



## ***Emericella nidulans* (4DP5), *Cladosporium herbarum* (7DF12) and *Bacillus subtilis* improve the nutritional value of palm kernel cake (PKC) through solid-state fermentation (SSF)**

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Received 9 November 2021; Received in revised form 26 May 2022; Accepted 27 June 2022

### **ABSTRACT**

**Aims:** Palm kernel cake (PKC) is a high-protein, high-energy food that is widely utilized in the animal feed business. However, the high fibre and limited amino acid content of untreated PKC were the main issues for it to be used as animal feed, particularly in non-ruminants. To improve the quality of PKC, this study combined the use of solid-state fermentation (SSF) and consortia of fungi and bacteria to treat the PKC.

**Methodology and results:** Two fungi, *Emericella nidulans* (4DP5) and *Cladosporium herbarum* (7DF12) and three strains of bacteria, *Bacillus subtilis*, which were active mannanase producers, were used in different combinations to reduce the hemicellulose content and improve the crude protein content of PKC in a lab-scale solid-state fermentation. PKC inoculated separately with five types of mixed culture treatments were allowed to ferment. The fermentation conditions were 20% inoculum (w/v), 85-92% humidity, pH 7.0 and PKC particle size 0.8 mm. PKC treatments with two fungi, *E. nidulans* (4DP5) and *C. herbarum* (7DF12), as well as a fungus-bacterium combination, *E. nidulans* (4DP5) and *B. subtilis*, outperformed the other three treatments. The crude protein levels were increased by 3.34% and 1.86%, respectively, due to these treatments. Furthermore, the level of aflatoxins produced increased marginally but remained within the permissible limits.

**Conclusion, significance and impact of study:** The treated PKC has more sugar and crude protein and less than 20 parts per billion (ppb) of aflatoxin, making it appropriate for animal consumption. The SSF technique of combining fungi and *Bacilli* enhanced the nutritional and market value of PKC substantially, which can be upscaled.

**Keywords:** Palm kernel cake, fungi, *Bacillus*, solid-state fermentation

### **INTRODUCTION**

Palm kernel cake (PKC) is a valuable by-product of the oil palm industry that is widely used in the animal feed industry. PKC is found to be a high-energy feed with a crude protein content of 16% (CP). PKC is a fibre with a hemicellulose content of 78% and a cellulose content of 12%. Mannose is the most abundant neutral sugar in the cell wall (56.4%), followed by glucose (11.6%), xylose (3.7%) and galactose (3.7%). Copper (Cu) levels range from 0.02 to 0.03%, calcium (Ca) is 0.3% and phosphorus (P) is 0.7% (Malaysian Palm Oil Council, 2002). However, PKC has poor amino acid content, especially lysine, methionine, cysteine and tryptophan (Aspar, 2001).

The main problem faced in the application of PKC as animal feed, especially in the non-ruminants is the high fibre content of untreated or raw PKC. Mannan as the major fibre component in PKC is an anti-nutritive agent that inhibits the utilization and absorption of amino acids

(Daud and Jarvis, 1992; Jana and Kango, 2020). Currently, the use of commercial enzymes to improve the availability of nutrients in PKC is not cost-effective because of the relatively high enzyme costs when compared to the price of treated PKC used in animal feed. One alternative to curb this problem is by targeting microbes with the ability to produce fibrolytic enzymes, especially mannanase, cellulase and xylanase, to degrade the fibrous materials in PKC.

Solid-state fermentation is the preferred approach for bioconversion of PKC into poultry feed using good microorganism strains since it encourages the natural environment for microorganisms to grow and there is less waste at the end because the entire product can be used directly as animal feeds (Illuyemi *et al.*, 2006; Azizi *et al.*, 2021). Thus, good strains of  $\beta$ -mannanase producing microorganisms are crucial to improving the quality of PKC through SSF. The ability to produce additional fibrolytic enzymes such as cellulase and xylanase are

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additional advantages that will remove fibre more effectively. Findings from the literature show that many strains of  $\beta$ -mannanase producing microorganisms screened were reported to be *Bacillus* sp., which includes *Bacillus* sp. KK01 (Hossain *et al.*, 1996); *Bacillus licheniformis* (Feng *et al.*, 2003); *Bacillus* sp. (Abe *et al.*, 1994); *Bacillus subtilis* ATCC 3366 (Chin, 2006); *Bacillus circulans* (Pangsri *et al.*, 2015) and *Bacillus* sp. N16-5 (Liu, 2021).

