Iranian Society of Animal Science

Journal of Livestock Science and Technologies

Shahid Bahonar University of Kerman

ISSN: 2322-3553 (Print) ISSN: 2322-374X (Online)

Paper type: Original Research

The effect of fermentation by *Bacillus subtilis* and *Aspergillus niger* on the nutritional value of date palm kernels

Javad Bayat kouhsar[1], Farkhondeh Rezaii*[2], Naser Mahmoudnia[1] and Farzad Ghanbari[1]

[1]*Department of Animal Science, Faculty of Agriculture, Gonbad Kavous University, Gonbad, Iran.*
[2]*Department of Agriculture, Payame Noor University, Iran.*

*Corresponding author:
E-mail address:
frezaii@pnu.ac.ir

Received: 02 Mar. 2021,
Accepted: 07 Jun. 2021,
Published online: 24 Aug 2021,

Abstract This study was performed to investigate the effect of fermentation by *Bacillus subtilis* (*B. subtilis*) and *Aspergillus niger* (*A. niger*) and alkaline processing on the nutritional value and chemical composition of date palm kernels (DPK). DPK was fermented for 28 days under solid-state fermentation culture with two different microorganisms (*B. subtilis* and, or *A. niger)*. Alkaline processing was performed by soaking DPK in NaOH solution for 24 hours. In this experiment, eight treatments were used: DPK (control), DPK fermented by *B. subtilis,* and *A. niger* separately or in combination, and processed with NaOH, alone or with *B. subtilis* or *A. niger* or both. Digestibility and gas production of fermented and processed DPK were performed using batch culture and gas production techniques. The results showed that there was an increase in crude protein (CP) and a decrease in crude fiber concentrations due to the fermentation of DPK with *B. subtilis* and *A. niger* (P<0.05). Total phenol content in fermented DPK (FDPK) with *B. subtilis* and *A. niger* was significantly lower than the control. Gas production significantly increased in FDPK compared to control and NaOH-treated samples (P<0.05). All treatments significantly increased the *in vitro* digestibility of DM (IVDOD) and OM (IVOMD) compared to the control (P<0.01). Among the treatments, simultaneous fermentation of DPK with *B. subtilis* and *A. niger* had the most significant effect on increasing the microbial CP (MCP) and its efficiency (EMCP; P<0.01).

Keywords: gas production, fermentation, bacteria, fungi, NaOH.

Introduction

The date palm (*Phoenix dactylifera*) belongs to the family Palmae (Arecaceae) and is one of the oldest trees grown by humans (Chandrasekaran and Bahkali, 2013). Algeria, Egypt, Saudi Arabia, Iran, and Iraq are the primary producers of dates (Al-Farsi and Lee, 2008). According to the FAO report, the world production of dates in 2012 was about 8.5 million tons, of which about one million tons were produced in Iran (ASIA, 2013). The date palm kernel (DPK) contains 10–15% of the total date palm weight (Chandrasekaran and Bahkali, 2013). Therefore, more than 850 thousand tons of DPK are produced every year in the world. The use of agricultural wastes in animal feed is undoubtedly economical and also reduces the environmental pollution.

According to research, DPK has nutritional value for livestock and poultry (Al-Farsi and Lee, 2008; Chandrasekaran and Bahkali, 2013; Aboragah et al., 2019). DPK consist of carbohydrates (2.4–4.7%), lipids (5.7–8.8%), protein (4.8–6.9%), ash (0.8–1.1%), moisture (8.6–12.5%), and fiber (67.6–74.2%) (Afiq et al., 2013). Rezaeenia et al. (2014) stated that DPK could be used as a source of fiber and energy (due to the rel-

atively high content of ether extract) in the diet of ruminants. Indeed, DPK is characterized by high lignocellulose content, poor digestibility, low protein content, and low palatability (Abid et al., 2019).

Many technologies were utilized to increase the nutritional values of poor-quality by-products and make them more digestible. Chemical (Obese et al., 2001) and biological processing (Mirnawati et al., 2013, Rizal et al., 2013), or combination of these methods (Sinurat et al., 2013) were used to improve the nutritional value of DPK. Chemical processing can break the bonds between cellulose, hemicellulose, and lignin (Shawrang et al., 2013).

Fermentation is another way to improve the nutritional value of the agricultural wastes, which imparts distinctive aroma and flavor to the product, and improves the feed digestibility. It also removes some toxins from the feed in the lignocellulosic products (Yadi and Yana, 2011). In the nature, DPK also undergoes complex microbial degradation and transformation.

Bacillus subtilis (*B. subtilis*), a spore-forming probiotic bacterium, is an aerobic organism that consumes large amounts of free oxygen in the gastrointestinal tract when proliferating. It can firmly restrain the growth of aerobic pathogens and promote the proliferation of anaerobic probiotics, including yeast, *Lactobacillus,* and *bifidobacterium* (Guo et al., 2017). Studies have shown that fermentation by *B. subtilis* increases crude protein (CP), and decreases crude fiber in cotton kernel meal (Jazi et al., 2017), and increases feed quality and diet digestibility (Li et al., 2014).

Aspergillus niger (*A. niger*), a filamentous fungus, is one of the most important microorganisms used in biotechnology and industrial production of fermented foods, organic acids, and enzymes. The addition of *A. niger* fungi in palm kernel cake has been shown to decrease the cellulose and hemicellulose contents while increasing soluble sugars, CP, phosphorus, and energy contents (Lawal et al., 2010). Marini et al. (2005) showed that fermentation of DPK with *A. niger* decreased its NDF contents from 76% to 53%, and increased the soluble protein from approximately 1 to 15 mg/g. Therefore, the main objective of this research was to evaluate the effects of chemical (NaOH) and biological (fermentation by *A. niger* and *B. subtilis*) treatments on the chemical composition and gas production parameters of DPK.

Materials and methods

DPK preparation

Raw DPK (Kebkab variety) was collected from an Iranian date cake factory, soaked in a water tank for 24 hours to remove any residual date palm flesh, and then dried at 65 °C for 48 hours before being milled with a hammer mill (Yousuf and Winterburn, 2016).

