sequence homology and score with *Rhizopus oryzae* (99% E value).

Figure 4 shows the phylogenetic analysis of the 18S rRNA amplitude of a localised isolated fungal strain with its neighbours. The tree was based on a final alignment of 1171 base pairs of the strain. According to Lee *et al.* (2000), the dendrogram generated by genetic distance data separating strains has been clearly studied into specific genera. Similarly, this study classified the unknown strain into *R. oryzae* under *Rhizopus* genera through phylogenetic analysis.

Chemical Compositions of Fermented PKE and Fermented PKC

Ether Extract Content (EEC). Figure 5a shows the effect of SSF by *R. oryzae* ME01 on EEC in fermented PKE (fPKE) and fermented PKC (fPKC). There was a significant decrease ($p < 0.05$) of EEC from Day 1 to Day 10 of fermentation against PKE compared to control, and the lowest EEC was recorded on Day 7, at 1.29% (w/w). Although fermentation of PKC also showed a decrease of EEC up to 0.01% (w/w) on Day 7 at 1.27% (w/w); the decrease was not significantly different ($p > 0.05$) than that of control.

The decrease in EEC showed that the *R. oryzae* ME01 assimilated oils in PKE and PKC for its metabolite use. In another study, Alshelmani *et al.* (2016) contended that EEC digestibility had increased in broilers fed with the fermented PKC. The ability of *R. oryzae* ME01 to lower EEC has opened up new opportunities in extracting the oil

through the use of enzymes besides the commonly used mechanical and chemical extraction methods. Extraction of oil from hazelnut containing 10%-12% (w/w) of oil by pectinase and cellulase enzyme achieved 98% oil extraction by producing a product containing only 1.5% (w/w) oil (Zúñiga *et al.*, 2003).

The presence of high EEC in PKE at 3.44% (w/w) compared to PKC (1.27% w/w) was a major determinant of successful nutritional improvement in SSF studies and it was found that products with high EEC were able to increase crude protein content (CPC), ash content and crude fibre content (CFC) compared to PKC through SSF. This is in line with the study of Gao *et al.* (2013) which found that SSF on soybeans by *A. niger* was able to increase CPC and CFC as a result of the use of soybean oil by the fungus.

Ash content. The ash content increased daily from Day 1 for both fermented products (Figure 5b). Fermentation that occurred had altered mineral composition in fermented products or in rumen animals (Pino and Heinrichs, 2016). For fPKE, the highest ash content was recorded on Day 10 at 7.81% (w/w), whereas from Day 1 to Day 4, the increase of ash content was not significantly different ($p > 0.05$) from the control (PKE Day 0) at (3.94% w/w). The same condition occurred in fPKC where the increase of ash content from Day 1 to Day 4 did not differ from Day 0 but from Day 5, the increase of ash content was significantly different ($p < 0.05$) from that of control PKC (3.52%

w/w). Day 9 recorded the highest ash content at 6.47% (w/w). The increase in ash content was due to an increase in fungal biomass and this result could be an indirect method to determine fungal growth (Koutinas *et al.*, 2003). It also showed that the mineral contents in fermented products were increased due to fungal biomass. The increment of mineral would decrease inorganic mineral supplementation in the animal feed.

Crude fibre content (CFC). Figure 5c shows the effect of SSF by *R. oryzae* ME01 on CFC in fPKE and fPKC. There was an increase in CFC from Day 1 to Day 10 of SSF on PKE and the increase was significantly different ($p < 0.05$) from that of control PKE. SSF on PKC from Day 1 to Day 10 also showed an increase but no significant difference ($p > 0.05$) with PKC without fermentation. Therefore, it was found that fermentation of PKE affected the CFC compared to the PKC and this was due to a very low EEC at 1.27% (w/w) in the PKC which caused deficiency of the fungus to use the oil. This is evident in the study by Gao *et al.* (2013) in which oil and starch became the contributing factor in increasing the CFC and CPC. The increase of CFC in the fermented PKE and PKC was contributed indirectly by fungal biomass and this finding showed that *R. oryzae* was not a good candidate to break down anti-nutritional

