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Improvement of Quality and Digestibility of *Moringa Oleifera* Leaves Feed via Solid-State Fermentation by *Aspergillus Niger*

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Abstract:
The *Moringa oleifera* leaf is an important source worldwide with a high nutritional value and functions in food and feed that may also treat a myriad of ailments but the leaf has low organoleptic properties and digestibility. To overcome this shortcoming, a novel *Aspergillus niger* was isolated from the *Moringa* leaf material. The fungal strain grows well on moist *Moringa* leaves and requires no additives. After performing a single factor test for temperature, moisture, inoculation size, and fermentation, the optimized condition was determined by using a response surface method, followed by a small-scale production test. The pleasant, sweet smelling aroma in the fermented leaves was then generated, supplementing than its native repulsive smell. The protein content and digestibility of the leaves increased by 23.4 % and 54.4 %, respectively; the direct-fed microbes reached up to 1.99×10^9 CFU per gram of fermented freeze-dried *Moringa* leaves. Digestive lignocellulolytic enzymes were substantially produced with 2.97 ± 0.24 U.g^{-1} of filter paper activity and 564.9 ± 37.4 U.g^{-1} of xylanase activity. Moreover, some functional components, such as flavonoids and γ-Aminobutyric acid content, were also significantly increased compared to that of the unfermented leaves. In conclusion, the feed quality and digestibility of *Moringa oleifera* leaves were greatly improved via solid-state fermentation by *Aspergillus niger*. Fermented *Moringa oleifera* can be used as a potentially high- quality feed alternative for the animal industry.

Keywords: *Aspergillus niger*, *Moringa* leaf, biological feed, solid-state fermentation, odor, crude protein content

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1 Introduction

In recent decades, demand for animal products has increased dramatically due to human population growth and consumer tastes (Salter 2017). Animal-derived protein currently accounts for almost 40 % of total human protein consumption. The Food and Agriculture Organization of the United Nations (FAO) expects a world meat production of 410 billion kg per year by 2050 (Boland et al. 2013; Spiegel, Noordam, and Fels-Klerx 2013). On a worldwide basis, corn and soybean meal are the main staples in the diet of pigs and poultry, providing most of the energy and nutrients needed (Lindberg 2014; Salter 2017). However, population growth, climate change, volatile energy prices, soil erosion, and water scarcity threaten to make food sources more difficult and more expensive to produce (Fraser et al. 2016). Moreover, the use of cereals in animal diets creates a competitive conflict with human nutrition, and the use of soybean is expensive (Vasta et al. 2008). Therefore, animal scientists need to exploit alternative feedstuffs in order to meet these challenges. New feed resources should have high nutritive value and conversion efficiency, be able to optimize animal product quality, and use land and water efficiently (Poppi and McLennan 2010). *Moringa* leaves are potential new feed sources which have generated public attention over the past few decades.

There are 13 species in the monogeneric genus *Moringa* of *Moringaceae* family, which are indigenous to north-west India, Africa, Arabia, Southeast Asia, the Pacific, Caribbean Islands and South America (Paliwal, Sharma, and Pracheta 2011). *Moringa oleifera* Lam. (*M. oleifera*) is the most widely cultivated species of the *Moringaceae*

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in the world (Leone et al. 2015; Paliwal, Sharma, and Pracheta 2011), due to its low production cost (Teixeira et al. 2014). *M. oleifera* leaves (MOLs) are rich in antioxidants, minerals, beta-carotene, and proteins (Falowo et al. 2018; Leone et al. 2015; Radovich 2011). HPLC analysis indicates the presence of phenolic acids (gallic, chlorogenic, ellagic and ferulic acid) and flavonoids (kaempferol, quercetin, and rutin) in MOLs. The high phenolic content of MOLs generates potent antioxidant properties both *in vitro* and *in vivo* (Verma et al. 2009). MOLs thus have an impressive range of medicinal uses with high nutritional value (Anwar et al. 2007). The leaves of *M. oleifera* can be used for treating myriads of ailments including typhoid fever, asthma, cough, parasitic diseases, arthritis, diabetes, cuts, skin swellings and skin diseases, genito-urinary ailments, hypertension, body pains and weakness; MOLs also have the robust ability to challenge terminal diseases such as HIV/AIDs, chronic anemia, cancer, malaria and hemorrhage (Abe and Ohtani 2013; Anwar et al. 2007; Caceres et al. 1991; Popoola and Obembe 2013; Ramachandran, Peter, and Gopalakrishnan 1980; Sivasankari, Anandharaj, and Gunasekaran 2014). Crude protein (CP) content in MOL forage reaches up to 25.1–29.1 % (Gopalakrishnan, Doriya, and Kumar 2016; Makkar and Becker 1996, 1997) and the amino acid profile of its leaves meet the standards of the World Health Organization (WHO) (Makkar and Becker 1996). Accordingly, MOL is regarded as having great potential as an animal feedstuff (Nouman et al. 2014).

There have been a few animal feeding trials of MOL by zoologists. MOL has been used as a sustainable feed resource for Rhode Island Red hens (Mohammed et al. 2012), cows (Sánchez, Spörndly, and Ledin 2006), lactating goat (Kholif et al. 2015) and *Nile tilapia* (Richter, Siddhuraju, and Becker 2003). However, MOL meal is less than 15 % of the replacing level of sesame meal in lactating goat's diets and less than 10 % of the replacing level for fish meal in *Nile tilapia*'s diets for improving production (Kholif et al. 2015; Richter, Siddhuraju, and Becker 2003). The limited use of MOL may be due to its unpleased smell and difficult/poor digestibility at only 31.8 % total protein digestibility *in vitro* (Teixeira et al. 2014). Therefore, A process for improving MOL feed quality is needed. Fermentation can help to improve the smell, increase the CP content and generate digestive enzymes in order to improve the feed quality of forages (Salgado et al. 2015; Wang et al. 2013). However, MOLs contain lots of antimicrobial components, for example, reaching up to 5.3 % per dry weight gram in polyphenols (Vongsak et al. 2013). Therefore, a safe and efficient strain is distinctly important for the fermentation of MOLs. *Aspergillus niger* (*A. niger*) is a mycelial fungus and is accepted as a direct-fed microbial for feed production in some countries including China and the USA. *A. niger* is more suitable for the fermentation of leaf material than bacteria due to its higher levels of lignocellulase secretion (Schuster et al. 2002; Wang et al. 2013). In this present study, 13 number of *A. niger* strains were screened for selection and the strain lm_01 was employed to process MOL *via* solid-state fermentation to improve its feed quality. Flavor and the content of CP, amino acid, digestive enzymes and protein digestibility were widely investigated in the fermented MOL compared with the non-fermented one for evaluating charges of quality.