Fermentation of DPK

B. subtilis (PTCC-1156) and *A. niger* (PTCC-5010) were obtained as lyophilized vials from the Iranian Scientific and Industrial Research Organization's Fungus and Bacteria Collection Center. Bacterial and fungal lyophilized vials were activated at 37 °C and 25 °C, in modified Rogosa broth media and potato dextrose agar broth, respectively. One kilogram of DPK powder was inoculated with one liter of distilled water and the inoculum (containing at least 10^5 colony-forming units per mL). A mixture of DPK and fungal or bacterial inoculi was preserved in plastic nylon bags at 25 °C for 21 days to complete the fermentation process.

Alkaline pretreatment procedure

Alkaline processing was performed by soaking DPK in an alkaline solution (2 g NaOH per 25 mL distilled water per 100 g DM) for 24 hours. The DPK was treated with 10 mL of 12 N hydrochloric acid to clear the NaOH residue. Then 4 liters of water were applied to fully spread the acid around the DPK. After one hour, the DPK was removed from this solution and dried at 60 °C (Squires et al., 1992).

Chemical analysis

Ten replicates were taken from each sample for determination of the pH, bulk density, and chemical composition. DM, CP, crude fat, crude fiber, and ash were determined by proximate analysis (AOAC, 2005), and ADF and NDF by standard methods (Van Soest et al., 1991).

Measurement of total phenol

To determine the total phenol content, 0.1 g of DPK powder was ground with 10 mL of 80% hot ethanol, and the resultant mixture was centrifuged at 1000 rpm for 10 minutes.

The supernatant was then placed in a hot water bath to concentrate (about 2 mL remained). One mL of the condensed fluid was then diluted to 50-mL volume with distilled water. In the next step, 0.5 mL of the solution was mixed with 2.5 mL distilled water. The solution was then treated with 0.5 mL of 50 percent Folin Ciocalteu reagent. Two mL of 20 percent sodium carbonate was added after 3 minutes. The solution was placed in a boiling water bath for 10 minutes. After cooling, the amount of light absorption at the absorption point of 650 nm was determined using a spectrophotometer. Finally, the amount of total phenol in the sample (mg/g DM) was measured using the Gallic acid standard curve (Malik and Singh, 1980).

Determination of bulk density

Bulk density is the space occupied by the feed mass (in terms of weight). The standard unit for measuring this factor is kg/ m^3 and depends on its size and compactne-

ss. For bulk density measurement, a 1000- mL cylinder was first filled with the test sample and shaken for 15 seconds in a rotating motion (without squeezing). The created space was refilled with the sample, and the total weight of the sample was then recorded. The bulk density of the sample was measured in g/cm^3 (Shelton et al., 2005).

In vitro ruminal gas production assay

DPK powder (control) and DPK after 28 days of fermentation with *B. subtilis* and *A. niger* and processed with NaOH were used as substrates in the current *in-vitro gas production* experiment. The gas production test was carried out according to Menke's (1988) approach.
Rumen fluid was collected from four male sheep (45±2 kg live weight) with ruminal fistula one hour before morning feeding, and quickly transferred to the laboratory. The animals were fed a diet containing 70 percent forage (equal proportions of alfalfa and corn silage) and 30 percent concentrates (barley, bran, cottonseed meal, and supplement) and had free access to water. The rumen fluid was then filtered through a special cloth and poured into an Erlenmeyer flask while bubbling with carbon dioxide, incubated in a 39 °C water bath.

Treated materials (200 mg DM samples) were transferred into glass vials. Four replicates of each sample were used as the test, while four vials without samples were considered as the control. A 2:1 mixture of artificial saliva and rumen fluid was prepared, and 30 mL of the mixture was transferred to glass vials containing a 0.2 g sample or monitor. Immediately, each vial was bubbled with carbon dioxide for 10 s and completely sealed using rubber stoppers and an aluminum cover. The vials were transferred to a shaking water bath at 39 ^{o}C and incubated for 2, 4, 6, 8, 12, 24, 36, 48, 72, and 96 h. A pressure gauge was used to measure gas production (GP). The experiment was carried out three times. The cumulative gas production was calculated according to Orskov and McDonald's method (1979). The organic matter digestibility (OMD), metabolizable energy (ME), and net energy (NE) contents were determined according to Menk et al. (1979). The short-chain fatty acid (SCFA) concentration was calculated using Makar's equation (2004).

$$OMD\ (\%) = 14.88 + 0.889\ GP + 0.45\ CP + 0.0651\ ash$$

$$ME\ (MJ / kg\ DM) = 2.20 + 0.136\ GP + 0.057\ CP + 0.0029\ CF$$

$$NE\ (MJ / kg\ DM) = -0.36 + 0.114\ GP + 0.0054\ CP + 0.0139\ EE - 0.0054\ ash$$

$$SCFA\ (mmol) = 0.0222\ GP - 0.00425$$

Where:
GP: Net gas production after 24 hours (per 200 mg sample DM)
CF: crude fiber (%)
EE: ether extract (%)

Gas production parameters were estimated as described by Orskov and McDonald (1979):

$$y = b\ (1 - e^{-ct})$$

Where:
y: the gas produced at the time of incubation
b: gas production from an insoluble fermentable fraction
e: Euler's number
c: gas production rates for b
t: incubation time

The data were analyzed in a completely randomized design using the SAS statistical software (version 9.1) (SAS, 2003).

In vitro digestibility of DM and OM

In vitro digestibility of DM and OM was measured using the batch culture method (Theodorou et al., 1994). The artificial saliva preparation process, ruminal fluid preparation method, and basal diet and treatments were all identical to the gas production test. In this experiment, 500 mg of DM-based samples were transferred into glass vials. Every vial was filled with 50 mL artificial saliva and ruminal fluid (2 volumes artificial saliva, 1 volume ruminal fluid). After that, a buffer was used to increase the pH to 6.8. The vials were then capped with a plastic cap and aluminum cover and kept in a water bath at 39 °C for 24 hours. At the end of the incubation period, the vials were placed in cold water to inactivate the microbial activity. pH was measured using a pH meter (Model 691, Metrohm Company).

