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Rough Protein Bioconversion and Phorbol Ester Biodegradation on *Jatropha Curcas* L. Seed Cake Fermented with Mold Consortium for Broiler Chicken Feed

Tuti Kurniati

Department of Biology Education, Faculty Tarbiyah and Teacher Training, UIN Sunan Gunung Djati Bandung
tuti.kurniati@uinsgd.ac.id

Abstract

Jatropha curcas L. seed cake is waste residue from the extraction process of Jatropha curcas L. oil. It has potential as a feed ingredient because it contains high crude protein, but on the other hand the Jatropha curcas L. seed cake contains toxic phorbol ester compounds. Fermentation process was carried out to reduce phorbol ester level and makes protein easier to digest. The research on protein bioconversion and biodegradation of phorbol ester through fermentation of Jatropha curcas L. seed meal by mold consortium has not been studied, so the purpose of this study was to produce Jatropha curcas L. seed meal with the highest protein content and the lowest phorbol ester content. Aspergillus niger and Neurospora sitophila consortium fermentation treatment with inoculum dose of 3g and 96 hours fermentation time had the highest crude protein increase of 16.88% and reduced 79.6% phorbol ester levels and had a metabolic energy of 3849 kcal / kg. The fermentation treatment by consortium Aspergillus niger and Neurospora sitophila can increase crude protein and reduce phorbol ester level. It can be used as a mixture of broiler chicken feed.

Keywords: *Bioconversion, Biodegradation, Crude Protein, Phorbol ester, Jatropha curcas L. Seed cake, Mold.*

1. Introduction

Jatropha curcas L. is belong to family Euphorbiaceae that is widely distributed in the tropics regions. It is found in Central and South Africa, Southeast Asia and India. *Jatropha curcas* L. was promoted as biofuel-producing plants with a rough calculation from 25% dry weight of seeds can be obtained (1,875 -2,5) tons of extracted oil / ha / year. The seed pressing process produces Crude Jatropha Oil (CJO) and residual pulp in the form of seed cake. The seed cake contain high protein which potentially as a feed ingredient, but *Jatropha curcas* L. seed meal also contains phorbol ester which is a toxic compound[1] and also a cause of cancer.[2-5] Some research to degrade phorbol ester has been done by Makkar, et al..[6,7,8,9]

Fermentation by bacteria can reduce the levels of phorbol ester in *Jatropha curcas* L. seeds.[10,11] Likewise fungi can accelerate the degradation of phorbol esters.[12,13] In addition, phorbol esters that found in castor oil mixed with soil have been degraded by several things such as sun exposure, [14] high temperature and humidity.[15]

Jatropha curcas L. seed cake contains protein and ash content higher than soybean cake. Crude protein can produce essential amino acids that recommended by FAO.[7]

A biological test was performed on broiler chickens to discover the benefits of bioconversion products through the determination of metabolic energy. The potential value of bioconversion products can be determined by proximate analysis. The real value is shown from the part that lost after the food has been metabolized.[16]

The energy needs depend on the size, age and type of chicken and chicken activity. The total metabolic energy is between 70% -90% of the gross energy.

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One of several methods to estimate the results of bioconversion in *Jatropha curcas* L. seed cake is determining its metabolic energy. Metabolic energy is a standard value that is most widely used, because it is practical in applications, especially for compiling composition of poultry feed.

2. Materials and Methods

2.1. Material

2.1.1. Fermentation Substrate

Jatropha curcas L. seed cake substrate was obtained from PT. Rajawali Nusantara Indonesia Jatitujuh, Majalengka. Located at 108.12 '-108.25' east longitude and 6.43 '-7.03' south latitude. The temperature average is 22^{o}C-29^{o}C; rainfall intensity is 209.84 mm / year, with humidity 57-81%, at height of 19,857 meters above sea level. The research was conducted in the Laboratory of Nutrition Non-Ruminant Poultry and Animal Food Industry Faculty of Animal Husbandry, Padjadjaran University (UNPAD) Jatinangor Sumedang.

2.1.2. Types of Microorganisms

The microorganisms used were *Rhizopus oryzae, Aspergillus niger and Neurospora sitophila*. Those Microorganisms were obtained from the Microbiology Laboratory of Bandung Institute of Technology (ITB).

