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Performance of locally isolated microorganism in degrading palm kernel cake (PKC) fibre and improving the nutritional value of fermented PKC

[Keupayaan mikroorganisma yang diasingkan dari persekitaran dalam penguraian gentian hampas isirung kelapa sawit (HIKS) dan peningkatan mutu pemakanan HIKS terawat]

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Key words: local isolates, fermented PKC, solid substrate fermentation

Abstract

Palm kernel cake (PKC) was fermented with a few types of microorganism using solid substrate fermentation (SSF) technique for the degradation of fibrous compounds and nutritive value improvements. The microorganisms were locally isolated to get specific enzymes for degrading the fibrous compound and improving the nutritional value of the raw material. After screening, identification and characterization of selected microorganisms, only a few microorganisms with insignificant toxicity problem were used as an inoculum in SSF of PKC. Results showed that the nutritive value of treated PKC with fungi increased and it could be used to substitute certain amounts of corn in poultry feed.

Introduction

Oil palm, *Elaeis guineensis*, is an important crop of Malaysia. Since the 1970s, Malaysia has been the largest producer and exporter of palm oil products in the world, namely palm oil and palm kernel oil (PKO). On top of these, there are a number of useful by-products such as oil palm fronds (OPF), oil palm trunks (OPT), palm press fibre (PPF), empty fruit bunches (EFB), palm kernel cake (PKC), palm oil mill effluent (POME; also called sludge and decanter cake) and palm kernel shells (PKS). These by-products are obtained in stages from the harvesting of the fruits, the extraction of palm oil or the refining of PKO. PKC and OPF in particular are suitable for feeding beef and dairy animals. They are abundantly produced throughout the year in Malaysia and this guarantees their supply and availability as major ingredients for livestock feeding. These materials are also being utilized as base materials in the manufacturing of specific industrial products, furnitures and organic fertilizers. Current research emphasizes on the use of PKC for poultry feed.

PKC is a low energy feed with moderate amount of crude protein (14–16%) and high in crude fibre (17%). It contains 31% acid detergent fibre (ADF) and 72% neutral detergent fibre (NDF). Other nutrients such as carotene, vitamin E, calcium, phosphorus and trace minerals are also available. In the total cell wall, mannose is the principle neutral sugar

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(56.4%), followed by glucose (11.6%), xylose (3.7%) and galactose (1.4%) (Anon. 2002). The amount of residual oil in PKC depends on the type of extraction used; either solvent-extracted or expeller-pressed. The oil content in the solvent-extracted PKC is low, around 0.5–3%, while the expeller-pressed PKC contains between 5–12% oil.

The PKC comprises mainly cell wall. This cell wall consists largely of polysaccharides such as mannan which is responsible for the low digestibility of PKC by poultry. Mechanical and chemical processes could increase digestibility of feedstuffs without much altering its nutrient content. Through biological processes such as fermentation, there are also possibilities for the improvement of digestibility, protein efficiency ratio and amino acid availability. During fermentation, improvements in vitamin content and destruction of anti-nutritional factors could also be achieved due to the presence of related enzymes.

Selective enrichment technique was conducted to isolate PKC degrading microorganisms from rotting PKC and the environment in order to obtain specific enzymes to degrade the fibrous compound in PKC. The application of selective enrichment medium, which contains PKC as a carbon and nitrogen source, supported the growth of desired microorganisms and inhibited the growth of the unwanted ones. Commercial substrates were selected, based on the type of compounds in PKC fibre like mannan (large proportion), cellulose and small quantities of galactomannan and xylan (Daud and Jarvis 1992; Dusterhoft et al. 1992) for screening processes.

A previous study (Daud et al. 1997) showed that PKC-based diet supplemented with mannanase produced higher ME values than the straight PKC-based diet. It was also reported (Yahya et al. 2000) that by mixing Hemicell enzyme (high in mannanase) with 10–15% palm kernel expeller (PKE) in broiler chicken feed, growth and feed efficiency of the birds increased. However, these commercial enzymes were not specific to PKC. Hence, it was expected that by using biological fermentation, microorganisms would produce enzymes that are related to the substrates.