The bottle's contents were filtered through a nylon cloth (42-mm pore size), to separate the undigested contents from the liquid phase. Five mL of the liquid form was combined with an equivalent volume of 0.2 N hydrochloric acid and placed in a freezer at -20 °C to measure the ammonia nitrogen concentration. The phenol-hypochlorite procedure was used to calculate the ammonia nitrogen content of the samples (Broderick and Kang, 1980). A spectrophotometer (Biotech-novaapec-England) was used to measure the optical absorption at 630 nm. The undigested contents of the vials were dried in a 60 °C oven for 48 hours to determine the DM disappearance. The remaining DM was then kept in the oven at 540 °C for six h, and the ash content was measured. Gas pressure was recorded using pressure indicators at 2, 4, 6, 8, 10, 12, 24 hours after incubation, and the accumulated gas was released. The following equation was used to estimate the gas production efficiency (Getachew et al., 2004).

$$G_y = GP_{24} / (0.5 - \text{DM weight after oven drying})$$

Where:
G_y = Gas production efficiency
GP_{24} = the gas production after 24 h of incubation

The microbial mass production was estimated using the following equation (Blümmel et al., 1997).

$$MCP\ (mg) = (GP \times PF) - 2.2$$

Where:
MCP= Microbial mass production
GP= pure gas Production after 24 hours (mL)
PF= Partitioning factor (mg of OM digested/ mL of pure gas volume)

The efficiency of microbial protein was estimated using the following equation:

Microbial mass production efficiency = MCP/ disappeared OM

The data were analyzed using the GLM procedure of SAS statistical software version 9.1 in a completely randomized design (SAS, 2003).

Results

Chemical composition

Fermentation of DPK with *B. subtilis*, *A. niger,* or a combination of both significantly reduced (Table 1) the crude fiber concentration compared to the control (23.45, 23.21, 22.05 vs. 26.8 percent, respectively). The simultaneous fermentation of *B. subtilis* and *A. niger* imparted the greatest effect on crude fiber reduction. Fermentation of DPK with *B. subtilis* and *A. niger* or processing with NaOH did not have any significant effect on DM, non-protein nitrogen (NPN), crude fat, crude ash, ADF, and NDF contents. Fermentation of DPK with a combination of *B. subtilis* and *A. niger* caused a significant increase in CP by almost 20 percent (from 6.28 to 7.52 percent) (P<0.05). The FDPK bulk density decreased under *B. subtilis* and *A. niger* processing, while NaOH treatment significantly increased it (P<0.05).

Table 1. Effect of fermentation with *Bacillus subtilis* and *Aspergillus niger* and NaOH processing on date kernel chemical composition

Treatments	DM (%)	Crude fiber (%)	ADF (%)	NDF (%)	CP (%)	NPN (%)	EE (%)	Ash (%)	BD (g/ cm^3)
Control	90.7	26.8^{a}	45.1	70.5	6.28^{b}	1.2	6.75	2.29	0.67^{bc}
Bacillus subtilis	90.7	23.4^{bc}	41.2	68.4	6.58^{b}	1.49	7.3	2.38	0.67^{bc}
Aspergillus niger	87.7	23.12^{bc}	42.2	70.0	6.67^{b}	0.72	6.52	2.04	0.68^{bc}
Bacillus subtilis and *Aspergillus niger*	88.0	22.05^{c}	39.8	65.8	7.52^{a}	0.48	6.35	2.68	0.64^{c}
NaOH	87.5	25.5^{ab}	42.2	73.6	6.86^{ab}	1.16	5.45	2.43	0.70^{ab}
NaOH and *Bacillus subtilis*	87.5	27.2^{a}	43.1	71.0	7.04^{ab}	1.75	6.95	2.24	0.73^{a}
NaOH and *Aspergillus niger*	89.0	27.8^{a}	43.6	72.2	6.98^{ab}	1.07	7.22	2.26	0.69^{ab}
NaOH, *Bacillus subtilis* and *Aspergillus niger*	89.2	27.0^{a}	41.4	72.0	6.28^{b}	1.12	5.97	2.19	0.73^{a}
SEM	0.10	1.1	1.68	2.00	0.26	0.36	1.13	0.18	0.01
P-Value	0.19	0.004	0.48	0.22	0.03	0.63	0.94	0.43	0.008

DM: Dry matter; ADF: Acid detergent fiber; NDF: Neutral detergent fiber; CP: Crude protein; NPN: Non-protein nitrogen; EE: Ether extract; BD: Bulk density; SEM: Standard error of the mean.
[a,b.] Within column, means with common superscripts do not differ (P>0.05).

Simultaneous fermentation of DPK with *B. subtilis* and *A. niger* also decreased the total phenol content compared to the control (P<0.05), while in other treatments, this effect was not significant (Figure 1).

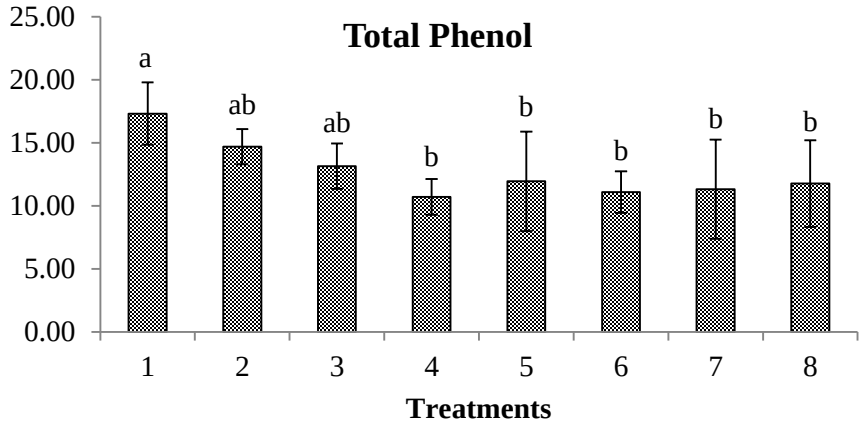

Figure 1. Effect of fermentation with *Bacillus subtilis* and *Aspergillus niger* and NaOH processing on total phenol (mg/ gDM) in date kernel.
Treatment 1= Control; Treatment 2= *Bacillus subtilis*; Treatment 3= *Aspergillus niger*; Treatment 4= *Bacillus subtilis* and *Aspergillus niger*; Treatment 5= NaOH; Treatment 6= NaOH and *Bacillus subtilis*; Treatment7 = NaOH and *Aspergillus niger*; Treatment 8= NaOH, *Bacillus subtilis* and *Aspergillus niger*.

Gas production parameters

Treatments had no significant effect on gas production potential and the rate of DPK (P> 0.05; Table 2). In comparison with the control and fermented products, the treatments containing NaOH (NaOH alone or in combination with *B. subtilis*, *A. niger,* or both) significantly decreased SCFA (P<0.05).