2 Materials and methods

2.1 *Moringa Oleifera* leaves

MOLs used in this study were harvested from August to September in 2016 at Baoshan city, Yunnan province in China. A nature drying process was executed in the native sunning ground. Dried leaves were ground, and the particle size was controlled to less than 840 μm using a classifying screen. The leaf meal had a water content of 9.82 ± 0.03 % and was stored at indoor temperature until use.

2.2 Isolation and identification of black *Aspergillus*

For harvesting black *Aspergillus* from *M. oleifera*, 100 g of wet MOLs with 50 g of water was put into a 1000 ml beaker to gather microbes for three days without a cover. 10 g of wet culture was then suspended in 100 ml of distilled water and another five dry leaf flours were suspended in the same volume of distilled water. The suspension liquid was stirred at 300 rpm for 10 min in order to establish a dilution series; refer to the description by Ulrich and Wirth (1999). After a 15 min suspension, the liquid was serially diluted from 10^{-4} - 10^{-6}. 0.4 ml of supernatant liquor was spread on 10 cm plates containing Martin's agar for enumeration of the black *Aspergillus* colony-forming units (CFUs). This was performed in 10 replicates. All plates were incubated at 28°C for 7 days. Under a 10× magnifying glass, the conidia colonies that changed from brown to black were sub-cultured on a new agar plate for harvesting pure colonies. Malt extract agar with 10 mg/L boscalid (MEA-B) plate was used to distinguish *A. niger* from *A. carbonarius* combine with conidia (Samson et al. 2007).

These strains were identified by using a 18S rDNA sequencing method (Wang et al. 2013). Briefly, 0.1 g of hyphae was frozen with liquid nitrogen and finely ground using a pestle, followed by purification of the DNA

with a fungal DNA Kit (Omega, USA). 50 μl of the reaction mixture contained 1× PCR buffer, 5 mM $MgCl_2$, 2 mM dNTPs, 2.5U of Taq polymerase from Fermentas, 1 μl of DNA template and each 10 μM primers (EF4f (5′-GGA AGG G[G/A]T GTA TTT ATT AG- 3′) and Fung5r (5′-GTA AAA GTC CTG GTT CCC-3′)). The thermal cycler was heated to 94°C for 3 min; then, 35 cycles were run at 94°C for 45 s, 55°C for 45 s, and 72°C for 1.5 min; and finally, it was settled at 72°C for 7 min. The purified product was examined by 1 % agarose gel electrophoresis, and the DNA sequence was determined by Sangong Biotech (Shanghai, China) Co., Ltd With the fungal morphological features, the GenBank sequence most similar to the clone was used for classifying the fungal strains to a specific genera. Evolutionary analyses were conducted in MEGA7 (Kumar, Stecher, and Tamura 2016). The conidial morphology of lm_01 was observed with an SU-70 scanning electron microscope (Hitachi, Japan).

2.3 Solid-state fermentation

Dry *Moringa* leaves and water without additive**s** were mixed using an SM-25 screw mixer (Sanyo, Japan) with a shred/water mass ratio of 1:1 for 20 min. The mixture was rationed in 250 ml triangular flasks with 60 g of the mixture in each bottle, consuming about 40 % of the bottle's volume. These bottles were sealed with a hydrophobic fluoropore membrane (Tianke, China) and autoclaved at 115°C for 15 min. Inoculation of three loops (0.5 cm in diameter) of an agar plate of each strain in each bottle, the flask was inverted 10 times to combine the mycelium. These flasks were tilted downward in the incubator at 28°C for 5 days. The experiment was performed in triplicates. All of the samples were frozen dry for assays by a two-step operation. Fermented samples were frozen at a −80°C refrigerator for 12 h, then were lyophilized in the 18 L lyophilizer (Labconco de, USA) until the pressure fell to 0.01bar.

2.4 Small-scale production test

The optimized conditions were used for a small-scale production test. The MOLs with water rate 49 % were autoclaved for 15 min at 121°C. Synchronously, plastic trays with reticular holes (39.5 × 31 × 10 cm) were sterilized by spraying and painting 75 % ethanol solution, then two-layer sterile gauze was cover up the trays on the BCM-100A clean bench (Antai, China). The cool MOLs inoculated were tiled into the trays with about 6 cm thickness, then fermenting in the sterile constant temperature and humidity incubator with humidity 80 % for 65 h at 33°C.

2.5 Odor assessment

The scent experiment was carried out by a team of eight assessors using a sensory analysis ranking method (Zhang, Xu, and Wang 2006). Briefly, each fermented and unfermented MOLs sample was marked by a three-digit number for sensory analysis. All samples were randomly placed on a white plate. The assessor smelled the odors one by one and described the features and intensity of the sample odor under the marked number. The odor features were decided by the frequentness using for detailing MOLs odor and the odor intensity was decided by a ranking method.

2.6 Ochratoxin a assay

For extracting Ochratoxin A (OTA), 5.0 g of fermented and unfermented MOLs was immersed with 20 ml of a methanol solution (70 % v/v) in a 50 ml centrifuge tube, then vortexed for 3 min. The filtered liquid was collected for analysis. OTA assay was executed by an Ochratoxin A ELISA test Kit (Wise, China).