This study was conducted to evaluate the performance of selected microorganisms in degrading and improving the nutritive values of PKC using solid substrate fermentation (SSF). From previous studies, it was found that bacteria, yeast and fungi could grow on solid substrates. Filamentous fungi are the most commonly used microorganisms in SSF because they are able to grow on solid materials with low water content (Pandey 1992). Bacteria are mainly involved in composting, ensiling and some food processing (Doelle et al. 1992). Yeast could be used for ethanol and food or feed production (Saucedo-Castaneda et al. 1992).

Materials and methods

Isolation of PKC fibre degrading microorganism

Soil and compost samples were collected from oil palm plantations and oil palm feed mill areas (fresh and rotting PKC) in order to obtain potential microorganisms, which could produce specific enzymes such as mannanase, glucanase and xylanase to degrade the fibrous compound in PKC. The samples (5.0 g) were added onto 50 ml mineral media (Arisan et al. 1993) with 10% PKC as a carbon source. The cultures were incubated at 30, 40 and 50 °C on an orbital shaker at 150 rpm. After seven days of incubation, 1.0 ml of the culture (designated as inoculum) was transferred onto fresh media. Serial transfers of the cultures to fresh media were conducted until the microbial culture became stable or the same organisms were obtained and grew on nutrient agar (NA) and potato dextrose agar (PDA). Microorganisms which survived in selective enrichment media and formed clear zones on commercial substrates were selected for further experimentation.

Screening of microbial activities

Screening of PKC fibre-degrading microorganisms was conducted using commercial substrates from Megazymes: Azo-Carob Galactomannan (S-ACGLM), Azo-xylan (S-AXYO) and Azo-Cm-Cellulose (S-ACMC) in formulated media (Kusakabe and Takahashi 1986). Overnight microbial cultures in liquid media were used for screening of endo-1,4-ß-D-mannanase, endo-1,4-ß-D-glucanase and endo-1,4-ß-D-xylanase activities correlated to the major compounds in PKC. Diameters of clearing zones were measured after 24, 48 and 72 h of incubation on the formulated solid media at 37 °C. After screening enzyme activities of the stable cultures, a few cultures were selected for further study. The selection was based on the diameter of clearing zones produced by the microbes.

Characterisation and identification of selected microorganism

Identification of selected bacteria cultures was done using biochemical analyses, microscopic morphological examinations and API kits. For fungal and yeast cultures, microscopic morphological examinations were conducted using the microslide culture technique and identifications were made using Biolog Microlog Identification System. Characterisations of mixed cultures were done using biochemical analyses and microscopic morphological examinations. For actinomycete cultures, microscopic morphological characterisation were made using Environmental Scanning Electron Microscope (ESEM).

Toxicity study

Toxicity studies of the fungal isolates were carried out to determine the possibility of the potential microorganisms producing mycotoxins as a secondary metabolite. In this study, aflatoxin was determined using an analytical method (Goto et al. 2002). A semi-quantitative TLC method was used to analyse aflatoxin in the samples with a detection limit of 5 ppb. Aflatoxin was extracted from the culture with 80% acetonitrile, separated and cleaned-up with multifunctional cleaning column (Romer No. 226/228) and evaporated to dryness with N_2 gas on a heating block (50 °C). The mycotoxin was dissolved in 100 µl toluene:acetonitrile (98:2) and spotted on TLC plate (Merck No. 5721). A mixture of aflatoxin (Sigma, A 9441) was spotted as a control. The plate was then developed for 40 min in toluene, ethyl acetate and formic acid (TEF), dried in a dark place at room temperature for 40 min before illumination under long-wavelength (365 nm) UV light in a dark cabinet. Analyses of ochratoxin A in the sample were done with a commercial kit (RIDASCREEN-FAST Mycotoxin) assay utilising enzyme immunoassay techniques for detection of toxin samples of untreated PKC (raw material) and fermented PKC.

Evaluation of selected microorganism in SSF of PKC

The performance of selected cultures in degrading PKC fibre and improving nutritional value of PKC after fermentation was evaluated. Several analyses were conducted in the study including reducing sugar test, fibre analyses, mannanase activity, soluble and crude protein analyses. In chemical analyses of fibre, neutral detergent fibre (NDF) and acid detergent fibre (ADF) were analysed (Van Seost 1966). NDF represents total fibre in plant cell walls and consists of lignin, cellulose and hemicellulose, while ADF represents the less digestible components which consist of lignin and cellulose.