The greatest reduction was recorded by treating the materials with NaOH and *A. niger* (More than 25% reduction).

The effect of fermentation with *Bacillus subtilis* and *Aspergillus niger* and NaOH processing on date kernel gas production is shown in Table 2. DPK gas production was increased by fermentation with *A. niger* and *B. subtilis* (alone or in combination), while alkali processing did not affect DPK gas production. In addition, as compared to the control, all four DPK treatments involving NaOH significantly reduced ME and OMD.

Table 2. Effect of fermentation with *Bacillus subtilis* and *Aspergillus niger* and NaOH processing on date kernel gas production parameters

Treatments	(a+b)	C	SCFA	ME	OMD
Control	238.4 ± 8.618	0.0461 ± 0.0024	0.658 ^{ab}	10.50 ^{a}	41.43^{ab}
Bacillus subtilis	271.5 ± 2.263	0.0348 ± 0.0013	0.643 ^{b}	10.31 ^{a}	40.84^{b}
Aspergillus niger	306.4 ± 2.997	0.0330 ± 0.0007	0.706 ^{a}	10.60 ^{a}	43.36^{a}
Bacillus subtilis and *Aspergillus niger*	339.9 ± 8.017	0.0256 ± 0.0012	0.661 ^{ab}	10.72 ^{a}	41.58^{ab}
NaOH	255.2 ± 3.874	0.0303 ± 0.0010	0.561 ^{c}	8.77 ^{c}	37.57^{c}
NaOH and *Bacillus subtilis*	258.6 ± 4.303	0.0275 ± 0.0010	0.532 ^{cd}	9.50 ^{b}	36.39^{cd}
NaOH and *Aspergillus niger*	206.4 ± 3.969	0.0350 ± 0.0016	0.491 ^{d}	9.38 ^{bc}	34.76^{d}
NaOH, *Bacillus subtilis* and *Aspergillus niger*	250.8 ± 4.163	0.0298 ± 0.0011	0.543 ^{c}	9.06 ^{bc}	36.83^{c}
SEM	-	-	0.016	0.233	0.646
P-Value	-	-	<0.0001	<0.0001	<0.0001

(a+b): Gas production potential (ml/gDM); C: Gas production rate (ml/h); SCFA: Short chain fatty acids (mmol); ME: Metabolizable energy (Mj/Kg); OMD: Organic matter digestibility (% DM); SEM: Standard error of mean.
$^{a,b.}$ Within column, means with common superscripts do not differ (P>0.05).

Digestibility, pH, ammonia nitrogen, and fermentation parameters

All treatments significantly increased the *in vitro* digestibility of DM and OM compared to the control (P< 0.01; Table 3). This increase was substantial (more than 100 percent) when DPK has simultaneously fermented with *B. subtilis* and *A. niger* compared to the control (19.33 and 21.03 vs. 40 and 43.31, respectively for DM and OM digestibility). All treatments (except fermentation with *B. subtilis* and processing with NaOH) caused a significant reduction in pH (P< 0.05). The greatest decrease in pH was observed under simultaneous fermentation with *B. subtilis* and *A. niger*. Fermentation with *A. niger* alone or in combination with *B. subtilis* increased ammonia nitrogen concentrations (P< 0.05; Table 3).

Table 3. Effect of fermentation with *Bacillus subtilis* and *Aspergillus niger* and NaOH processing on the digestibility and fermentation parameters of date kernel

Treatments	IVDOD	IVOMD	pH	N-NH_3	Gas $yield_{24}$	PF	MCP	EMCP
Control	19.33^{d}	21.03^{d}	6.29^{ab}	4.32^{bc}	341.67^{ab}	3.11^{bc}	30.40^{e}	0.293^{bc}
Bacillus subtilis	28^{bc}	32.41^{bc}	6.32^{a}	4.71^{abc}	231.67^{d}	3.96^{a}	7.07^{bcd}	0.414^{ab}
Aspergillus niger	24.66^{cd}	28.71^{c}	6.22^{e}	5.38^{ab}	385.86^{a}	3.01^{de}	38.33^{de}	0.257^{c}
Bacillus subtilis and *Aspergillus niger*	40^{a}	43.31^{a}	6.21^{e}	6.04^{a}	241.99^{cd}	4.40^{a}	105.73^{a}	0.498^{a}
NaOH	24.66^{cd}	28.09^{cd}	6.26^{bc}	4.08^{bc}	288.68^{bc}	3.85^{ab}	58.65^{cde}	0.429^{ab}
NaOH and *Bacillus subtilis*	31.33^{bc}	34.95^{bc}	6.22^{de}	4.63^{abc}	249.10^{cd}	4.36^{a}	84.37^{abc}	0.495^{a}
NaOH and *Aspergillus niger*	28.66^{bc}	30.22^{c}	6.23^{cde}	3.24^{c}	280.56^{c}	3.66^{abc}	58.88^{cde}	0.398^{ab}
NaOH, *Bacillus subtilis* and *Aspergillus niger*	34^{ab}	38.19^{ab}	6.25^{cd}	4.43^{bc}	248.96^{cd}	4.45^{a}	94.70^{ab}	0.504^{a}
SEM	2.336	2.393	0.115	0.536	17.802	0.265	11.102	0.0453
P-Value	0.0005	0.0003	<0.0001	0.0645	0.0001	0.0061	0.0020	0.0076

IVDOD: In vitro Dry Matter Digestibility (%); IVOMD: In vitro Organic Matter Digestibility (%); N-NH_3: Ammonia nitrogen (mg/dl); Gas $yield_{24}$: The amount of gas production after 24 hours of incubation (ml); PF: Partitioning factor (mg OM truly degraded/ml gas produced in 24 h); MCP: Microbial crude protein (mg); EMCP: Efficiency of Microbial crude protein; SEM: Standard error of the mean.
$^{a,b.}$ Within column, means with common superscripts do not differ (P>0.05).

All treatments except fermentation with *A. niger* and processing with NaOH significantly reduced the *in vitro* gas production (P< 0.05; Table 3 and Figure 2). The partitioning factor (PF) was increased in all treatments except fermentation with A. *niger*, and the greatest effect was observed in FDPK with a combination of *B. subtilis* and *A. niger* with or without NaOH (P< 0.05; Table 3). Fermentation and processing also increased (except fermentation with *B. subtilis*) MCP, and the greatest effect was observed in FDPK with *B. subtilis* and *A. niger*,

which was more than three times compared to the control (30.40 compared to 105.73 mg). Furthermore, the greatest effect on the EMCP was found when FDPK was co-cultured with *B. subtilis* and *A. niger* with or without NaOH.