However, bacterial cells alone may not be able to penetrate fibrous PKC efficiently and have less efficiency in the mineralization of complex polymeric compounds such as lignin because they lack the invasive properties that hyphae, mycelium or rhizoid provide. Therefore, fungi play an important synergistic role in forming mycelium to penetrate and weaken the structure of PKC. Several strains of fungi have been studied for their potential to improve the nutritive value of PKC, including *Trichoderma koningii*, *Trichoderma harzianum*, *Trichoderma longibrachiatum*, *Sclerotium rolfsii*, *Aspergillus niger* and *Rhizopus azygosporus*. Fungal culturing of PKC also brought about an increase in the level of unsaturated fatty acids and a decrease in the level of saturated fatty acids (Illuyemi *et al.*, 2006; Arsy *et al.*, 2020). However, filamentous fungi like *Fusarium*, *Aspergillus* and *Penicillium* produce mycotoxins as secondary metabolites (Binder, 2007). Aflatoxins are the most known mycotoxin as they reduce growth and feed efficiency, and cause liver and kidney damage (Bintyihok, 2002; Coppock *et al.*, 2018). The Food and Drug Administration (FDA) in the United States has set a maximum permitted aflatoxin concentration of 20 ppb in general animal feed (Kumar *et al.*, 2020; Marshall *et al.*, 2020). As a result, the aflatoxin level in fungal-treated PKC must not exceed this limit. This work aims to analyze the reducing sugar,  $\beta$ -mannanase, crude protein and aflatoxin content in the treated and untreated PKC from SSF by employing different mixed culture treatments.

## MATERIALS AND METHODS

### Fungal and bacterial strains

Two species of fungi, *Emericella nidulans* (4DP5), *Cladosporium herbarum* (7DF12) and three strains of bacteria, *Bacillus subtilis* strain F121112, *Bacillus subtilis* strain PY79 and *Bacillus subtilis*, which are good  $\beta$ -mannanase producing strains were isolated and identified from oil palm empty fruit bunch compost (Remi, 2009). Fungi and *Bacillus* spp. were routinely grown on potato dextrose and Luria Bertani agar (PDA and LB), respectively. The fungi were sub-cultured every four days and incubated at 30 °C, while the *Bacillus* spp. were sub-cultured every two days and incubated at 37 °C.

### Inoculum preparation

Locust Bean Gum (LBG) was used as the carbon source in the preparation of the bacteria inoculum. The media

was prepared according to Abe *et al.* (1994). Fresh bacteria culture was inoculated in 40 mL of sterilized LBG broth medium. Fermentation was carried out in an incubator shaker at 30 °C for 24 h. CFU counts on plates were performed in triplicates using the serial dilution method to determine the number of cells per mL inoculation. Fungi culture was grown on a PDA plate for 3-7 days to get a fully sporulated culture at 30 °C. The spores were harvested using a sterilized cotton swab and the spores were suspended in 40 mL of 0.1% Tween-80 in distilled water. The spore count was performed by plating the serially diluted spores onto PDA medium to obtain a  $10^6$  CFU mL<sup>-1</sup> inoculum. Inoculum for individual treatment was prepared by mixing the isolates [*B. subtilis* F121112, *B. subtilis* PY79, *B. subtilis*, *E. nidulans* (4DP5) and *C. herbarum* (7DF12)] in a 1:1 ratio to a total volume of 80 mL.

### Substrate preparation

Palm kernel cake (PKC) was obtained from a palm oil estate in Sandakan, Malaysia. It was ground and sieved through standard mesh sieves of 0.6 mm and 1.0 mm to obtain particles of approximately 0.8 mm in size (Saw *et al.*, 2006).