2.1.3. Phorbol Ester Levels

Dichloromethane p.a for extraction of *Jatropha curcas* L. seed cake sample, acetonitrile HPLC Grade, aquabidest, H_3PO_4 p.a. for mobile phase. The standard phorbol ester used was phorbol 12-miristate 13-acetate (PMA) with 100% purity, purchased from SIGMA (St. Louis, MO, USA).

2.1.4. Experiment Livestock

The livestock used in the experiment were 35 days old final stock strain Cobb broiler chicken as many as 15 individuals. An average of initial body weight was 1.23 kg/ individual with coefficient of variation 2.65%. Chickens are placed in 15 units of metabolic cages randomly. Each cage consists of one chicken.

3. Method

Proximate test was carried out to determine the crude protein content of *Jatropha curcas* L. seed cake fermented with different treatments by *Rhizopus oryzae, Aspergillus niger* and *Neurospora sitophila,* based on dose and time.

The experiment used a completely randomized design (CRD) with 3 x 3 factorial arrangement replicated 3 times. The factorial pattern namely dose factor (d): d1 = 2g, d2 = 3g and d3 = 4g, and time factor (w): w1 = 72 hours, w2 = 96 hours and w3 = 120 hours. Phorbol ester levels were measured based on the highest protein increase.

3.1. Data Analysis

The crude protein content data from the proximate analysis was tested with Analysis of Variance (ANOVA), if there were differences, further tested by Duncan's double range test with a significance level of 5% .[17] or with a mathematical model below:

$$\mathbf{Yijk = \mu + Wi + Dj\ (i) + WDij + \varepsilon ijk}$$

Yijk = Parameters measured by the effect of the i-fermentation time and the j-inoculum dose.
μ = average
Wi = Effect of i-fermentation time.
Dj (i) = Effect of j-inoculum dose.

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WDij = Effect of interaction between the effect of the i-fermentation time with the j-inoculum dose
εijk = Effect of the k- experimental unit due to a combination of treatments (ij).

Phorbol ester levels are calculated by the following formula:

$$\textbf{Phorbol ester levels (µg / gram)} = \frac{C \times V}{W}$$

C = Concentration obtained from the calculation of the regression equation (µg / ml)
V = sample volume (ml)
W = Weighing sample weight (grams)

3.2. Metabolic Energy

The effect of the tested treatments on the observed variables was analyzed with the statistical model "Test t-Student".

$$t = d/\mathrm{sd}$$
$$sd = \frac{\sum \mathrm{di2} - \frac{d}{n(\Sigma di)^2}}{n-1}$$
$$d = \frac{\Sigma di}{n}$$
$$sd = sd/\sqrt{n}$$

With:
t = Different treatment
d = Average
sd = Standard deviation
di = Difference between treatments
n = Repetition

The calculation of metabolic energy refers to the Sibblald and Morse modification method with the following formula:

$$Emn\left(\frac{kkal}{kg}\right) = \frac{(Ebr \times K) - (Je \times Ebe) - \{\frac{(K \times Nr)}{100} - \frac{Je \times Ne}{100}\}}{K} \times 8,22$$

Emn = Metabolic energy of fermented product corrected by nitrogen retention (kcal / kg).
Ebr = Gross energy of fermented product (kcal / kg)
Ebe = gross energy of excreta (kcal / kg)
K = Number of fermented products consumed (kg)
Je = Number of excreta (kg)
Nr = Nitrogen fermented product (%)
Ne = Nitrogen
8.22 = Energy constant value (kcal / g)

3.3. Jatropha curcas L. Seed Cake Fermentation

The seed cake of *Jatropha curcas* L. was sterilized by using an autoclave at 121^{o}C at a pressure of 1 atm for 20 minutes, and then drained until it reaches 30^{o}-35^{o} C. Then the consortium microorganisms was inoculated to the seed cake of *Jatropha curcas* L. with dose of 2 g; 3 g; 4 g. After that *Jatropha curcas L.* seed cake was incubated in a fermentation chamber at 30^{o}C for 72 hours, 96 hours and 120 hours.