2.7 Protein and amino acid assay

Crude protein (CP) was assessed according to the Kjeldahl method (Bremner et al. 1996) using a set of Gerhardt Kjeldahl determination devices (Buchi, Switzerland). In brief, 0.5 g of MOLs with 0.15 g $CuSO_4$, 4.5 g K_2SO_4, and 15.0 ml of concentrated sulfuric acid were digested in a K-438 digestive tube at 200°C for 30 min and then raised to 400°C until the liquid became light green. 200 ml of liquid was collected on a K-350 distilling apparatus according to instrument instructions and titrated with 0.05 mol/L hydrochloric acid solution until light red

with phenolphthalein as the endpoint indicator. The usage amount of hydrochloric acid was calculated, and the content of crude protein was expressed as per-centum. Three duplicates for each sample were performed.

Free and total amino acids were analyzed using an S433D automatic amino acid analyzer (Sykam, Germany). In brief, 1.0 g MOLs was extracted by boiling them three times for 20 min in 100 ml of a 75 % ethanol solution inside a 250 ml conical flask. The filtrates were combined, and ethanol was removed through evaporation. The residuum was transferred to a 25 ml volumetric flask in divided doses using 0.02 mol/L hydrochloric acid solution and a constant volume. The supernatant was used for analysis of free amino acids. 0.10 g MOLs with 10.0 ml of 6 mol/L were hydrolyzed at 110°C for 22 h in the sealed digestion tube. After filtering, 1.0 ml of filtrate was dried under vacuum conditions and diluted appropriately for the analysis of total amino acids by the automated program.

2.8 Extraction and assay of flavonoids

Flavonoid extraction and assay refer to Wang et al. (2013). 70 % (v/v) aqueous ethanol was used as an extraction solvent. Ten grams of fermented or unfermented MOLs were placed in a 250 ml conical flask with a cover, 200 ml of the solution was added, and the sample was immersed in the solution with gentle shaking. The extraction was executed with an optimized ultrasonic-assisted program at a power of 100 W, an ultrasonic frequency of 25 kHz, for a time period of 45 min, and a temperature of 60°C. After filtration, the filtrate was collected into a clean receiving flask, and the filtrate cake was subjected to the above extraction process once again. Twice-filtered liquid samples were combined for determining the flavonoid content.

In brief for assay, 0.5 ml of the double-dilute extract or the quercetin standard solution was mixed with 3.0 ml of methanol, 0.2 ml of 10 % aluminum chloride (distilled water as blank), 0.2 ml of 1 M potassium acetate, and 5.6 ml of distilled water. After 30 min of incubation at 25°C, the absorbance at 415 nm was determined against a distilled water blank on a UV mini-1240 spectrophotometer (Shimadzu, Japan). All samples were prepared in triplicates, and the mean values of the flavonoid content were expressed as milligrams of quercetin equivalents per gram of dry weight (dw), calculated according to the standard calibration curve.

2.9 Analysis of filter paper activity (FPA) and xylanase activity

Five-gram samples were extracted with 200 ml of citrate buffer (0.05 mol/L, pH 5.0). The suspension was magnetically mixed for 30 min at room temperature and followed by 24 h of incubation at 4°C. After filtration, the filtrate was collected for analysis of FPA and xylanase activity. FPA and xylanase assay refer to the description by Wang et al. (2013) and Souza, Roberto, and Milagres (1999), respectively. For FPA, 50 mg of quantitative paper (Xinhua, China) was soaked with 1.0 ml of citrate buffer in a 25 ml test tube, mixed with 0.5 ml of the enzyme (autoclaved enzyme liquid as control), and incubated at 37°C for 60 min. For xylanase, 0.5 ml of the enzyme was mixed with 1.5 ml of 1.0 % of xylan (Gentihold, China), and incubated at 50°C for 30 min. All the reactions were terminated by adding 2.0 ml of dinitrosalicylic acid (DNS). The reducing sugar content was estimated by the DNS method (Miller 1959). One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of reducing sugars per minute.

2.10 *In Vitro* protein digestibility assay

A multi-enzyme system was used for an *in vitro* protein digestibility assay,which consisted of 23,360 U trypsin, 149 U α- chymotrypsin and 133 U peptidase per ml (Alonso, Aguirre, and Marzo 2000). Five milliliters of the multi-enzyme solution was added to 50 ml of suspension medium containing. One gram of fermented, or unfermented *Moringa* meal, then adjusted to a pH 8.0 and incubated at 37°C. The pH change after a ten minutes period (pH10 min) was recorded while the mix was stirred. The percent of *in vitro* protein digestibility (IVPD) was calculated with the value equal to "210.46–18.10 pH_{10min}".

2.11 Box-behnken design (BBD) and statistical analysis

Response surface methodology (RSM) was used to optimize the pretreatment conditions for the CP content of MOLs. Three factors and three levels were selected, the actual ranges and levels of the independent variables investigated in this study are shown in Table 1. The BBD was formed by SYSTAT 12 (Systat Software, Inc., USA). The software was also used to analyze the data, describe the response surface, and generate the response surface

plots. The goodness of fit of the second-order polynomial model equation was indicated by an F test at the 5 % level of significance, the determination coefficient R^2, and the lack of fit. Response surface contour plots were generated to indicate the CP content fluctuation of MOLs. The most optimized conditions were decided by the "Response Surface Method – Optimize - Canonical" program of SYSTAT 12. The statistical analysis was carried out in the one-way ANOVA program of the SAS system for Windows 8.02 (SAS Institute Inc., USA). Duncan's multiple-range test was selected as the comparison method in the program and the significance level was set at 0.05.