### Solid-state fermentation

Erlenmeyer flasks of 250 mL containing 20 g (dry weight) of PKC were autoclaved at 121 °C for 20 min and cooled to room temperature. Eighteen mL of mineral salt medium (2 g/L KH<sub>2</sub>PO<sub>4</sub>, 5 g/L NH<sub>4</sub>NO<sub>3</sub>, 1 g/L NaCl, 1 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O) with sodium hydroxide (NaOH) was added and vortexed briefly. The vortexing step is to ensure the mineral salt solution and the NaOH solution cover the substrate. Subsequently, the contents were transferred to a sterilised stomacher plastic bag and homogenised in a stomacher for 10 min. The stomacher thoroughly mixed the content to maximize the homogeneity. The homogenised mixture was reintroduced into the Erlenmeyer flasks. The size of the inoculum was fixed at 20% in all experiments (dry weight basis). Hence, each flask comprised of 20 g PKC (dry weight) required 4 mL of inoculum. The mixture was incubated at 30 °C for seven days. The humidity in the incubator was maintained by using a beaker of distilled water (humidity of 85-92%). The inoculum consisted of different combinations of isolates were: Treatment 1 consisted of *B. subtilis* + *B. subtilis* strain PY79 + *B. subtilis* strain F121112; Treatment 2 consisted of *C. herbarum* (7DF12) + *E. nidulans* (4DP5); Treatment 3 consisted of *C. herbarum* (7DF12) + *E. nidulans* (4DP5) + *B. subtilis* + *B. subtilis* strain PY79 + *B. subtilis* strain F121112; Treatment 4 consisted of *C. herbarum* (7DF12) + *B. subtilis* and Treatment 5 consisted of *E. nidulans* (4DP5) + *B. subtilis*. Three replicas were used for the fermentation. Sampling was performed on day 0 and day 7 analysed, and the data generated were compared.

### Sample extraction

At each sampling time, an Erlenmeyer flask containing fermented PKC was removed from the incubator. The contents in the flask were then mixed thoroughly. One gram of fermented PKC was transferred into a sterilized universal bottle and 9 mL of sterile sodium phosphate buffer (50 mM; pH7.0) was then added. The PKC mixture was vortexed for 5 min and then centrifuged at 8000× g for 2 min. The supernatant was subjected to reducing sugar analysis and β-mannanase assay. For the determination of pH and CFU counts on the plate, the above steps were repeated by substituting buffer solution with sterile distilled water without the centrifugation step. Another one gram of fermented PKC was serially diluted with 9 mL phosphate buffer up to 10<sup>-7</sup> for CFU determination and 50 μL of the 10<sup>-5</sup> to 10<sup>-7</sup> dilutions were plated into three PDA and three LB agar media each. The plates were incubated for two days at 30 °C and 37 °C, respectively. The numbers of colonies formed were counted. The CFU is expressed as colony-forming units per gram of dry PKC (CFU/g).

### PKC analysis

Treated PKC was subjected to reducing sugar analysis, β-mannanase assay, crude protein analysis and aflatoxin determination.

### Reducing sugar analysis

Reducing sugars (mannose equivalent) were measured using the method of Miller (1959). A volume of 660 μL of DNS reagent was added to 660 μL of supernatant in a 15 mL falcon tube. The mixture was heated to 100 °C for 5 min in a boiling water bath to develop red-brown colour. The mixture was left to cool down to room temperature before adding 13.2 mL of distilled water and the absorbance of the mixture was recorded with a spectrophotometer at 540 nm. The reaction mixture for blank (supernatant was replaced by sodium phosphate buffer of 50 mM and pH 7.0). The reducing sugar content of the sample was determined from the mannose standard curve. The assay was conducted in triplicates.

### β-mannanase assay

A reaction mixture consisted of 600 μL 1% LBG solution and 60 μL of the sample (crude enzyme) were mixed. The reaction mixture was then incubated at 30 °C for 30 min in a water bath. Immediately after 30 min, 660 μL of DNS reagent was added to the reaction mixture and the reaction was stopped by immersing the reaction tube in a boiling water bath for 5 min. After cooled to room temperature, 13.2 mL of distilled water were added to the reaction mixture and vortexed. Reducing sugars (mannose equivalent) liberated in the reaction mixture was measured by reading the absorbance of the mixture at 540 nm with a spectrophotometer and the reducing sugar content of the sample was determined from the

mannose standard curve. The reaction mixture for blank (crude enzyme was replaced by sodium phosphate buffer). One unit (U) of β-mannanase is defined as the amount of enzyme releasing 1 μmol mannose equivalent per min under the assay condition.