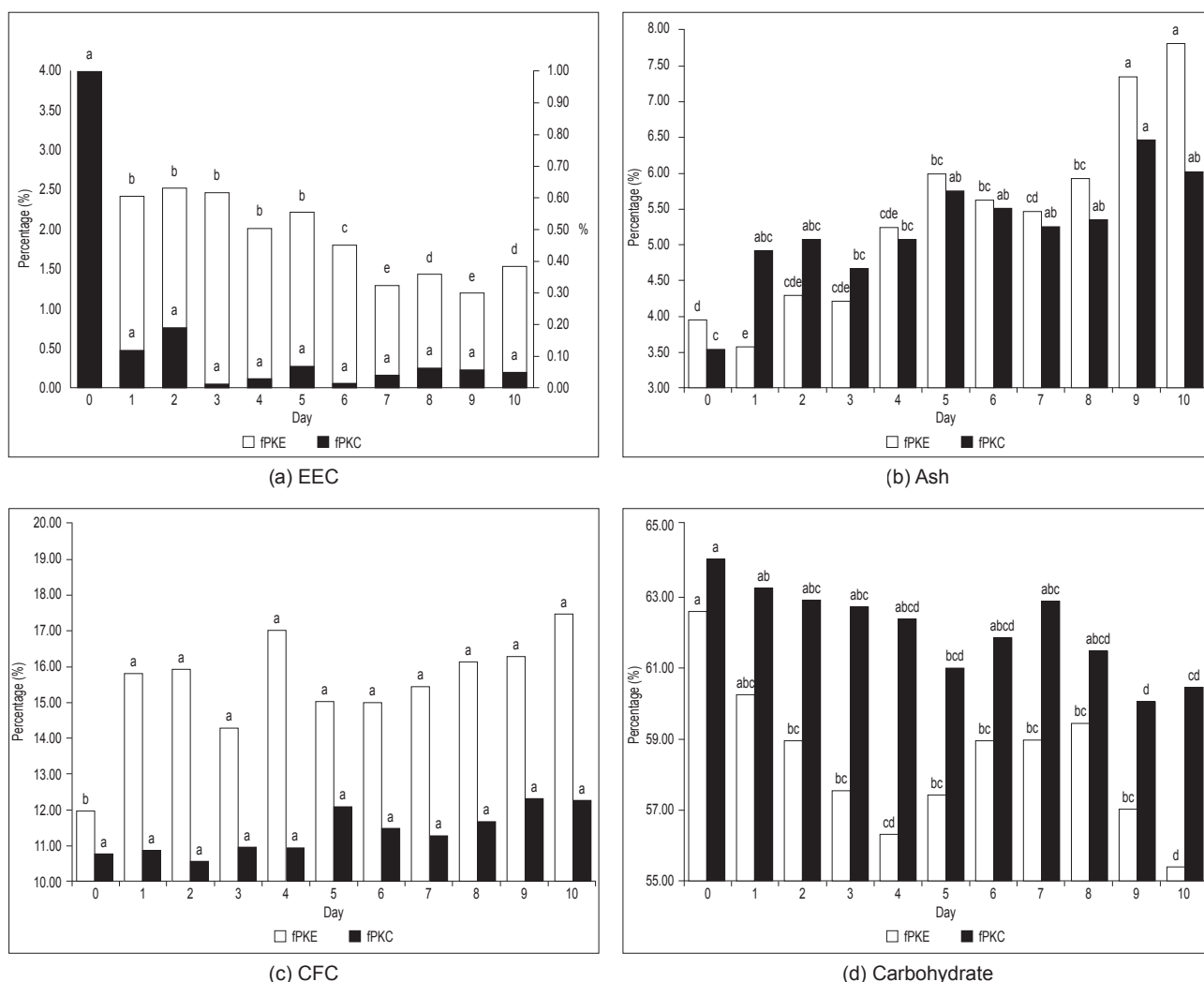
properties in PKE and PKC. In addition, PKC indeed contained high fibre content for livestock (Agunbiade *et al.*, 1999; Perez *et al.*, 2000).