3 Results

3.1 Isolation of black *Aspergillus* fungi and pre-experiment of fermenting *Moringa Oleifera* leaves

By investigating the spore color, five out of 20 isolates for probable black *Aspergillus* were obtained from the Martin's agar plate. Among the five isolates, two were obtained from the plate of wet *Moringa* leaf culture and three were from the agar plates of the dry leaf. Fourteen of the isolates and strains were selected for the fermentation of MOLs for five days, and the odor, fermentation efficiency and OTA of the cultures were then investigated (Table 1). Except for XA-8, the others could grow well in the MOLs medium. The isolate lm_01 had the strongest fermentation efficiency and covered the whole medium in less than 24 h with a significantly improved sweet aroma replacing the unpleasant *Moringa* odor. Lm_04 and Gyx086 also had fast growth in MOLs with the hyphae covering the culture in 24–36 h but brought different odor intensity. The others had a slower growth than the foregoing three and the MOLs had a lightly sweet-aroma odor. The sweet-aroma odor gradually weakened when the fermentation time was over 72 h. Furthermore, black conidia could be found in some of the cultures. Notably, black conidia were observed at 60 h in lm_01 culture. OTA was not detected in all cultures.

Table 1: Odor and fermentation efficiency comparison of five black *Aspergillus* isolates from *Moringa oleifera* leaves with other eight *Aspergillus* strains.

NO.	Strains and isolates	FE* (h)	Odor feature (36 ~ 72 h)	Sweet-aroma intensity **	OTA (μg/g)	Fungi source (Species)
1	Un-inoculation	N [a]	Unpleased moringa odor	-	0 [b]	N
2	lm_01	< 24	Sweet aroma	+++	0	This study (*A. niger*)
3	lm_02	36	Sweet aroma	+	0	This study (— [c])
4	lm_03	36	Sweet aroma	+++	0	This study (—)
5	lm_04	24–36	Sweet aroma mixed with unpleased odor	++	0	This study (—)
6	lm_05	36	Sweet aroma	+	0	This study (—)
7	XA-8	N	Unpleased moringa odor	-	0	Lab store (*A. niger*)
8	XA-10	> 48	Sweet aroma mixed with moringa odor	+	0	Lab store (*A. niger*)
9	Tu-A	> 36	Sweet aroma	+	0	Lab store (*A. niger*)
10	Tu-B	> 36	Sweet aroma mixed with moringa odor	+	0	Lab store (*A. niger*)
11	Tu-C	36	Sweet aroma mixed with moringa odor	++	0	Lab store (*A. niger*)
12	Gyx003	> 36	sweet aroma and moringa odor	+	0	(Wang et al. 2013) (*A. niger*)
13	Gyx017	> 36	sweet aroma	+	0	(Wang et al. 2013) (*A. awamori*)
14	Gyx086	24–36	sweet aroma	++	0	(Wang et al. 2013) (*A. niger*)

* FE (fermentation efficiency) was assessed by the need time of fungal hypha covering the moringa leaf cultures; ** different signs are significantly different: thick sweet aroma +++, sweet aroma ++, light sweet aroma +, - no sweet aroma; [a] The N means nothing; [b] the zero means without detectability of OTA; [c] — black *Aspergillus* was isolated by morphological identification which produces the black conidia.

3.2 Identification of the New Isolate Lm_01

Isolates of lm_01 were able to grow fast in Martin's agar plate and to sporulate brown-black spores but were not able to grow in a malt extract agar plate with 10 mg/L boscalid after 48 h, when incubated at 28°C. This species produced smooth conidia in the size range of 3–5 µm. The 18S rDNA sequencing of lm_ 01 was 100 % of ident value with those fungi in the Gene Bank that are identified as *A. niger* (The sequence data in GenBank MG889596). The phylogenetic tree of lm_01 was constructed as shown in Figure 1, and the spores of lm_01 were 3–4 µm in size.

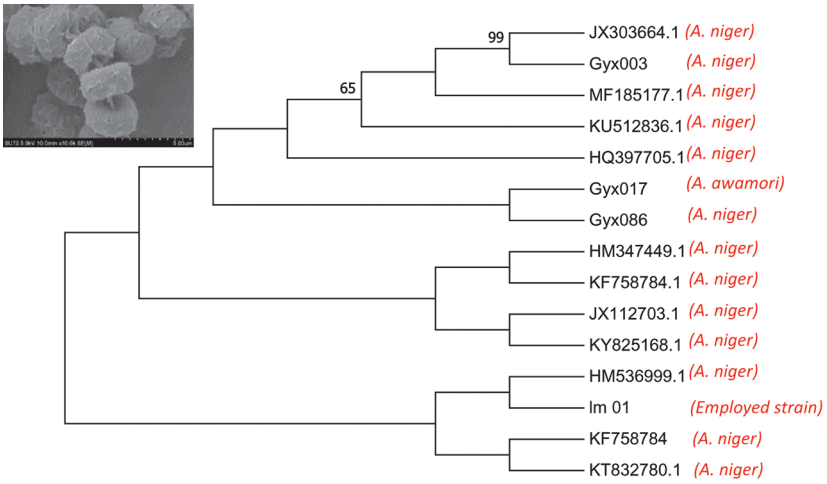

Figure 1: Bootstrap consensus tree of lm_01 com by Neighbor-joining alignment and its conidia figure by SEM. Note Those codes are the numbers of fungi strains in the GeneBack database.

3.3 Protein content affected by fermentation temperature, time, moisture and inoculation size of medium

Fermentation temperature, time, moisture and inoculation size greatly affected the growth of fungi lm_01 on the MOLs. Crude protein content was significantly increased by the fermentation process ($p < 0.05$) as shown in Figure 2. Temperatures of 28–31°C were effective for stimulating fungal growth, and increasing the content of CP in MOLs cultures (Figure 2(a, b). A moisture content of about 50 % and over three loops of inoculation size for per 30 g of MOL dried matter were appropriate for the same purpose (Figure 2(c-f). Moreover, less than 72 h of fermentation time was relatively more appropriate based on the expected production efficiency and CP content. As mentioned above, temperature, moisture and fermentation time were determined by the index to further optimize the experiment and to increase the CP content of the culture.