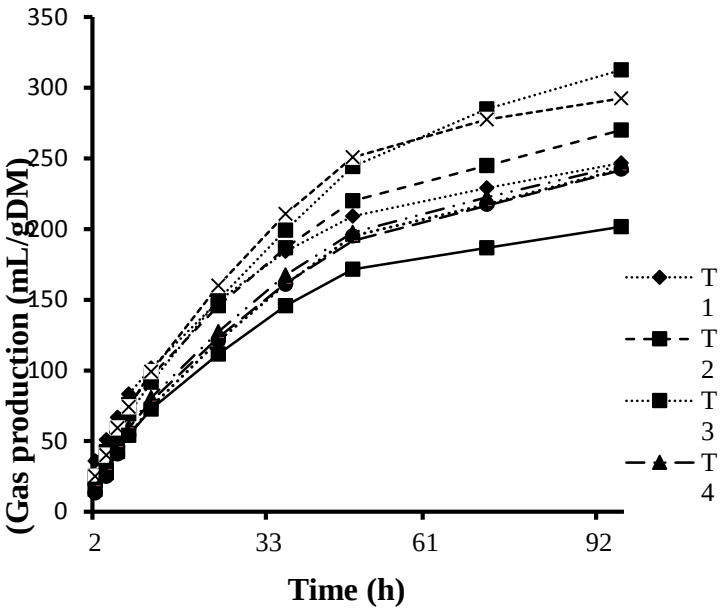

Figure 2. Effect of fermentation with *Bacillus subtilis* and *Aspergillus niger* and NaOH processing on date kernel gas production after 96 h incubation.
Treatment 1= Control; Treatment 2= *Bacillus subtilis*; Treatment 3= *Aspergillus niger*; Treatment 4= *Bacillus subtilis* and *Aspergillus niger*; Treatment 5= NaOH; Treatment 6= NaOH and *Bacillus subtilis*; Treatment 7= NaOH and *Aspergillus niger*; Treatment 8= NaOH, *Bacillus subtilis* and *Aspergillus niger*.

Discussion

Chemical composition

In this study, fermentation with *B. subtilis* and *A. niger* reduced DPK crude fiber, being more substantial in simultaneous fermentation with both organisms. This is in agreement with the published studies reporting that the processing or fermentation can reduce fiber contents in agricultural by-products (Dusterhoft et al., 1992, Iluyemi et al., 2006, Mirnawati et al., 2013, Rizal et al., 2013, Jazi et al., 2017, Abid et al., 2019, Aboragah et al., 2019). Non-starch polysaccharides are abundant in oil-free DPK (palm kernel cake) (Dusterhoft et al., 1992). Mannans, the most important component of hemicellulose, constitute the majority of DPK's dry weight (Araujo and Ward, 1990). Many fungi are capable of producing mannan-degrading enzymes (Abdeshahian et al., 2010). Different species of *Aspergillus* produce a wide range of microbial enzymes (Gao et al., 2008). *Aspergillus niger* has been shown to have a high capacity to produce active cellulase and mannanase in the culture medium as compared to other *Aspergillus* species (Ademark et al., 1998, van Zyl et al., 2009). *B. subtilis* has also been found to generate fiber-degrading microbial enzymes such as cellulases, mannanase, and amylase (Hossain et al., 1996, Chuan et al., 2006, Pangsri and Pangsri, 2017). Since *A. niger* and *B. subtilis* have strong cellulase activity and transform cellulose to glucose (Mirnawati et al., 2011), fiber reduction happens at the end of fermentation; therefore, the decline in DPK crude fiber was attributed to the cellulolytic properties of fungi and bacteria (Rizal et al., 2013). The amount of enzyme produced by fungi and bacteria depends on the fermentation conditions such as temperature, humidity, oxygen, etc. (Chuan et al., 2006, Abdeshahian et al., 2010). Furthermore, the substantial reduction in crude fiber levels due to fermentation with *A. niger,* and *B. subtilis* demonstrated that the fermentation conditions in this study were effective.

The treatments did not affect the ADF and NDF content (Table 1). Contrary to these results, Lawal et al. (2010) found that fermentation or processing with alkali reduced ADF and NDF. The alkaline agents such as NaOH, ammonia, or CaOH could be absorbed into the cell wall and chemically break down linkages between lignin and hemicellulose (Jackson, 1977). However, in this experiment, biological methods were more effective than chemical methods in breaking the fiber bonds.
In the present experiment, the CP content increased in FDPK treated with *B. subtilis* and *A. niger*. Increased CP

concentration in DPK resulting from fermentation with *A. niger* has been reported in other studies (Iluyemi et al., 2006, Lawal et al., 2010, Ramin et al., 2010). Ramin et al. (2010) used three species of fungi (*A. niger*, *Trichoderma harizianum*, and *Rhizopus oryzae)* to treat palm kernel cake, and found that only two species (*A. niger* and *Rhizopus oryzae),* increased the CP concentrations (18 to 27%). Lawal et al. (2010) observed that the DPK CP concentration increased from 12 to 20% by fermentation with *A. niger*. Rizal et al. (2013) investigated the effect of fermentation by four fungal species on DPK and observed that *A. niger* increased the CP content more than other fungi. During fermentation, the growth of fungi and bacteria occurs in the substrate (DPK), and fungi are reported to contain large amounts of CP (40 to 60 percent) (Crueger et al., 1990). As a result, some of the increase in DPK CP is due to the growth of *A. niger* and *B. subtilis*. In other words, the increase in CP content of the substrate after fermentation is associated with the process of protein production in fungi and single cells, which cannot be easily separated into fungal and substrate proteins (Carlile et al., 2001). Fermentation of DPK with *B. subtilis* and *A. niger* increased the concentration of N-NH_3 and MCP compared to the control and treatment with NaOH. Similar to these findings, Kholif et al. (2015) found that the concentration of N-NH_3 in DPK increased due to cellulase activity from *A. niger* compared to the control. Aziz (2020) also found that applying various enzymes to palm leaves increased the ammonia nitrogen and microbial CP production. Increased N-NH_3 concentration due to fermentation with *B. subtilis* and *A. niger* may be due to improved digestibility of OM and DM (Table 3).