The soluble protein and reducing sugar were measured using Bio-rad protein microassay procedure based on the Bradford method (Bradford 1976) and dinitrosalicylic acid (DNS) method (Miller 1959), respectively. Crude protein was analysed using near infrared reflectance spectroscopy model 6500 (NIRSystems, MA, USA) and the spectra were processed using NSAS software. ß-Mannanase activity was determined using pure *Aspergillus niger*

ß-mannanase as standard (McCleary 1978). One unit of ß-mannanase activity is defined as the amount of enzyme required to release one micromole of mannose reducing sugar equivalents per minute under the defined assay conditions i.e. per ml filtrate (U/ml). Mannose was determined using the value of linear regression of mannose standard.

Results and discussion

Isolation, screening and characterisation of PKC fibre degrading microorganism

By using enrichment culture technique for the isolation, undesired organisms were eliminated from the culture, through competitive exclusion or dilution. After screening enzyme activities of the stable cultures from every batch of isolation, only a few cultures were selected for further study. Six yeast-like fungal cultures were isolated and selected for further study. The selection was based on the diameter of clearing zones produced by the yeast. The selected cultures were G3, G1 and H3, which were isolated from soil samples; F2 isolated from rotting PKC; D1 from PKC mixed with waste; and C3 from co-agulated PKC samples. All cultures showed similar diameter sizes of their respective clearing zones on the three substrates *(Table 1).*

The colonies also showed similar morphological characteristics on PDA plate with pleomorphic yeast-like cells, rapid growing, raised, folded and cream coloured colonies. Microscopic morphological examinations showed that the cells could produce septate hyphae while the arthroconidia were unicellular and elongated in shape. The above cultures were identified using Biolog YT Microplate and the results were automatically read by Biolog Microlog MicroStation. The cultures were identified as *Trichosporon beigelii B (Plate 1).*

Table 1. Diameter of clearing zones (after three days incubation) of selected yeasts on formulated media

Cultures	Mannan (cm)	Cellulose (cm)	Xylan (cm)
G1	3.9 ± 0.10	3.4 ± 0.06	–
G3	4.0 ± 0.00	3.5 ± 0.10	–
F2	4.0 ± 0.00	3.6 ± 0.00	–
D1	4.0 ± 0.06	3.5 ± 0.06	–
C3	4.0 ± 0.00	3.5 ± 0.12	–
H3	4.0 ± 0.06	3.5 ± 0.00	–

Values represent means (± STD) and (–) represent no clearing zone

Two types of actinomycetes, three types of bacteria and two fungal cultures; which were isolated from oil palm by-products compost and soil, were selected for further study. From microscopic examinations and biochemical analyses, the three types of bacteria cultures (P1, P2 and P3) were gram positive, rod shape and gave positive results for catalase and oxidase analyses. These bacteria were identified using API 50 CH strips and API 50 CHB medium (bioMérieux, Lyon, France). The results in biochemical profiles were interpreted using APILAB software.

After 48 hours of incubation at 37 °C, the results showed that P1 is *Bacillus circulans (Plate 2),* P2 is *B. subtilis* and P3 is *B. licheniformis* with identification percentages of 95.6%, 95.6% and 93.0%, respectively. From the results, all bacteria showed similar diameter of clearing zone on the three substrates except that there was no activity of cellulase by P2 and xylanase activity by P3. After two days of incubation, the diameters of clearing zone of P1, P2 and P3 on mannan substrate were all 3.7 cm. Clearing zones of P1 and P3 on cellulose substrate were 2.8 and 3.0 cm, respectively while the diameters for P1 and P2 on xylan substrate were both 1.5 cm.