### Crude protein determination

Protein assay was done using the Lowry-Folin method. Fifty microliters of sample or standard were added with 50 μL of 2 N NaOH and 1 mL of Lowry-Folin Reagent 1 (2% Na<sub>2</sub>CO<sub>3</sub>, 1% CuSO<sub>4</sub> and 1% KNaC<sub>4</sub>H<sub>4</sub>O<sub>6</sub>.4H<sub>2</sub>O) in a 1.5 mL centrifuge tube. The reaction mixture was mixed by vortexing the tube before it was incubated at room temperature for 10 min. The Folin reagent was prepared at this time interval. Two hundred mL of Folin reagent were added to the mixture and incubated again at room temperature for another 30 min. After that, the optical density (OD) of the reaction mixture was read at 570 nm using a spectrophotometer. Bovine Serum Albumin (BSA) was used as the standard for this assay. The formula to calculate the β-mannanase activity is as follows:

$$\beta\text{-mannanase activity (Unit/mg)} = \frac{\text{Enzyme activity (Unit/mL)}}{\text{Protein concentration (mg/mL)}}$$

### Aflatoxin determination

Aflatoxin levels in PKC samples in treatments using fungal strain were determined using the Transia Plate Total Aflatoxins by Diffchamb, France, according to the manufacturer's protocol. Five hundred mg of the ground PKC was weighed into a clean universal bottle and mixed with 2.5 mL of fresh 70% methanol solution. The suspension was vortexed for 3 min and the samples were allowed to settle for 2-3 min. The extract was filtered into another clean universal bottle. Fifty μL of conjugate (provided together with the kit) was added to each well. Fifty μL of standards and samples were added to the assigned wells. The reaction mixtures were incubated at room temperature for 10 min. Holding the plate firmly, the contents of the plate were shaken out over a waste container by briskly flicking the wrist. Each well is rinsed using at least 300 μL of distilled water per well. The washing was repeated four times. One hundred μL of the substrate was added to each well. The plate was incubated at room temperature in the dark for 10 min to allow the blue colour to develop in the well. One hundred μL of stop solution was added to each well. The contents of the wells were mixed thoroughly to ensure complete colour conversion as the blue contents turned yellow. The optical density was measured at 450 nm using a microplate reader. The aflatoxin level was determined from the aflatoxin standard curve. The crude protein and aflatoxin contents differ slightly between samples due to the different inoculum combinations used in each sample, with the inoculum itself containing some crude protein and aflatoxin, and hence aflatoxin in all samples was measured.

**Table 1:** The maximum threshold of aflatoxin level permitted in food and feed.

For	Maximum Level	Commodities
Humans	20 ppb	All food except milk
Animal species	20 ppb	All food (exceptions below)
Exceptions:		
Breeding cattle, Breeding swine, Mature poultry	100 ppb	Corn
Finishing swine (>100 lbs)	200 ppb	Corn
Finishing beef cattle	300 ppb	Corn
Finishing beef cattle, swine poultry	300 ppb	Cottonseed meal

\*ppb = parts per billion.

Sources: FDA (2019) and Marshall *et al.* (2020)

### Statistical analysis

The collected data were analyzed using One-way ANOVA in SPSS (SPSS Inc. Chicago, USA) to determine the effect of SSF on reducing sugar, crude protein, aflatoxin production and mannanase activity.

### RESULTS AND DISCUSSION

In this study, microbes' consortia consisting of fungi and *Bacilli* were used in different combinations to carry out SSF to improve the nutritive value of PKC, by reducing the insoluble crude fibre (CF), increasing the crude protein (CP) and sugar levels and ensuring that the aflatoxin level of treated PKC was within acceptable limits of 20 ppb (Table 1) set by the FDA for animal feed (Marshall *et al.*, 2020). The use of *E. nidulans*, *C. herbarum* and *B. subtilis* to ferment PKC is novel, as this combination has never been used before and most other PKC research has not investigated aflatoxin levels after treatment. Furthermore, the fact that those microbes originated from oil palm empty fruit bunch compost, which is oily in nature, is an advantage because it ensures that the microbes can grow on PKC, as opposed to microbes isolated elsewhere in other works, which may not prefer oily substrates such as PKC.

#### Analysis of reducing sugar

Reducing sugar content of PKC during SSF employing mixed culture was monitored to determine the level of degradation of hemicellulose in PKC. In comparison to untreated PKC, treated PKC gave a significant ( $p \leq 0.05$ ) increase in reducing sugar. *Emericella nidulans* (4DP5) + *B. subtilis* (Treatment 5) showed the highest percentage of increase in reducing sugar content of 418.3% on day 7 in PKC during SSF against the initial concentration of reducing sugar in comparison to all the other four treatments (Figure 1). The increase in reducing sugar content in PKC with the use of various combinations of bacteria and fungi suggests that hemicellulose degrading enzymes were actively secreted during the SSF. The lowest increase in reducing sugar was obtained from treatment of two fungi (93.27%; Treatment 2) indicating that the enzymes degrading the mannan were probably contributed by the *B. subtilis*. This is consistent with the reports by others that *Bacillus* spp. are strong producers