An important feature of biomass materials is their bulk density, related to transportation and storage costs (Jeguirim et al., 2012). The low density is not desirable because it hurts energy density, transportation costs, and storage capacity for the producer and the final user (Obernberger and Thek, 2004). The processing of DPK with NaOH increased the mass density from 0.67 to 0.73 (g/cm^3), indicating that the NaOH-processed DPK required less storage space per unit weight than other treatments. Similar to these results, other studies have shown that the incubation of DPK increased the bulk density compared to unincubated DPK (Ghehsareh et al., 2011). Increased bulk density may be related to lower fiber content (as a result of fermentation and NaOH processing).

The present treatments had no significant effect on the ether extract (EE) concentration. Aziz (2020) found that several enzymes, including cellulase, fibrolytics, taninnase, or their combinations did not affect the EE level in palm leaves. Contrary to these observations, Rizal et al. (2013) observed that fermentation of DPK with *A. niger* reduced the EE content. *A. niger* may produce lipase during which can reduce the EE content (Falony et al., 2006, Mirnawati et al., 2011).

A reduction in phenolic compounds was previously reported in feed fermented with *A. niger* (Dei et al., 2008), as well as fermented rapeseed meal with a combination of *B. subtilis* and *A. niger* (Ashayerizadeh et al., 2017). Asgari et al. (2013) also showed that alkaline pH could accelerate the degradation of phenolic compounds. The reduction in phenolic compounds may be due to the production of tannase by *A. niger* and *B. subtilis*, which cause the breakdown of phenolic compounds and hydrolyzable tannins into glucose and gallic acid (Pinto et al., 2001). It can also be attributed to enzymes synthesized by *A. niger* (such as fiber degrading enzymes: hemicellulases, hydrolase, and pectinase; phytase and hydrolyze tannins) (Ong et al., 2004; Abdeshahian et al.; 2010, Rizal et al., 2013), and *B. subtilis* (such as cellulase and phytase) (Schallmey et al., 2004).

Gas production parameters

The results of this experiment showed that fermentation of DPK with *A. niger* and *B. subtilis*, but not NaOH, increased gas production, while NaOH processing did not affect it. The potential and rate of gas production also were not affected by the treatments. Similar to these observations, Abid et al. (2019) found that the use of exogenous fibrolytic enzymes increases gas production and gas production potential in DPK. Contrary to these observations, Ramin et al. (2010) reported that the volume of gas released when DPK was fermented with *A. niger,* and *Rhizopus oryzae* was lower than the control. The amount of gas production is essential to identify the digestibility, fermentation process, and microbial protein synthesis from raw materials by rumen microorganisms (Sommart et al., 2000). Increased gas production (as a result of fermentation with *A. niger* and *B. subtilis*) is probably due to the effect of cellulase and an increase in digestible OM (Table 3).

Alkaline treatment of DPK, but not fermentation with *B. subtilis* and *A. niger*, reduced the concentration of SCFA. Contrary to these findings, Kholif et al. (2015) found that using the cellulase (derived from *A. niger*) increased the total concentration of SCFA in DPK as compared to the control.

Digestibility, pH, ammonia nitrogen, and fermentation parameters

Fermentation with *A. niger* or *B. subtilis* alone or in combination with NaOH significantly increased the digestibility of DM and OM (up to more than 100 percent). Similarly, Kholif et al. (2015) found that treating DPK with *A. niger* cellulolytic enzymes improved DM and OM *in vitro* digestibility (from 16 to 30 percent for DM and 19 to 35 percent for OM). The increase in the OM and DM digestibility of the DPK treated with NaOH in the current study is in line with the findings reporting increases in fiber digestion of alkaline-pretreated low-quality forages (Rad et al., 2015; Aboragah et al., 2019). Studies have also shown that fermentation of DPK with *A. niger* significantly increased the CF digestibility (Mirnawati et al., 2011; Rizal et al., 2013; Abid et al., 2019).

The improved digestibility of date palm kernel DM and OM is probably due to *A. niger* cellulolytic enzymes, which convert cellulose to simple sugars (Mirnawati et al., 2011). Processing lignocellulosic materials with chemical or microbial compounds breaks the lignin-hemicellulose linkages, resulting in carbohydrate hydrolysis, and increased digestibility (Rad et al., 2015; Sun et al., 2016; Aboragah et al., 2019). In general, removing significant amounts of lignin from low-quality forage makes it easier for ruminal microorganisms to bind to structural carbohydrates, and fiber degradation improves as microbial colonization increases (Aboragah et al., 2019). Pretreatment with NaOH could also reduce the strength of intermolecular hydrogen bonds in the cellulose fibers and improved cellulose digestion (Behera et al., 2014). The reduction of phenolic compounds may have contributed to the increased DM and OM digestibility in FDPK with *B. subtilis* and *A. niger* (Figure 1; (Abid et al., 2019; Aboragah et al., 2019).

DPK fermentation with *B. subtilis* and *A. niger* alone or with NaOH resulted in a significant reduction in pH. Since fibrolytic enzymes have a strong effect on the fermentation of structural and non-structural carbohydrates, as well as the synthesis of SCFA, the decrease in pH may be due to *B. subtilis* and *A. niger* fibrolytic enzymes (Kholif et al., 2015).

Conclusions

Overall, this study showed that fermentation with *B. subtilis* and *A. niger* improved the nutritional value of DPK, which was more effective than alkaline treatment. The best results for increasing OM and DM digestibility, ammonia nitrogen, CP, MCP, EMCP, gas production, and reduction of phenol concentration and crude fiber content were obtained when DPK was fermented simultaneously with *B. subtilis* and *A. niger*.

Conflict of interest

The authors declare that there are no conflicts of interest.

References

Abdeshahian P., Samat N., Hamid A.A., Yusoff W.M.W., 2010. Utilization of palm kernel cake for production of β-mannanase by *Aspergillus niger* FTCC 5003 in solid substrate fermentation using an aerated column bioreactor. *Journal of Industrial Microbiology & Biotechnology* 37**,** 103-109.

Abid K., Jabri J., Beckers Y., Yaich H., Malek A., et al. 2019. Influence of adding fibrolytic enzymes on the ruminal fermentation of date palm by-products. *Archives Animal Breeding* 62**,** 1-8.