From microscopic morphological examinations of the two selected fungal cultures, both fungi were identified as *Aspergillus* sp. One of the fungi was a thermophilic fungus and it could grow in an environment of up to 50 °C. The culture was fast growing on PDA and colonies showed typical blue-green surface pigmentation with a suede-like surface consisting of a dense felt of conidiophores. The conidial heads were typically columnar and uniseriate. The

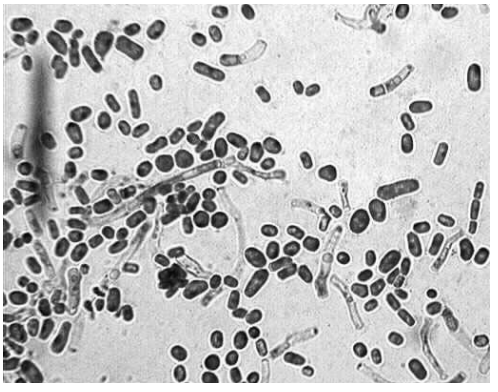

Plate 1. ***Trichosporon beigelii B***

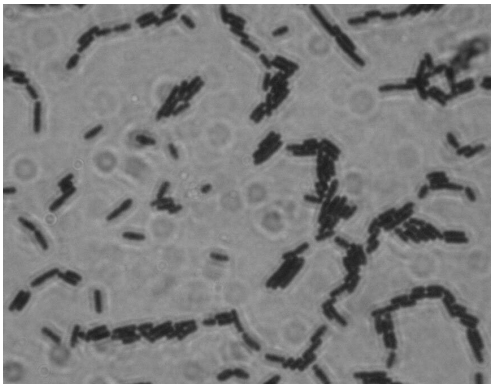

Plate 2. ***Bacillus circulans***

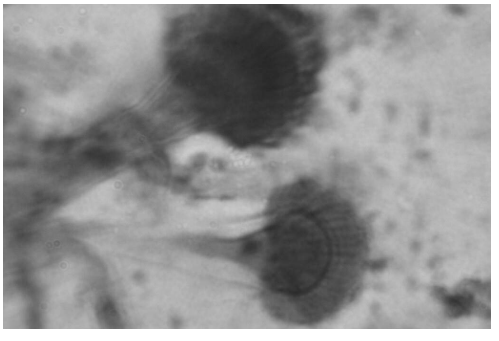

Plate 3. ***Aspergillus fumigatus***

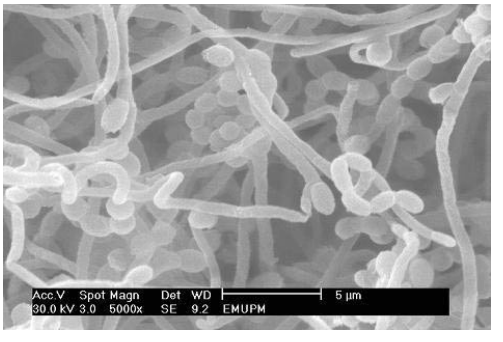

Plate 4. ***Streptomyces*** *sp.*

conidiophores were short, smooth-walled and had conical-shaped terminal vesicles, which supported a single row of phialides on the upper two thirds of the vesicle. The second fungus culture was a mesophilic fungus, with an optimum growth temperature of between 26 and 30 °C. It produced yellowish white mycelia with large black conidia from biseriate phialides on PDA media. The fungi culture was sent to CABI Bioscience, United Kingdom for confirmation of the species. The thermophilic fungus was identified as *A. fumigatus (Plate 3)* while the mesophilic fungus was identified as *A. niger*.

For actinomycete cultures, microscopic morphological examinations with ESEM showed that Z-50 belongs to *Streptomyces* sp. and Z-30 belongs to *Nocardia* sp. The micrograph of Z-50 showed the coiled or spiral-fashions conidia and mycelia of *Streptomyces* sp. *(Plate 4)*. Micrograph of Z-30 showed the vegetative hyphae were fragmented into bacteroid, rod-shaped and cocoids as the cells matured and shared by external forces. *Streptomyces* sp. is a thermophilic actinomycete and can grow in an environment of up to 50 °C, while *Nocardia* sp. belongs to mesophilic group with an optimum growth temperature of 30 °C. From macroscopic morphology examinations, both actinomycete cultures appeared dry, tough and leathery in texture on water agar. The extensively branching mycelia formed also gave stronger degree of adherence to solid medium.