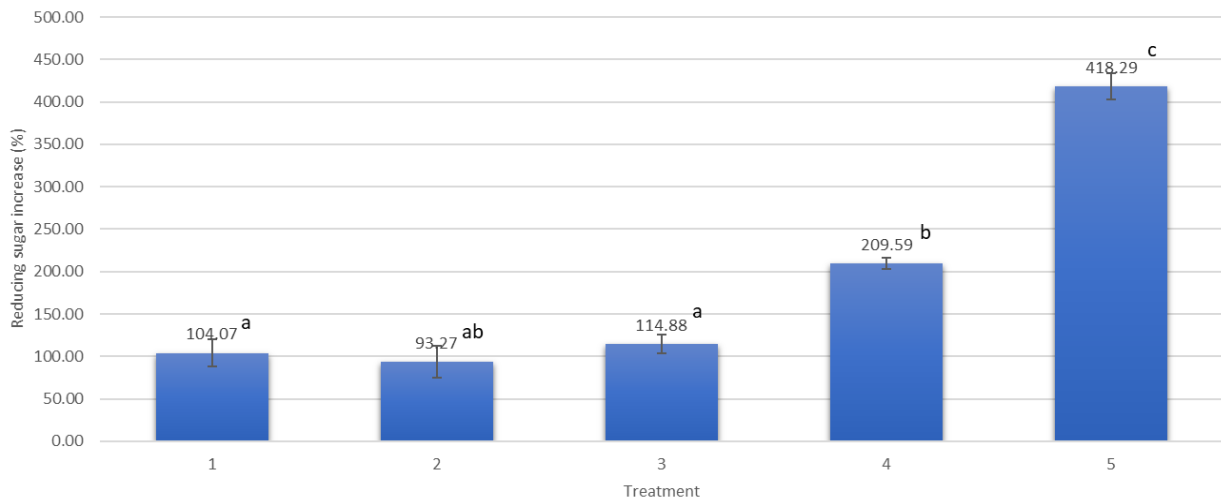
of  $\beta$ -mannanase (Abe *et al.*, 1994; Chin, 2006; Pangsri *et al.*, 2015; Gomez-Osorio *et al.*, 2022).

#### $\beta$ -mannanase assay

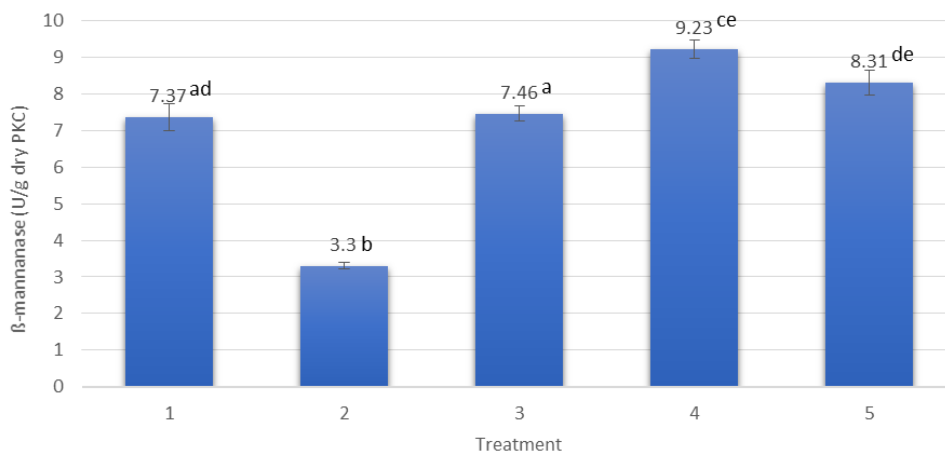
$\beta$ -mannanase level in PKC was also monitored to determine the highest  $\beta$ -mannanase produced in PKC during SSF. In comparison to untreated PKC, treated PKC gave a significant ( $p \leq 0.05$ ) increase in  $\beta$ -mannanase content. The highest  $\beta$ -mannanase reading in U/g dry PKC for the five treatments can be observed in Figure 2. Treatments with a mixed culture of Treatment 4 [*B. subtilis* (7DY7) + *C. herbarum* (7DF12)] and Treatment 5 [*B. subtilis* (7DY7) + *E. nidulans* (4DP5)] showed higher  $\beta$ -mannanase production in comparison to the other three treatments by producing 9.23 U/g dry PKC and 8.31 U/g dry PKC of  $\beta$ -mannanase activity, respectively. The lowest  $\beta$ -mannanase produced was obtained from Treatment 2 consisting of the two fungi (3.30 U/g dry PKC). This confirms that *B. subtilis* produced higher  $\beta$ -mannanase in comparison to the two fungi, *C. herbarum* (7DF12) and *E. nidulans* (4DP5) because all the other treatments consisting of *Bacillus* spp. had higher  $\beta$ -mannanase than Treatment 2 consisting of the two fungi. Several studies also reported that *B. subtilis* has high  $\beta$ -mannanase activities and is capable of hydrolysing hemicellulose (Cheng *et al.*, 2016) including those in PKC (Gomez-Osorio *et al.*, 2022). Interestingly, Treatment 1 consisting of only three *Bacilli* spp. had significantly lower  $\beta$ -mannanase than Treatment 4 indicating that *C. herbarum* (7DF12) provided synergistic effects to enhance the overall  $\beta$ -mannanase activity. This effect is not seen in Treatment 5 which consisted of the other fungal, *E. nidulans* (4DP5). However, it is not clear whether the production of higher mannanase is translated to the production of more crude protein in PKC. Hence, we have determined the crude protein contents of the five treatments.