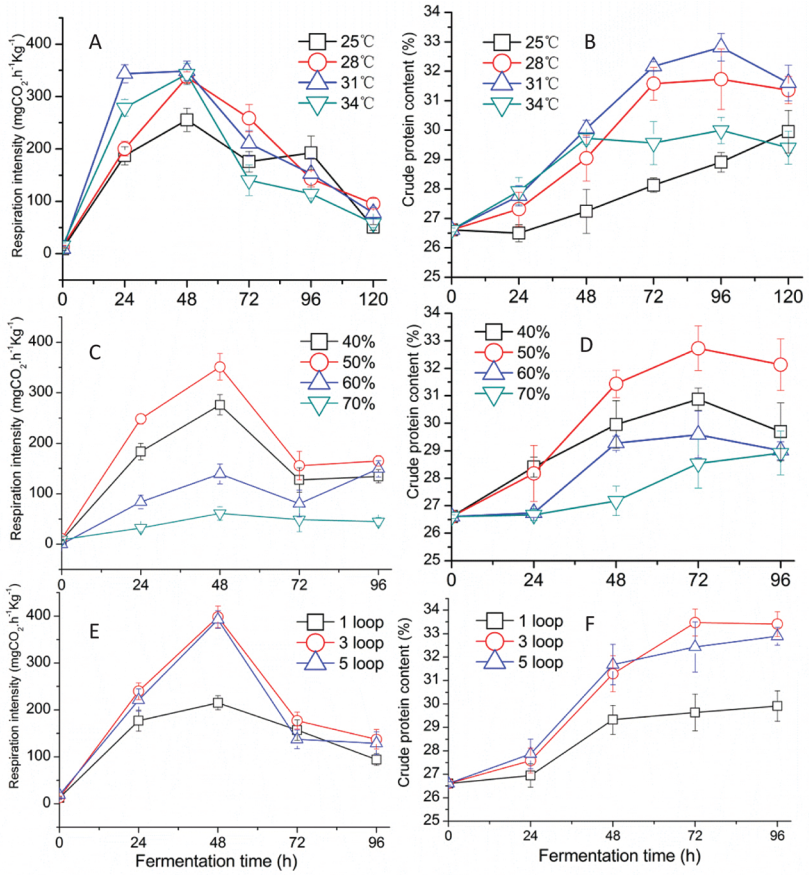

Figure 2: Fungal growth and CP contents in MOL cultures fermented under different conditions of temperature, moisture, inoculation size, and fermentation time.

3.4 Optimization of CP content in fermented MOL culture by an RSA method

The results of the Box-Behnken experiment are presented in Table 2. The "RSM -Estimate Model" program of the SYSTAT 12 was used to analyze the data with a full quadratic model. The results of the variance analysis (ANOVA), regression coefficients analysis and lack of fit test are shown in Table 3. Table 3(a) shows the values of regression coefficients and the corresponding P for the quadratic model. X1- X3 represents the coded variables and Y stands for the response variables of the CP content. The model can be mathematically expressed by using regression coefficients as coded factors (CP content = 32.573 + 0.118X1 - 0.788X2 + 0.855X3 + 0.03X1X2 −0.24X1X3 + 0.105X2X3 −0.739$X1^2$ −2.104$X2^2$ −0.379$X3^2$). The R^2 of the fitted model was 0.987. The lack of fit test produced a p-value of 0.377 (as shown in table 3(b), indicating it was not significant ($p > 0.05$). These values suggest that a full quadratic model was appropriate for the trial.

Table 2: Box–Behnken design matrix for optimization of the CP content during the state-solid fermentation of the lm_01.

Run	X_1 Temperature(°C)	X_2 Moisture(%)	X_3 Time(h)	CP content (% dw)	Predict value
1	−1 (28)	−1 (45)	0 (60)	30.37 ± 0.19	30.221
2	1 (32)	−1	0	30.79 ± 0.22	30.606
3	−1	1 (55)	0	28.61 ± 0.47	28.794
4	1	1	0	29.15 ± 0.20	29.09
5	−1	0 (50)	−1 (48)	30.84 ± 0.25	30.587
6	1	0	−1	30.62 ± 0.17	30.613
7	−1	0	1 (72)	32.08 ± 0.07	32.087
8	1	0	1	32.28 ± 0.13	32.533
9	0 (30)	−1	−1	29.59 ± 0.37	29.783
10	0	1	−1	28.62 ± 0.16	28.687
11	0	−1	1	32.04 ± 0.13	31.973
12	0	1	1	30.11 ± 0.42	29.917

13	0	0	0	32.74 ± 0.31	32.573
14	0	0	0	32.67 ± 0.14	32.573
15	0	0	0	32.31 ± 0.23	32.573

Table 3 Response surface ANOVA of the quadratic model of the CP content

a The regression coefficients ANOVA of the response surface quadratic model.

Effect	Coefficient	Standard Error	t	p-value
CONSTANT	32.573	0.162	201.247	0.000
X1	0.118	0.099	1.185	0.289
X2	−0.788	0.099	−7.945	0.001
X3	0.855	0.099	8.626	0.000
X1*X1	−0.739	0.146	−5.066	0.004
X2*X2	−2.104	0.146	−14.422	0.000
X3*X3	−0.379	0.146	−2.599	0.048
X1*X2	0.03	0.14	0.214	0.839
X1*X3	−0.24	0.14	−1.712	0.148
X2*X3	0.105	0.14	0.749	0.488

b The variance analysis ANOVA and the lack of fit test for the response surface quadratic model.