Toxicity study

After identification of selected cultures, six yeast-like fungal cultures *(T. beigelii B)* and one fungal culture *(A. fumigatus)* could not be used as an inoculum for production of fermented PKC because they were pathogenic to human and animals. Analyses of toxins in the raw material and fermented PKC showed that the levels of aflatoxin and

ochratoxin A detected were insignificant and below the detection limit (5 ppb). The recovery of aflatoxins from samples spiked at 20 ppb (mixed aflatoxins – B_1, B_2, G_1 and G_2) was 70% to 80%. However, aflatoxins were produced and detected in sterilized PKC sample that was inoculated with a potent aflatoxin producing strain; *A. flavus* and *A. parasiticus*.

Therefore, precautions must be taken to prevent contamination from these types of fungi on PKC since under the right conditions (temperature and moisture content), the fungi could grow and produce toxin. The contamination could happen during processing, at farm level or during storage of feeds. Mould contamination can be controlled when the moisture content is less than 13%. Storage under good conditions and over a reasonable period of time would minimize further elaboration of the toxin by these toxigenic fungi.

Evaluation of selected microorganism in SSF of PKC

Results from fermentation of selected miroorganisms in PKC using SSF technique showed that only fungi cultures could grow and degrade certain amount of fibre in the raw material. The reduction of NDF and ADF in fermented PKC with *A. niger* were 30.39% and 14.58%, respectively *(Table 2)*. For fermented PKC with *A. fumigatus*, the reductions of NDF and ADF were 30.33% and 13.36%, respectively.

Although there was a significant reduction of fibre in fermented PKC with *A. fumigatus*, the culture could not be used as inoculum for fermented feed because it is pathogenic to animals and human (Latge 1999). Based on the same work (Daud et al. 2003), the metabolisable energy (ME) of PKC treated with a different strain of *A. niger* increased from 6.20 MJ/kg to 9.0 MJ/kg. For PKC treated with actinomycete cultures (*Nocardia* sp. and *Streptomyces* sp.), there was no significant reduction of fibre (% NDF) in the produce *(Table 3)*.

The study of bacteria cultivation on PKC using SSF technique showed that the selected bacteria cultures could grow in the substrate with 60% moisture content. However, results showed that there was no significant reduction of fibre in the produce *(Table 4)*. It is well known that the strains isolated and developed for use in SSF

Table 2. Chemical analyses of treated PKC with fungi cultures

Cultures	% NDF	% ADF	Reducing sugar (mg/g substrate)	Soluble protein (mg/g PKC)	Mannanase (U/g PKC)
Control (untreated PKC)	76.86 ± 2.07a	45.00 ± 2.34a	3.84 ± 0.34a	1.31 ± 0.24a	18.47 ± 0.71a
Aspergillus niger	53.50 ± 0.63b	38.44 ± 1.31b	3.56 ± 0.54a	15.61 ± 3.00b	60.47 ± 4.33b
Aspergillus fumigatus	53.55 ± 4.21b	38.99 ± 2.59b	2.14 ± 0.22b	6.36 ± 1.33c	20.84 ± 7.94a

Data are presented in the mean values ± STD. Values with different letters within a column are significantly different (p <0.05)

Table 3. Chemical analyses of treated PKC with actinomycete cultures

Cultures	% NDF	% ADF	Reducing sugar (mg/g substrate)	Soluble protein (mg/g PKC)	Mannanase (U/g PKC)
Control (untreated PKC)	85.20 ± 3.49a	51.55 ± 0.62a	1.40 ± 0.16a	0.74 ± 0.01a	4.06 ± 0.02a
Nocardia sp.	93.19 ± 3.88a	51.30 ± 1.17a	2.15 ± 0.62a	0.84 ± 0.10a	4.29 ± 0.12a
Streptomyces sp.	89.67 ± 0.34a	57.22 ± 0.02b	1.63 ± 0.34a	1.06 ± 0.13a	5.14 ± 0.71a

Data are presented in the mean values ± STD. Values with different letters within a column are significantly different (p <0.05)

Table 4. Fibre analyses of treated PKC with selected bacteria cultures

Cultures	% NDF	% ADF
Control (untreated PKC)	76.53 ± 0.92	40.04 ± 0.08
Bacillus circulans	76.21 ± 1.90	39.59 ± 1.27
Bacillus subtilis	78.45 ± 1.40	40.40 ± 0.29
Bacillus licheniformis	79.13 ± 0.62	40.66 ± 0.16