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The fermentation product was sterilized by using an autoclave at 121^{o}C at a pressure of 1 atm for 15 minutes, and then dried. After that the crude protein content was tested based on the modification of the AOAC (Association of Official Agricultural Chemists) method.[18]

3.4. Analysis of Crude Protein Content

3.4.1. Crude Protein Content (Kjeldahl Method)

The sample was weighed as much as 6 g and then put into a Kjeldahl flask and degraded with 20 ml of H_2SO_4. After that the sample was heated until clear solution occurs. That Solution was diluted and distilled with the addition of 10 ml of 10% NaOH. The distillate was collected in 25 ml of 3% H_3BO_3 solution then titrated with a standard HCl solution. Methyl red was used as an indicator. From the results of this titration the total nitrogen can be known. The crude protein content of the sample was calculated by multiplying the total nitrogen and the correction factor.

3.4.2. Phorbol Esther Content

Phorbol ester levels were measured by the HPLC method, with the following steps:

a. The sample was extracted using the modified Makkar et al.[7] method. Samples were weighed as much as 0.2 grams and extracted with 10 ml of dichloromethane for 2 × 24 hours, then filtered. The 7 ml filtrate was dried. The residue from the drying product was re-dissolved with 0.5 ml / L acetonitrile: 1.75 ml / L H_3PO_4 (1: 1).
b. The stationary phase HPLC (column) used was the C 18 back phase column (LikChosper). The mobile phase used was (A 1.75 ml) phosphoric acid (85%) in one liter of aquabidest and B (acetonitrile). The isocratic elution system used was 35% and H_3PO_4 65%.

The test was carried out at room temperature with a flow velocity of 1 ml / minute the wavelength used was 280 nm. Phorbol ester levels can be determined by comparing the area of the sample with the area of the standard 12-miristate 13-acetate (PMA) phorbol that has known concentration.

4. Results

4.1. Bioconversion of Crude Protein of *Jatropha curcas* L. seed cake by *Rhizopus oryzae, Aspergillus niger,* and *Neurospora sitophila*

The single mold that used on bioconversion of crude protein in the treatment of *Jatropha curcas* L. seed cake fermentation process was:

k1. *Rhizopus oryzae*,

k2. *Aspergillus niger*,

k3. *Neurospores sitophila*.

While the consortium used were:

k4. *Rhizopus oryzae* and *Aspergillus niger*,

k5. *Rhizopus oryzae* and *Neurospora sitophila*,

k6. *Aspergillus niger* and *Neurospora sitophila*

k7. *Rhizopus oryzae*, *Aspergillus niger* and *Neurospora sitophila*.