Carbohydrate content. The lowest carbohydrate content was recorded on Day 10 of fermentation on PKE at 55.39% (w/w) (Figure 5d). The decrease in carbohydrate content of fPKE was found to be significantly different ($p < 0.05$) from that of PKE without fermentation (63.36% w/w), starting on Day 4. For fPKC, the decrease in carbohydrate content was significantly different ($p < 0.05$) from the control PKC from Day 5 of fermentation while Day 9 recorded the lowest carbohydrate content at 60.06% (w/w). However, carbohydrate content on Day 9 was not significantly different ($p > 0.05$) from Day 5 of fermented PKC. According to Gao *et al.* (2013), the reduction of starch through its use by microorganisms is a major cause for the increase in CPC.

Energy content. It was found that the energy content decreased from Day 1 to Day 10 for fPKE and fPKC and the decrease was significantly different ($p < 0.05$) from Day 0 (Figure 6a). The lowest energy content was recorded on Day 10 of fermentation (306.54 kcal/100 g) for fPKE while for fPKC, it was on Day 9 (325.17 kcal/100 g).



Figure 4. Phylogenetic analysis showed the relationship between amplitude A and its neighbours (the phylogenetic tree was based on the final alignment of the 1171 bp strain.) neighbour-joining phylogenetic tree showed maximum likelihood model showing the relationship based on 18S rRNA gene sequence alignments. The length at the branching points are the percentages of occurrence in hundreds bootstrapped trees.



Note: ^{a,b,c,d,e} Min with different superscript is significantly different ($p < 0.05$).