Values in the rows are not significantly different (p <0.05)

Table 5. NIR analyses of treated PKC with selected bacteria cultures and *Aspergillus niger* in sequential cultivation

Treatment	% NDF	% ADF	% Crude protein
Control (untreated PKC)	73.10 ± 1.74a	41.00 ± 0.81a	15.52 ± 0.31a
Aspergillus niger	43.98 ± 1.65b	32.88 ± 0.36b	19.78 ± 1.07b
A. niger + distilled water	47.15 ± 0.62bc	32.60 ± 0.63b	19.35 ± 1.61b
A. niger + *Bacillus circulans*	50.06 ± 2.73cd	33.74 ± 1.00b	19.47 ± 0.34b
A. niger + *B. subtilis*	44.87 ± 1.16bd	32.96 ± 0.15b	19.62 ± 0.36b
A. niger + *B. licheniformis*	46.96 ± 2.56bd	33.32 ± 0.68b	18.73 ± 0.60b

Data are presented in the mean values ± STD. Values with different letters within a column are significantly different (p <0.05)

processes are poor producers of enzyme in the submerged (SmF) system and vice versa. It seems that under the isolation technique which used liquid media in this study, the selected bacteria cultures could produce higher mannanase in SmF system (Jameáh et al. 2003) than in the SSF system. The requirement for critical moisture content by the microorganisms determines their ability to grow and produce the enzyme, or any other metabolite in the SSF system. With this type of raw material, the isolated microorganism, other than fungal strains, could be applied in the PKC fermentation process through co-culture or sequential cultivation mode.

A preliminary study was carried out using the bacterial cultures in sequential cultivation with fungi for PKC fermentation. After first fermentation with fungi, complex compounds in the PKC were partially degraded to simple compounds and at the same time could provide simple sugar for the bacteria to grow. In the study, the moisture content of the two-day fermented PKC with *A. niger* had been increased from 50–60% before the addition of bacterial culture. Results from the first experiment *(Table 5)* showed that the contributions of the three types of *Bacillus* sp. were not significant in PKC fibre degradation after comparing with positive control (fermented PKC with *A. niger* alone, with or without the addition of moisture content). Further experiments need to be carried out on adjustment and optimization of the temperature, moisture content and pH of the culture before the addition of the bacteria cultures.

Conclusion

From this study, it could be concluded that only fungi cultures contributed to fibre degradation and improved the nutritive value of fermented PKC. For the substitution of certain amounts of corn by the fermented PKC for poultry feed, further research involving animal trials and toxicology tests needs to be carried out to study poultry growth performance and to determine the side effects of secondary metabolites in the fermented PKC, which could cause acute or chronic toxicity to poultry. As for the locally isolated microorganisms, wild-type cultures usually could not produce the desired enzymes in commercially viable quantities compared to improved strains. Increased yields of enzymes may be achieved by

optimizing the culture medium and growth conditions, but this approach would be limited by the organism's maximum ability to synthesize the enzymes. This is because the potential productivity of the organism is controlled by its genome which needs to be modified to increase the potential yield. Therefore, further studies on strain improvement of wild-type cultures would help to improve the performance of local isolates.

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Abstrak

Hampas isirung kelapa sawit (HIKS) difermentasikan dengan beberapa jenis mikroorganisma menggunakan teknik fermentasi substrat pepejal bagi mengurai bahan-bahan yang berserabut dan meningkatkan mutu pemakanannya. Mikroorganisma berkenaan diasingkan dari persekitaran bagi memastikan enzim yang spesifik dihasilkan sewaktu proses fermentasi, agar serabut di dalam HIKS dapat diuraikan. Hanya beberapa jenis mikroorganisma yang tidak mempunyai masalah ketoksikan yang ketara sahaja dipilih selepas proses penyaringan, pengenalpastian dan pencirian. Mikroorganisma ini digunakan sebagai inokulum dalam fermentasi substrat HIKS. Hasil kajian menunjukkan nilai pemakanan HIKS yang telah difermentasikan dengan fungus didapati meningkat dan boleh digunakan untuk menggantikan sebahagian daripada jagung di dalam rangsum ayam.

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