Source	df	SS	Mean Squares	F-ratio	p-value
Regression	9	28.899	3.211	40.856	0.001
Linear	3	10.920	3.640	46.314	0.001
Quadratic	3	17.701	5.900	75.076	0.001
Interaction	3	0.278	0.093	1.179	0.405
Residual Error	5	0.393	0.079		
Total Error	14	29.292			
Squared Multiple R	0.987				
Adjusted Squared Multiple R	0.962				
Lack of Fit	3	0.286	0.095	1.794	0.377
Pure Error	2	0.106	0.053		
Residual Error	5	0.393	0.079		

The 2-D contour plots for the CP content were generated based on the model from the SYSTAT 12 software program "RSM-Contour/surface" and were depicted in Figure 3. Each subgraph presented the effect of two variables on CP content. There is some interaction between temperature and moisture. An elliptical contour plot was affected by CP content with a similar interaction between fermentation time, temperature, and moisture as shown in the plot. X1 (temperature) and X2 (moisture) had an arched curvilinear effect on CP content, but X3 (fermentation time) had an almost liner effect on the response during fermentation. The statistically optimal values of variables for CP content were verified from the canonical analysis of the response surface. The stationary point obtained by the SYSTAT 12 program "RSM-Optimize" is Maximum. The optimal values were 30.32°C, and 48.719 % moisture at 74.774 h. The optimized predicted value for CP content was 33.21 %. However, the sweet aroma of the fermented MOL would quickly decrease when the fermentation time was over 72h. Therefore, it was necessary to control the fermentation time to less than 72 h. A 65 h fermentation time was decided upon based on the contour plots (Figure 3(b-c) and the preceding trials (Table 2) in protecting the thick, sweet aroma. The predicted value of CP content in this fermentation condition was 33.0 % which is only slightly lower than the optimized predicted value of 33.21 %.

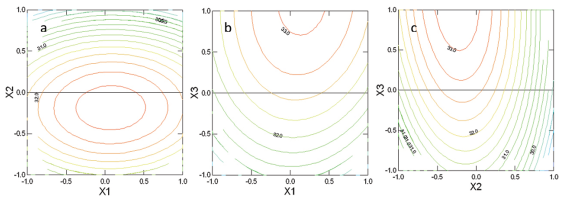

Figure 3: 2-D contour plots on interactive effects among temperature, moisture content, and fermentation time on CP content of MOL cultures fermented by lm_01.

3.5 Small-scale production test of fermented MOLs by Lm_01

Seven and a half kilograms of MOLs were fermented under proximate optimized conditions with a temperature of 33°C, 49 % water ratio, inoculation size of 3 loops per 30 g dw and a fermentation time of 65 h (Table 4). The CP content was 32.83 ± 1.07 % freeze-dried samples, which were very close to the predicted value and did not significantly differ from the test value when fermenting under a 50 % water ratio at 30°C for 60 h. The feed quality of the fermented MOLs was greatly improved with a significantly higher CP digestibility, lignocellulolytic enzyme activity, and flavor as shown in Table 4.

Table 4: The content of free amino acids and total amino acids in fermented MOLs and unfermented MOLs.

	Unfermented MOLs	Fermented MOLs		Unfermented MOLs	Fermented MOLs
Crude protein (%)	26.61 ± 0.07	32. 83 ± 0.11 [s]	**Odor**	Distasteful MOls smell	Thick pleasant sweet smell
CP digestibilitya (%)	31.56 ± 0.25	48.72 ± 0.16 [s]	**Totle flavonoids (mg/g dw)**	16.44 ± 0.15	18.31 ± 0.18[s]
Xylanase (U/g dw)	— b	564.93 ± 37.41	**γ-Aminobutyric acid (mg/g dw)**	3.20 ± 0.09	3.89 ± 0.31[s]
FPA (U/g dw)	—	2.97 ± 0.24	**OTA**	0	0
Reducing sugar (G mg/g)	42.47 ± 0.05	19.22 ± 0.16 [s]	**A. niger (*109 CFU/g dw)**	—	1.99 ± 0.27
pH	5.62 ± 0.09	2.87 ± 0.12 [s]	**Mass weight (kg)**	15.00	11.04

Note [a]*In vitro* protein digestibility shown as hydrolysis value the value of casein was 93.8; [b] — Not determinant was as zero; [s] a significantly different between fermented and unfermented MOLs (α = 0.05)

3.6 Aroma component of fermented MOLs

The chromatogram of GC-MS showed the components of headspace air from unfermented and fermented MOLs (Figure 4). There was a great change of peak in the two subgraphs which corresponded with a great change of aroma between fermented and unfermented MOLs. The 2-methyl-butanoic acid, furanoid, linalool and benzofuranone are main odor components which have been confirmed in unfermented MOLs, but in fermented MOLs toluene and styrene are the main odor components. However, there were only a few components that could be identified using the NIST standard reference with a probability value over 60.

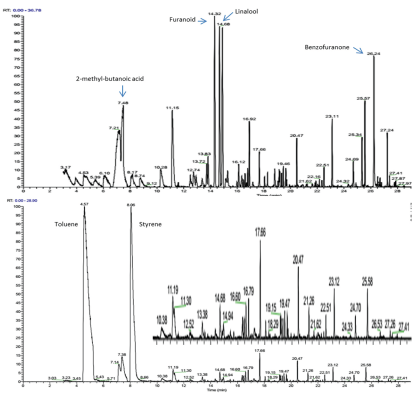

Figure 4: The chromatograms of GC-MS analysis from the headspace air of unfermented and fermented MOLs (a: unfermented MOLs; b: fermented MOLs).

3.7 Amino acids constitute of fermented MOLs

The amount of free and total amino acids in the MOLs fermented by lm_01 were 17.77 and 283.9 mg per gram, respectively (Table 5), and total amino acids were significantly higher than the unfermented one ($p < 0.05$). The essential amino acids were very rich in both the unfermented and fermented MOLs.

Table 5: The content of free amino acids and total amino acid in the fermented and unfermented MOLs.