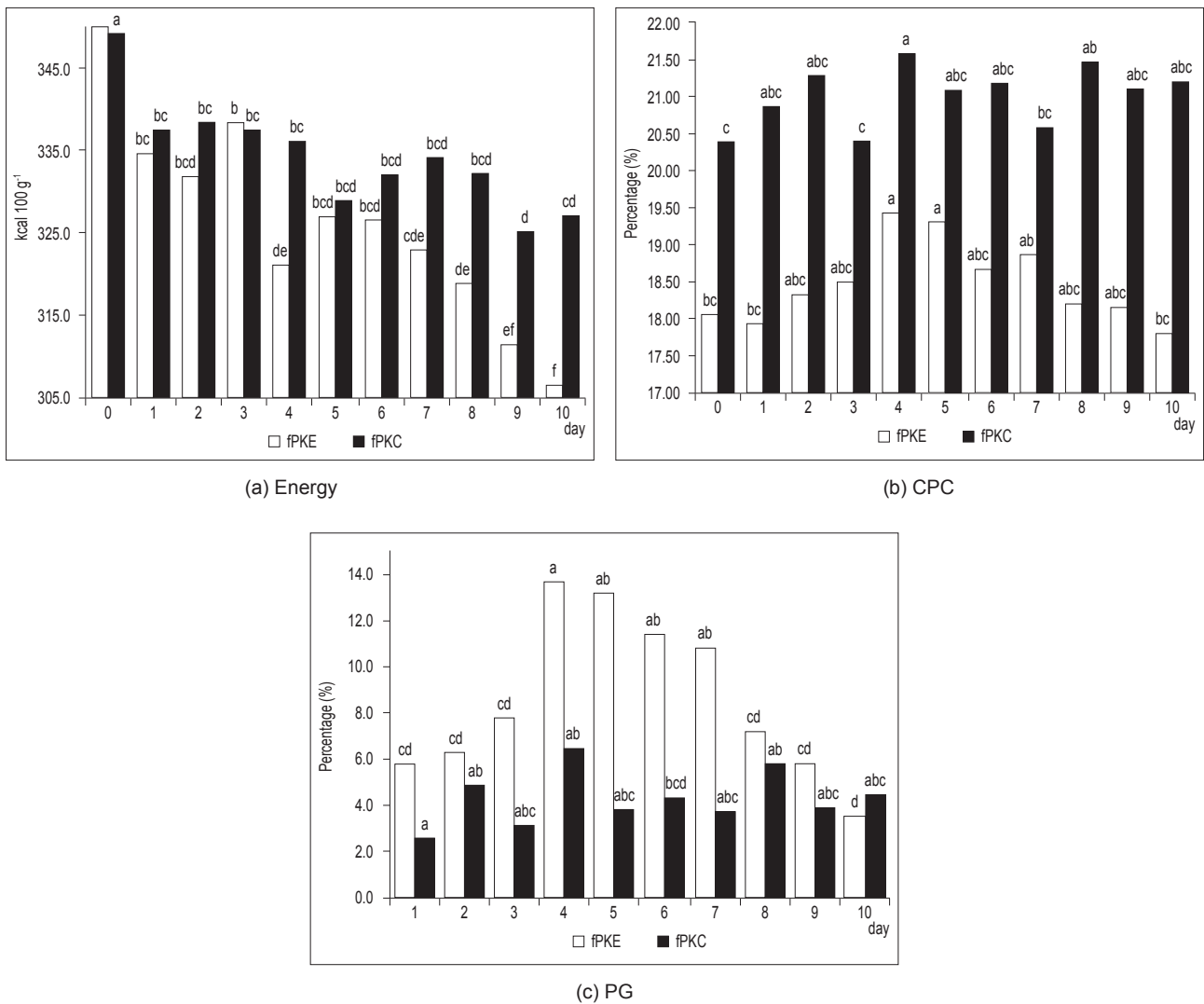
Figure 5. Effect of solid state fermentation by *Rhizopus oryzae* ME01 on (a) ether extract content (EEC), (b) ash, (c) crude fibre content (CFC), and (d) carbohydrate.

The determination for energy is due to three factors namely EEC, CPC and carbohydrates (Galla *et al.*, 2012). It was found that the decrease in energy content in this study was directly proportional to the decrease in EEC and carbohydrate content in fPKE and fPKC. Energy in fermented products was found to be depleted daily due to the metabolism of *R. oryzae* ME01 which utilised nutrients in PKE and PKC particularly, EEC and carbohydrates, for the production of its biomass. The depletion of energy in fermented products could affect the energy requirement of animal but it can be balanced by addition of other raw materials such as corn or palm oil in the animal feed.

Crude protein content (CPC). Figure 6b shows the effect of SSF by *R. oryzae* ME01 on the CPC in fPKE and fPKC. For fPKE, Day 4 and Day 5 of SSF recorded the highest CPC at 19.44% (w/w) and protein gain (PG) at 19.32% (w/w), respectively. These increases

were significantly different ($p < 0.05$) from other days as well as Day 0 (non-fermented PKE). Meanwhile, the CPC in fermented PKC from Day 1 to Day 10 was not significantly different ($p > 0.05$) from the control except Day 4 which showed highest protein increase of 21.59% (w/w). In a study by Swe *et al.* (2003) on PKC, it was found that protein increase was significant on Day 8, from 16.8% to 29.6% of SSF yield by *A. niger*.

One factor contributing to the difference in CPC between fPKE and fPKC in this study was the size of the PKC as substrates. PKC has uniform size and light colour appearance due to solvent extraction process. The size of PKC was as small as 0.2 mm, while PKE has a non-uniform size and larger than 0.2 mm. According to Schmidt and Furlong (2012), smaller substrate sizes will result in higher CPC than large-sized substrates. However, large sized substrates will have larger surface area and thus, increases the *R. oryzae* biomass.