Aboragah A., Embaby M., Günal M., AbuGhazaleh A., 2019. Effect of alkaline and sonication pretreatments on the rumen degradability of date palm seeds. *Tropical Animal Health and Production* 52**,** 771-776.

Ademark P., Varga A., Medve J., Harjunpää V., Drakenberg T. et al. 1998. Softwood hemicellulose-degrading enzymes from *Aspergillus niger*: purification and properties of a β-mannanase. *Journal of Biotechnology* 63**,** 199-210.

Afiq M.A., Rahman R.A., Man Y.C., Al-Kahtani H., Mansor T., 2013. Date seed and date seed oil. *International Food Research Journal* 20**,** 2035-2043.

Al-Farsi M.A., Lee C.Y., 2008. Nutritional and functional properties of dates: a review. *Critical Reviews in Food Science and Nutrition* 48**,** 877-887.

AOAC., 2005. Official Methods of Analysis. Vol. 1. No. 1. 18th ed. Association of Official Analytical Chemists. Washing Town, D.C.

Araujo A., Ward O.P., 1990. Extracellular mannanases and galactanases from selected fungi. *Journal of Industrial Microbiology* 6**,** 171-178.

Asgari G., Chavoshani A., Seid-Mohammadi A., Rahmani A., 2013. Removal of pentachlorophenol using microwave assisted persulfate from synthetic wastewater. *Journal of Water and Wastewater* 25-33.

Ashayerizadeh A., Dastar B., Shargh M.S., Mahoonak A.S., Zerehdaran S., 2017. Fermented rapeseed meal is effective in controlling Salmonella enterica serovar Typhimurium infection and improving growth performance in broiler chicks. *Veterinary Microbiology* 201**,** 93-102.

ASIA I. 2013. Food and Agriculture Organization of the United Nations. Rome.

Aziz H., 2020. Utilization of date palm leaves treated with enzymes in small ruminants feeding. *Egyptian Journal of Nutrition and Feeds* 23**,** 55-70.

Behera S., Arora R., Nandhagopal N., Kumar S., 2014. Importance of chemical pretreatment for bioconversion of lignocellulosic biomass. *Renewable and Sustainable Energy Reviews* 36**,** 91-106.

Blümmel M., Steingaβ H., Becker K., 1997. The relationship between in vitro gas production, in vitro microbial biomass yield, and 15 N incorporation and its implications for the prediction of voluntary feed intake of roughages. *British Journal of Nutrition* 77**,** 911-921.

Broderick G., Kang J., 1980. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media1. *Journal of Dairy Science* 63**,** 64-75.

Carlile M.J., Watkinson S.C., Gooday G.W., 2001. The fungi. Gulf Professional Publishing

Chandrasekaran M., Bahkali A.H., 2013. Valorization of date palm (Phoenix dactylifera) fruit processing by-products and wastes using bioprocess technology–Review. *Saudi Journal of Biological Sciences* 20**,** 105-120.

Chuan C.H., Krishnaiah K., Wong C.M., Janaun J.Palm kernel cake as substrate for βmannanase production by *Bacillus subtilis* ATCC3366 under submerged and solid

state fermentations. Proc. Proceedings of the 1st International Conference on Natural Resources Engineering & Technology. 2006.

Crueger W., Crueger A., Brock T.D., Brock T.D., 1990. Biotechnology: a Textbook of Industrial Microbiology.

Dei H., Rose S., Mackenzie A., Amarowicz R., 2008. Growth performance of broiler chickens fed diets containing shea nut (Vitellaria paradoxa, Gaertn.) meal fermented with *Aspergillus niger*. *Poultry Science* 87**,** 1773-1778.

Dusterhoft E.M., Posthumus M., Voragen A., 1992. Non-starch polysaccharides from sunflower (Helianthus annuus) meal and palm-kernel (Elaeis guineensis) meal—investigation of the structure of major polysaccharides. *Journal of the Science of Food and Agriculture* 59**,** 151-160.

Falony G., Armas J.C., Mendoza J.C.D., Hernández J.L.M., 2006. Production of Extracellular Lipase from *Aspergillus niger* by Solid-State Fermentation. *Food Technology & Biotechnology* 44**,** 235-240.

Gao J., Weng H., Zhu D., Yuan M., Guan F. et al. 2008. Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal *Aspergillus terreus* M11 under solid-state cultivation of corn stover. *Bioresource Technology* 99**,** 7623-7629.

Getachew G., DePeters E., Robinson P., 2004. In vitro gas production provides effective method for assessing ruminant feeds. *California Agriculture* 58**,** 54-58.

Ghehsareh A.M., Samadi N., Borji H., 2011. Comparison of date-palm wastes and perlite as growth substrates on some tomato growing indexes. *African Journal of Biotechnology* 10**,** 4871-4878.

Guo J., Dong X., Liu S., Tong J., 2017. Effects of long-term *Bacillus subtilis* CGMCC 1.921 supplementation on performance, egg quality, and fecal and cecal microbiota of laying hens. *Poultry Science* 96**,** 1280-1289.

Hossain M.Z., Abe J.-i., Hizukuri S., 1996. Multiple forms of β-mannanase from *Bacillus* sp. KK01. *Enzyme and Microbial Technology* 18**,** 95-98.

Iluyemi F., Hanafi M., Radziah O., Kamarudin M., 2006. Fungal solid state culture of palm kernel cake. *Bioresource Technology* 97**,** 477-482.

Jackson M., 1977. The alkali treatment of straws. *Animal Feed Science and Technology* 2**,** 105-130.

Jazi V., Boldaji F., Dastar B., Hashemi S., Ashayerizadeh A., 2017. Effects of fermented cottonseed meal on the growth performance, gastrointestinal microflora population, and small intestinal morphology in broiler chickens. *British Poultry Science* 58**,** 402-408.

Jeguirim M., Dorge S., Trouvé G., Said R., 2012. Study on the thermal behavior of different date palm residues: characterization and devolatilization kinetics under inert and oxidative atmospheres. *Energy* 44**,** 702-709.

Kholif A., Farahat E., Hanafy M., Kholif S., El-Sayed R., 2015. Utilization of cellulolytic enzymes to improve the nutritive value of date kernels and the investigation of the impact of adding these enzymes to lactating goat's diets on rumen fermentation and nutrients digestibility. *Asian Journal of Animal Science* 9**,** 441-447.