Name	Free amino acid/mg.g^{-1}		Total amino acid			
			Unfermented MOLs		Fermented MOLs	
	Unfermented MOLs	Fermented MOLs	mg.g^{-1}	mg/g CP	mg.g^{-1}	mg/g CP
Asp	0.36 ± 0.05	0.41 ± 0.08	20.24 ± 0.35	100.70	29.59 ± 0.29 ^{s}	104.2
Thr	0.80 ± 0.02	0.84 ± 0.03	10.19 ± 0.21	50.70	14.71 ± 0.17 ^{s}	51.81
Ser	1.04 ± 0.09	1.06 ± 0.14	9.85 ± 0.32	49.00	14.16 ± 0.41 ^{s}	49.87
Glu	1.15 ± 0.03	0.89 ± 0.07	30.53 ± 0.28	151.89	42.44 ± 0.33 ^{s}	149.49
Gly	0.18 ± 0.03	0.16 ± 0.02	11.23 ± 0.57	55.87	16.48 ± 1.09 ^{s}	58.04
Ala	1.80 ± 0.06	1.80 ± 0.05	13.49 ± 0.73	67.11	19.65 ± 0.50 ^{s}	69.21
Cys	0.04 ± 0.04	0.02 ± 0.06	1.41 ± 0.34	7.01	1.89 ± 0.13 ^{s}	6.66
Val	0.97 ± 0.00	1.04 ± 0.04	12.71 ± 0.49	63.23	18.18 ± 0.74 ^{s}	64.03
Met	0	0	2.44 ± 0.06	12.14	3.59 ± 0.17 ^{s}	12.64
Ile	0.74 ± 0.06	0.81 ± 0.10	9.89 ± 0.22	49.20	14.73 ± 0.50 ^{s}	51.88
Leu	0.92 ± 0.08	0.95 ± 0.15	18.81 ± 0.79	93.58	27.95 ± 1.05 ^{s}	98.45
Tyr	0.26 ± 0.01	0.27 ± 0.09	7.70 ± 0.48	38.31	11.02 ± 0.25 ^{s}	38.81
Phe	1.50 ± 0.07	1.19 ± 0.13	12.93 ± 0.12	64.33	18.51 ± 0.77 ^{s}	65.20
His	0.25 ± 0.01	0.19 ± 0.04	7.20 ± 0.27	35.82	10.11 ± 0.61 ^{s}	35.61
Lys	0.17 ± 0.05	0.18 ± 0.11	11.93 ± 0.19	59.35	13.52 ± 0.33 ^{s}	47.62
Arg	0.66 ± 0.01	0.60 ± 0.08	11.56 ± 0.09	57.51	17.02 ± 0.35 ^{s}	59.95
Pro	1.17 ± 0.09	1.07 ± 0.10	8.91 ± 0.18	44.33	10.33 ± 0.32 ^{s}	36.38
Total	17.34 ± 0.26	17.77 ± 0.45	201.0 ± 1.47		283.9 ± 1.85 ^{s}	

Note: ^{s} $\alpha = 0.05$ significantly higher than unfermented MOLs; The bold type mark for essential amino acid

4 Discussion

In this study, *Moringa* leaf was selected as the arboreal leaf resource for exploiting biological feed. Based on the limited alternatives for a high protein feed ingredient (Richter, Siddhuraju, and Becker 2003) with low digestibility (Teixeira et al. 2014) and an unpleasant smell, a fermentation process was expected to improve the feed quality. Therefore, the first step was to look for safe and highly effective microbes, notably fungal microorganisms, which have higher degradation ability for lignocellulose than the material (Wang et al. 2013). As shown in Table 1, lm_01 had the strongest fermentation ability in the medium which only included MOLs and water, and its fermented culture had a sweet aroma smell without OTA, therefore having value in its identification. The *A. niger* XA-8 could not survive in MOLs, likely due to the inhibiting effect of its active component (Gopalakrishnan, Doriya, and Kumar 2016). The isolate lm_01 with brown-black conidia usually was black *Aspergillus* (Schuster et al. 2002). However, there are 19 accepted taxa among black *Aspergilli* and the identification of the species is often problematic due to their extremely small differences in morphology and genetic makeup (Samson et al. 2007). *Aspergillus carbonarius*, a black *Aspergillus* groups, is a key species that produces nephrotoxic mycotoxin OTA (Esteban et al. 2004), which should be avoided for use in the food and feed industry. Lm_01 could not grow in MEA-B plate and could not produce OTA which indicates that the isolate is not an *Aspergillus carbonarius, A. sclerotioniger, A. homomorphus* or *A. sclerotiicarbonarius* with conidia size of 8–10 µm, and is likely an *A. ellipticus, A. niger, A. brasiliensis, A. vadensis, A. piperis* or *A. costaricaensis* due to their conidia size of 3–5 µm (Samson et al. 2007; Schuster et al. 2002). Moreover, the 18S rDNA sequence is 100 % identical to those of *A. nigers* in the Gene bank. Thus, the new isolate was identified as an *A. niger* vis morphological and genic characteristics. Under our knowledge, this affiliation of lm_01 is relatively reasonable. *A. niger* is a relatedly safe strain for food and feed production. FAO/WHO has accepted enzyme preparations from *A. niger* including the organism itself, and listed them with an acceptable Daily Intake of 'not specified' (Schuster et al. 2002). Moreover, *A. niger* has been affirmed as 'generally regarded as safe' (GRAS) by the FDA (Saleh et al.

2011), and is accepted in the list of direct-fed microbials in China and American. However, OTA production in *A. niger* is found in ca. 6 % of the strains (Samson et al. 2007). For that reason, OTA production needs to be assessed before a new *A. niger* may be used. In this study, none of the isolates and strains had produced OTA which indicates they are safe for fermenting MOLs.

The lm_01 was rapidly growing in MOLs from 0 to 48 h, following an improvement in the MOL odor from an unpleasant, native smell, to the pleasant, sweet-aroma (Table 2 and Figure 2). Good flavours lead to an increase in animal weight gain (Coleman and Moore 2003), and thus the odor is an important index for feed. The thick sweet-aroma smell was presented after 36 – 72 h of fermentation time, which is closely related to the amount of the fungal growth. For example, lm_01 with its thin hypha which covered the culture at 24 h, simultaneously released a light sweet aroma smell. Its later thick hypha was accompanied by a thicker sweet-aroma smell. A spot of black conidia was observed at 60 h which implied the conidia had started to age; the pleasant odor gradually reduced especially after 72 h. The extracellular enzymes from the *A. niger* during fermentation likely played a key role in forming more mono- molecule volatiles (Xu, Yan, and Zhu 2005). This is supported by the chromatogram of GC –MS (Figure 4). High peaks of chromatogram were presented within 10 min in the fermented MOLs, but the high peaks in the unfermented MOLs appeared after 10 min. The main volatile components in fermented MOLs were small molecules of toluene and styrene with "Area %"s of 29.5 and 20.9, respectively. Toluene has a sweet, pungent odor with an odor threshold of 2.9 ppm (Ghosh, Kim, and Sohn 2011). While styrene has a sweet smell, but higher concentrations have a less pleasant odor. The sweet-aroma odor is thus likely due to the presence of the toluene and styrene.