Note: ^{a,b,c,d,e,f} Min with different superscript is significantly different ($p < 0.05$).

Figure 6. Effect of solid state fermentation by *R. oryzae* ME01 on (a) energy, (b) crude protein content (CPC), and (c) protein gain (PG).

Figure 6c shows the effect of SSF by *R. oryzae* ME01 on PG in fPKE and fPKC. On Day 4 and 5, fPKE indicated the highest PG at 13.63% (w/w) and 12.82% (w/w), respectively. For fPKC, Day 4 recorded the highest PG at 6.46% (w/w). The relationship between CPC and PG was proportional. The highest CPC led to highest PG. Although fPKC recorded higher CPC than fPKE, the PG of fPKE exceeded that of fPKC by two folds. The increase in CPC could be due to the use of fungi and starch (Gao *et al.*, 2013). Since PKE contained higher EEC than PKC, the increase in CPC of fPKE was higher than that of control PKE at 7.64% (w/w), whereas changes in fPKC by 3.45% (w/w) did not differ from PKC and was even lower than fPKE (Table 3).

Results summarised in Table 3 showed significant changes for carbohydrate content, EEC and ash content with a decrease in carbohydrate and EEC content by 11.52% and 55% (w/w), respectively in fPKE. The reduction of carbohydrate is a good

indicator so that more fermented PKE and PKC could be added in animal diet due to lesser non-soluble starch in the animal feed but the decrease of fat has to be balanced by adding other fat sources such as palm oil. There was an increase in protein and ash content by 7.6% (w/w) and 200% (w/w), respectively, in fermented PKE by *R. oryzae* ME01. This suggested that the action of fungus which involved the hydrolysis of various enzymes including the action of lipolytic enzymes on lipids in PKE caused a change in nutrient composition in fPKE. Similar trends occurred for carbohydrate content, EEC, protein and ash content in fPKC which verified this fungal activity. It was found that there was a decrease in carbohydrate content by 2.32% (w/w) and EEC content by 100%. The protein and ash content increased by 3.45% (w/w) and 18.35% (w/w), respectively. Small changes in fPKC compared to fPKE are likely driven by differences in fat content as suggested by Gao *et al.* (2013).

TABLE 3. CHANGES IN PKC's NUTRITION CONTENT AFTER FERMENTATION

Nutrition type	PKE	fPKE	% Change	PKC	fPKC	% Change
Crude protein content (CPC) (%w/w)	17.25	19.44	+7.64	20.31	21.59	+3.45
Crude fibre content (CFC) (%w/w)	11.97	16.99	+41.94	10.77	12.35	+14.67
Carbohydrate content (%w/w)	63.36	55.39	-11.52	64.05	59.92	-2.32
Ether extract content (EEC) (%w/w)	3.44	1.29	-55	1.27	0.01	-100
Ash content (%w/w)	3.94	7.81	+200	3.52	6.47	+18.35
Energy content (kcal/100 g)	353.25	306.54	-13.22	335.51	325.17	-3.08

Note: PKC - palm kernel cake; fPKC - fermented palm kernel cake; PKE - palm kernel expeller; fPKE - fermented palm kernel expeller.

CONCLUSION

In conclusion, the enhancement in nutritional value for PKC particularly protein content through SSF using a locally isolated fungus was successfully conducted. The changes in fermented PKC nutrients showed that the ash and protein content increased by 18.26% and 3.45%, respectively. After 10 days fermentation, carbohydrate and fat content decreased significantly by 2.32% and 98%, respectively. The increase of ash and protein content for fermented PKE was significant ($p < 0.05$) where ash and protein content increased by 200% and 7.64%, respectively, while carbohydrate (11.52%) and fat content (55%) decreased after 10 days of SSF. Thus, PKE showed better performance for fermentation by *R. oryzae* ME01.

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