#### Crude protein analysis

The difference in the percentage of crude protein content before treatment (day 0) and after treatment (day 7) for mixed culture treatments on PKC in SSF is shown in Figure 3. Treatment 2 consisting of *E. nidulans* (4DP5) + *C. herbarum* (7DF12) increased the crude protein content of PKC significantly ( $p \leq 0.05$ ) by 3.34% whereas for other



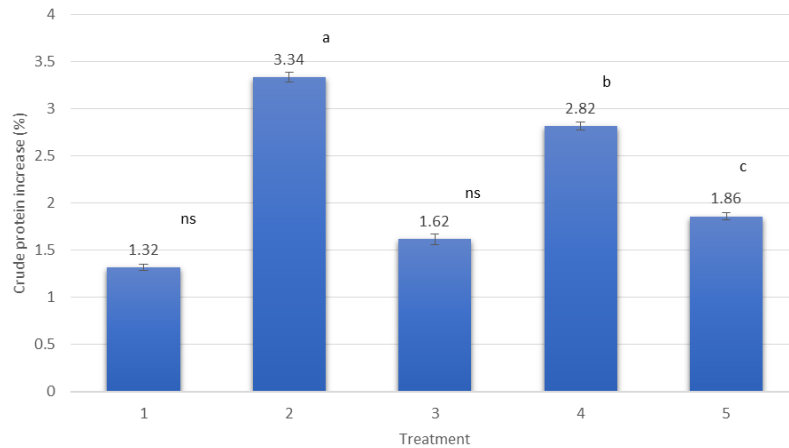
**Figure 1:** Percentage of reducing sugar increase in PKC measured after seven days of SSF with five types of treatments. Treatment 1, *B. subtilis* + *B. subtilis* strain PY79 + *B. subtilis* strain F121112; Treatment 2, *C. herbarum* (7DF12) + *E. nidulans* (4DP5); Treatment 3, *C. herbarum* (7DF12) + *E. nidulans* (4DP5) + *B. subtilis* + *B. subtilis* strain PY79 + *B. subtilis* strain F121112; Treatment 4, *C. herbarum* (7DF12) + *B. subtilis* and Treatment 5, *E. nidulans* (4DP5) + *B. subtilis*. Different superscripts indicate that the means are significantly different ( $p \leq 0.05$ ). Not significantly different is shown by the shared superscript.