In this study, the CP content in fermented MOLs was 26.61 ± 0.07 %. The value is very close to the result by Asante et al. (2014). However, other reports have found great difference with CP contents from 22.7 % to 30.29 % (Mohammed et al. 2012; Moyo et al. 2011; Ramachandran, et al. 1980; Richter, et al. 2003), likely due to the different leaf proveniences. The CP content in MOLs increased by 23.37 % during fermentation (Table 4), which indicates that the protein content could be significantly increased by a fermentation procession, as shown in Figure 2. The change in CP content is mainly due to a partial degradation of lignocellulose, which could then raise the proportion of protein (Bhat 2000; Salgado et al. 2015). However, the in vitro protein digestibility of unfermented MOLs was only 31.56 ± 0.25 % of hydrolysis value by the three-enzyme hydrolysis system, which does not significantly differ from the 31.83 ± 0.12 % of the two-enzyme system ($p > 0.05$) (Teixeira et al. 2014). The in vitro protein digestibility of fermented MOLs was 48.72 ± 0.16 %, which is 53.06 % higher than that of unfermented MOL. The likely reason is two-sided: hydrolysis of MOL protein from proteases secreted by the fungus and the degradation of cellulose results in the release of protein from the cell wall. The increase of protein digestibility is likely more important to monogastric animals than rumen animals, for about 64 % of MOL crude protein can be found to be degradable after 24 h in the rumen (Makkar and Becker 1997). Apart from CP content and protein digestibility, the amino acid composition in protein is well-known, which can serve as an important index for animal nutrition, particularly essential amino acids. In this study, free amino acids were not significantly different between unfermented and fermented MOLs as shown in the Table 5, but the latter has significantly higher amounts of amino acids ($p < 0.05$). All the essential amino acid amounts in MOL were higher than the amino acid pattern of the FAO reference protein and comparable to those in soyabeans (Makkar and Becker 1997). Comparing to the FAO/WHO/UNU amino acid requirement pattern (Schaafsma 2000), the ratios of fermented MOLs value/the requirement value of total aromatic amino acids, isoleucine, leucine, threonine, and valine were 104/63, 51.88/28, 98.45/66, 51.81/34 and 64.03/35, respectively. Such nutritional values are even sufficient for the amino acid requirements of preschool-age children.

The fermented MOL possessed not only high nutrition integration but direct-fed microbials, digestive enzymes and a functional component, which is a multi-functional biological feed. The CFU amount of *A. niger* is 1.99×10^9 per gram freeze-dried MOL as shown in Table 4. *A. niger* has been widely used for food and feed production, and enzyme preparations from *A. niger* including the organism itself have been accepted as an Acceptable Daily Intake of 'not specified' by FAO/WHO (Schuster et al. 2002). The cholesterol level of blood serum of broiler significantly reduced by feeding *A. niger* as a probiotic (Al-Kassie, Al-Jumaa, and Jameel 2008). *Gingko* leaves fermented by *A. niger* could effectively improve animal's growth performance, reducing the feed/gain ratio and benefit the immune function of the animal (Cao et al. 2012; Zhang et al. 2012). The enzyme activity of FPA and xylanase is 2.97 ± 0.24 and 564.93 ± 37.41 U per gram of freeze-dried fermented MOL, respectively. The enzymatic activity decreases only 7.2 % after three months in the freeze-dried MOLs with pH 2.87 by storage in at 4°C (data not shown). Cellulase and xylanase can degrade cellulose and hemicellulose into small molecular sugar, which is a benefit for decreasing the gastrointestinal viscosity and increasing the nutritional value of feed (Bhat 2000). The small molecular sugar can also improve the flavour of the feed. The reducing content sugar in fermented MOLs was significantly lower than that of the unfermented MOLs, given the impact of sugar consumption by *A. niger*. However, the total flavonoids and γ-aminobutyric acid in fermented MOLs was significantly higher than that of unfermented MOLs. Flavonoids are mainly a functional component existing in MOLs and are used in a wide range of medications for treating myriads of ailments (Anwar et al. 2007; Caceres

et al. 1991; Ramachandran, Peter, and Gopalakrishnan 1980; Sivasankari, Anandharaj, and Gunasekaran 2014). These natural antioxidants have been used as a supplement for animal feeds both to improve animal health and to protect animal products (Yilmaz 2006), as well as in various compounds involved in food flavour (El Gharras 2009). γ-aminobutyric acid (GABA) is a non-protein natural amino acid with many physiological activities, such as lowering blood pressure, promoting anti-aging and anti-anxiety (Wei et al. 2017). As a feed additive, GABA can effectively improve the activity of digestive enzymes, and reduce the absorption and immune functions of intestinal mucosa in heat-stressed chicken (Chen et al. 2014). Body weight, feed intake and relative spleen weight were significantly increased in chicks given GABA (Xie et al. 2012). Moreover, the fermented MOLs were easier to be broken into small size than the unfermented MOLs, which consists our previous research (Wang et al. 2018). The size further affects the flowability which is an important factor for pelletizing procession (Ali et al. 2017). As above, fermentation by the lm_01 is a great strategy to improve the feeding quality of MOL. In another project, a feeding test of *Nile tilapia* has been finished in this group, the fermented MOLs could be used to replace 20 % of fish meal without affecting the usual growth (data not shown), thus greatly reducing the production cost. In conclusion, palatability, the content of CP and amino acid, digestive enzymes, and protein digestibility were significant improvement by the fermenting procession of MOLs, and the fermented MOLs can be expected a better feeding effect for animals.

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