Lawal T., Iyayi E., Adeniyi B., Adaramoye O., 2010. Biodegradation of palm kernel cake with multienzyme complexes from fungi and its feeding value for broilers. *International Journal of Poultry Science* 9**,** 695-701.

Li W., Bai J., Li Y., Qin Y., Yu D., 2014. Effects of *Bacillus subtilis* on meat quality, nutrient digestibility, and serum biochemical parameters of broilers. *Chinese Journal of Veterinary Science* 34**,** 1682-1685.

Makar H., 2004. Recent advances in the in vitro gas method for evaluation of nutritional quality of feed resources. *FAO Animal Production and Health Paper* 55-88.

Malik C.P., Singh M., 1980. Plant enzymology and histo-enzymology. Kalyani Publishers, New Delhi, India.

Marini A., Daud M., Noraini S., Jame ah H., Azahan E.E., 2005. Performance of locally isolated microorganism in degrading palm kernel cake (PKC) fibre and improving the nutritional value of fermented PKC. *Journal of Tropical Agriculture and Food Science* 33**,** 311-319.

Menke K., Raab L., Salewski A., Steingass H., Fritz D. et al. 1979. The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor in vitro. *The Journal of Agricultural Science* 93**,** 217-222.

Menke K.H., 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. *Animal Research and Development* 28**,** 7-55.

Mirnawati, Djulardi A., Marlida Y., 2013. Improving the Quality of Palm Kernel Cake Through Fermentation by Eupenicillium javanicum as Poultry Ration. *Pakistan Journal of Nutrition* 12**,** 1085-1088.

Mirnawati Y.R., Marlida Y., Kompiang I.P., 2011. Evaluation of palm kernel cake fermented by *Aspergillus niger* as substitute for soybean meal protein in the diet of broiler. *International Journal of Poultry Science* 10**,** 537-541.

Obernberger I., Thek G., 2004. Physical characterisation and chemical composition of densified biomass fuels with regard to their combustion behaviour. *Biomass and Bioenergy* 27**,** 653-669.

Obese F., Osafo E., Okai D., 2001. Evaluation of the feeding value of palm press fibre using in vitro digestibility techniques. *Tropical Animal Health and Production* 33**,** 165-172.

Ong L., Abd-Aziz S., Noraini S., Karim M., Hassan M., 2004. Enzyme production and profile by *Aspergillus niger* during solid substrate fermentation using palm kernel cake as substrate. *Applied Biochemistry and Biotechnology* 118**,** 73-79.

Ørskov E., McDonald I., 1979. The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. *The Journal of Agricultural Science* 92**,** 499-503.

Pangsri P., Pangsri P., 2017. Mannanase enzyme from *Bacillus subtilis* P2-5 with waste management. *International Journal of Poultry Science* 138**,** 343-347.

Pinto G.A., Leite S.G., Terzi S.C., Couri S., 2001. Selection of tannase-producing *Aspergillus niger* strains. *Brazilian Journal of Microbiology* 32**,** 24-26.

Rad A.R., Ahmadi F., Mohammadabadi T., Ziaee E., Polikarpov I., 2015. Combination of sodium hydroxide and lime as a pretreatment for conversion of date palm leaves into a promising ruminant feed: an optimization approach. *Waste and Biomass Valorization* 6**,** 243-252.

Ramin M., Alimon A., Ivan M., 2010. Effects of fungal treatment on the in vitro digestion of palm kernel cake. *Livestock Research for Rural Development* 22**.**

Rezaeenia A., Naserian A., Valizadeh R., Tahmasbi A., 2014. Chemical composition, DM, and NDF degradation kinetics in rumen of eleven different date pits. *Annual Research & Review in Biology* 3524-3531.

Rizal Y., Nuraini, Mirnawati, Mahata M.E., 2013. Comparisons of Nutrient Contents and Nutritional Values of Palm Kernel Cake Fermented by Using Different Fungi. *Pakistan Journal of Nutrition* 12**,** 943-948.

SAS. 2003. SAS user's guide: Statistics. Sas Inst

Schallmey M., Singh A., Ward O.P., 2004. Developments in the use of Bacillus species for industrial production. *Canadian Journal of Microbiology* 50**,** 1-17.

Shawrang P., Sadeghi A., Ahmadpanah J., 2013. Ruminal degradation kinetics of wheat straw irradiated by high doses of electron beam. *Iranian Journal of Applied Animal Science* 25-29.

Shelton J., Dean D., Southern L., Bidner T., 2005. Effect of protein and energy sources and bulk density of diets on growth performance of chicks. *Poultry Science* 84**,** 1547-1554.

Sinurat A.P., T P., T P., 2013. Improving nutrient values of palm kernel cake (PKC) by reducing shell contamination and enzymes supplementation. *Jurnal Ilmu Ternak dan Veteriner* 18**,** 27-33.

Sommart K., Parker D., Rowlinson P., Wanapat M., 2000. Fermentation characteristics and microbial protein synthesis in an in vitro system using cassava, rice straw, and dried ruzi grass as substrates. *Asian-Australasian Journal of Animal Sciences* 13**,** 1084-1093.

Squires M., Naber E., Toelle V., 1992. The effects of heat, water, acid, and alkali treatment of tomato cannery wastes on growth, metabolizable energy value, and nitrogen utilization of broiler chicks. *Poultry Science* 71**,** 522-529.

Sun S., Sun S., Cao X., Sun R., 2016. The role of pretreatment in improving the enzymatic hydrolysis of lignocellulosic materials. *Bioresource Technology* 199**,** 49-58.

Theodorou M.K., Williams B.A., Dhanoa M.S., McAllan A.B., France J., 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. *Animal Feed Science and Technology* 48**,** 185-197.

Van Soest P.v., Robertson J., Lewis B., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science* 74**,** 3583-3597.

van Zyl P.J., Moodley V., Rose S., Roth R., Van Zyl W., 2009. Production of the Aspergillus aculeatus endo-1, 4-β-mannanase in A. *niger*. *Journal of Industrial Microbiology & Biotechnology* 36**,** 611-617.

Yadi P., Yana S., 2011. The influence of palm kernel cake and rice bran fermentation product mixture to the broiler carcass quality. *International Journal of Science and Engineering* 2**,** 1-3.

Yousuf R.G., Winterburn J.B., 2016. Date seed characterisation, substrate extraction, and process modelling for the production of polyhydroxybutyrate by *Cupriavidus necator*. *Bioresource Technology* 222**,** 242-251.