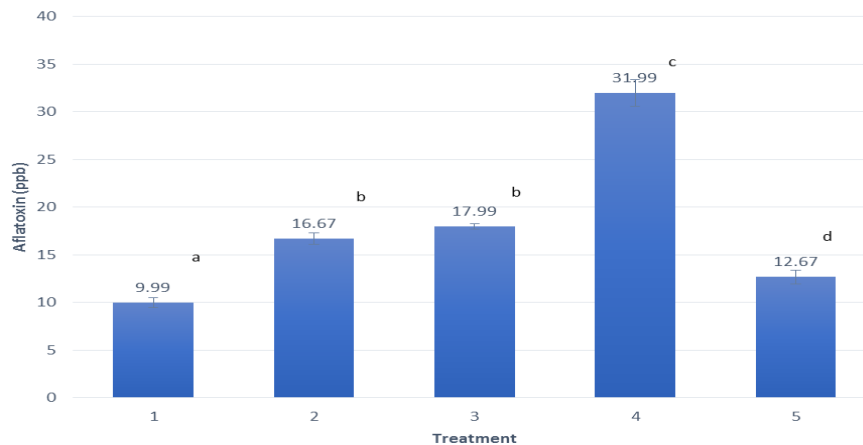
**Figure 2:**  $\beta$ -mannanase activity (U/g dry PKC) in PKC measured after seven days of SSF with five treatments. Treatment 1, *B. subtilis* + *B. subtilis* strain PY79 + *B. subtilis* strain F121112; Treatment 2, *C. herbarum* (7DF12) + *E. nidulans* (4DP5); Treatment 3, *C. herbarum* (7DF12) + *E. nidulans* (4DP5) + *B. subtilis* + *B. subtilis* strain PY79 + *B. subtilis* strain F121112; Treatment 4, *C. herbarum* (7DF12) + *B. subtilis*, and Treatment 5, *E. nidulans* (4DP5) + *B. subtilis*. Different superscripts indicate that the means are significantly different ( $p \leq 0.05$ ). Not significantly different is shown by the shared superscript.

combinations, *C. herbarum* (7DF12) + *B. subtilis* (7DY7) (2.82%), *E. nidulans* (4DP5) + *B. subtilis* (7DY7) (1.86%), also showed a significant increase. However, the treatment with 3 bacteria (1.32%) and 2 fungi + 3 bacteria (1.62%) did not produce any significant change (Figure 3). The overall results indicated that the two fungi are capable to increase the crude PKC protein content when compared to all the other combinations. Microorganisms, especially fungi are commonly used to convert the agro-industrial waste to obtain products with higher nutritive

value, especially the protein and vitamin contents and with increased digestibility. These microorganisms, such as *E. nidulans* (4DP5) + *C. herbarum* (7DF12) are very attractive feedstuffs because they can be cultivated on agro-industrial waste, with the production of large amounts of cells rich in proteins that commonly contain all the essential amino acids, serving as single-cell protein in addition to the favourably high vitamin and mineral levels (Kuhad *et al.*, 1997). The advantages of using microorganisms such as *E. nidulans* (4DP5) + *C.*



**Figure 3:** Percentage of crude protein increase in PKC measured after seven days of SSF with five types of treatments. Treatment 1, *B. subtilis* + *B. subtilis* strain PY79 + *B. subtilis* strain F121112; Treatment 2, *C. herbarum* (7DF12) + *E. nidulans* (4DP5); Treatment 3, *C. herbarum* (7DF12) + *E. nidulans* (4DP5) + *B. subtilis* + *B. subtilis* strain PY79 + *B. subtilis* strain F121112; Treatment 4, *C. herbarum* (7DF12) + *B. subtilis* and Treatment 5, *E. nidulans* (4DP5) + *B. subtilis*. Different superscripts indicate that the means are significantly different ( $p \leq 0.05$ ). Not significantly different is shown by the shared superscript.



**Figure 4:** Aflatoxin (ppb) in PKC was measured after seven days of SSF with five types of treatments. Treatment 1, Raw PKC (with no microbe inoculation); Treatment 2, *C. herbarum* (7DF12) + *E. nidulans* (4DP5); Treatment 3, *C. herbarum* (7DF12) + *E. nidulans* (4DP5) + *B. subtilis* + *B. subtilis* strain PY79 + *B. subtilis* strain F121112; Treatment 4, *C. herbarum* (7DF12) + *B. subtilis* and Treatment 5, *E. nidulans* (4DP5) + *B. subtilis*. Different superscripts indicate that the means are significantly different ( $p \leq 0.05$ ). Not significantly different is shown by the shared superscript.

*herbarum* (7DF12) are that they grow fast and produce protein in large amounts because the protein of microbial cells is very high, typically being around  $600 \text{ g kg}^{-1}$  (Villas-Bôas *et al.*, 2002). The PKC crude protein results showed that, despite producing less  $\beta$ -mannanase, the two fungi can grow well on PKC and increased the crude protein content, making PKC a better animal feed. However, the level of aflatoxin in the SSF after treatment will determine which is the better overall animal feed.

#### Aflatoxin determination

Aflatoxin contamination in feedstuff caused by fungi is a serious problem for livestock health and, as a result, has

an impact on agricultural economics. Therefore, the aflatoxin level in fungal treated PKC must be determined to ensure that the amount does not exceed the maximum threshold of 20 ppb (Table 1) acceptable in the general animal feed although some exceptions are given to feeds for some animals (Marshall *et al.*, 2020). Aflatoxin level in PKC was determined in treatments that contained fungal isolates to determine the percentage of increase in each treatment and whether that fell within the acceptable limit. The difference in the levels of aflatoxin before (day 0) and after treatment (day 7) with mixed cultures containing *E. nidulans* (4DP5), *C. herbarum* (7DF12) and *Bacilli* are shown in Figure 4. Aflatoxin levels increased to 12.67 ppb after treatment with *E. nidulans* (4DP5) + *B. subtilis*

(Treatment 5), 16.67 ppb after treatment with mixed cultures of two fungi (Treatment 2), 17.99 ppb after treatment with two fungi + three bacteria (Treatment 3) and 31.99 ppb after treatment with *C. herbarum* (7DF12) + *B. subtilis* (Treatment 4). The treatment with *E. nidulans* (4DP5) + *B. subtilis* gave the lowest increase in aflatoxin level of only 26.8% from 9.99 ppb (aflatoxin content in untreated PKC) to 12.67 ppb. The overall results show that all PKC treated with microbes' consortia containing fungi produced fermented PKC with aflatoxin levels less than 20 ppb and are appropriate for animal consumption except for Treatment 4.

In this study, the initial aflatoxin level in PKC without inoculation of fungi was also determined on day 0. It was found that the aflatoxin level in untreated PKC was 9.99 ppb. These considerable amounts are due to the contamination by aflatoxin producing microbes during storage of untreated PKC at room temperature before SSF. Pasha *et al.* (2007) reported that feed or grain for livestock and poultry could support fungal growth and aflatoxin formation. It is often the case that feedstuffs have been spoiled before harvest and already contained considerable amounts of mycotoxins; thus, it is sensible to store feedstuffs under conditions of temperature and humidity, which minimize fungal growth. Proper storage such as oven drying and storing in a cold room may become feasible approaches to produce end-products with lower aflatoxin contamination. The maximum threshold of aflatoxin levels acceptable in food and feed is listed in Table 1.

In the last decade, co-cultivation of microbes, including bacteria and yeasts has been successfully implemented to produce new natural products (Bertrand *et al.*, 2014). The food industry has been utilizing the co-culture method to make cheese (Martin *et al.*, 2001), yoghurt (Sodini *et al.*, 2000) and cacao beans (Schwan and Wheals, 2004) as well as whisky (van Beek and Priest, 2002). In this study, the implementation of co-cultivation of *E. nidulans* (4DP5) and *B. subtilis* also produced the desired outcome of the fermented PKC for use as an animal feed with higher crude protein and sugar.

## CONCLUSION

Finding the proper microbe combinations to treat PKC in an SFF to yield end products with increased crude protein and sugar content is a challenging task. On the one hand, we tried to maximize the bioconversion of crude fibre to protein, but at the same time, we need to ensure that the aflatoxins that are produced using the fermentation process fall below the allowable limit. The findings of this study revealed some exciting findings, such as the ability to produce fermented PKC that met the above standards using the proper combination of fungus and bacteria. Treatment of PKC in the SSF by using the two fungi in Treatment 2, *E. nidulans* (4DP5) and *C. herbarum* (7DF12) produced the highest overall increase in crude proteins, followed by Treatment 4 consisting of *C. herbarum* (7DF12) and *B. subtilis* and Treatment 5

consisting of *E. nidulans* (4DP5) + *B. subtilis*. Although Treatment 4 produced a relatively high amount of crude protein, it is not suitable for use as animal feed because the aflatoxin level in it is higher than the allowable limits.

We also observed that treating PKC with a combination of *E. nidulans* (4DP5) and *B. subtilis* (Treatment 5) resulted in the greatest increase in reducing sugar content in PKC (418.29%) despite the overall lower crude protein increase when compared to Treatment 2. As a result, it will be interesting to conduct a field trial to compare the feed conversion rates of the PKCs treated with the two fungi (Treatment 2) and those by *E. nidulans* (4DP5) and *B. subtilis* (Treatment 5) when used as chicken and ruminant feed.

## ACKNOWLEDGEMENTS

The research was funded by the Malaysian Ministry of Science, Technology, and Innovation's Science Fund (02-01-10-SF0005), and a Fundamental grant from Universiti Malaysia Sabah.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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