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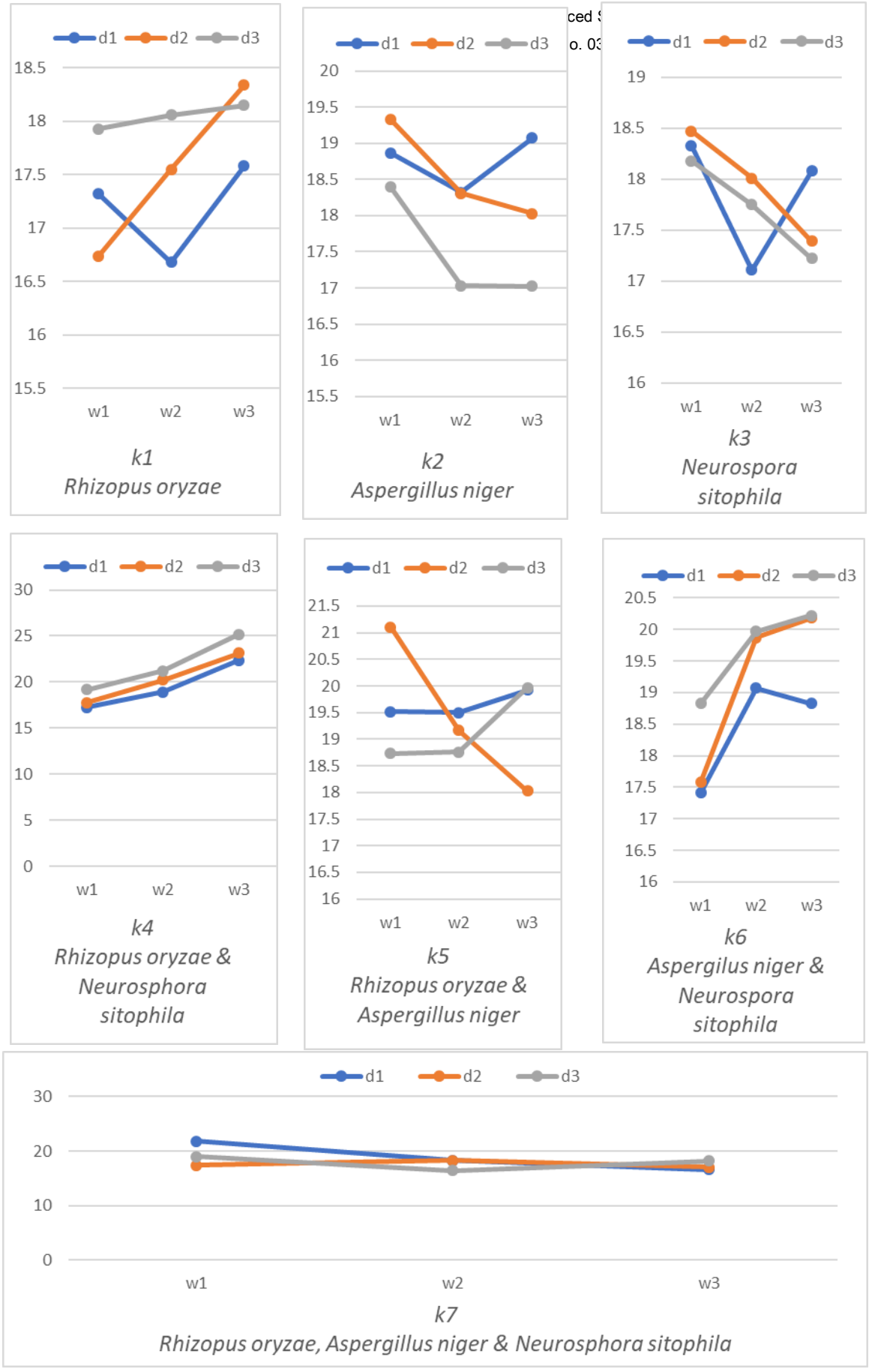

Figure 1. Bioconversion of the *Jatropha curcas* L. seed cake fermentation treatment process to crude protein content

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Protein is an organic compound containing elements of carbon, hydrogen, oxygen and nitrogen as its constituent. The use of crude protein content is better known in the analysis of animal feed ingredients, all the nitrogen elements present in a feed ingredient are counted as the crude protein contained in these ingredients. The higher nitrogen content of an ingredient, the higher amount of crude protein on it.

The results of the analysis of variance showed that the dose of inoculum and fermentation time significantly affected (α <0.05) on amount of crude protein content. To find out the difference in effect between treatments, Duncan's Multiple Range test was performed, the results were presented in Figure 1.

The dose level is related to the number of microbial populations used in the fermentation process. It determines the fermentation rate on the substrate. After the adaptation phase was passed by microbes, the microbes begin to enter the logarithmic phase, the phase where microbes experience the fastest growth and development.

The proximate analysis result of initial crude protein content was 17%. The highest crude protein content that obtained by bioconversion of *Jatropha curcas* L. seed cake fermented by k1 (*Rhizopus oryzae*) [20] was at a dose of 3 g and fermentation time of 120 hours with an increase of 7.88%. At k2 (*Aspergillus niger*) with a dose of 3 g and 72 hour fermentation time the increase in crude protein content was 13.71%. In k3 (*Neurospora sitophila)* with a dose of 3 g and 72 fermentation time, the increase in crude protein content was 7.82%. At k4 (*Rhizopus oryzae* and *Aspergillus niger*) [21] with a dose of 3 g and a 72 hour fermentation time with a crude protein the increase was 21.11%. At k5 *(Rhizopus oryzae* and *Neurospora sitophila*) [22] with a dose of 4 g and a fermentation time of 120 hours with a crude protein increase of 25.16%. At k6 (*Aspergillus niger* and *Neurospora sitophila*) with a dose of 2 g and a 72 hour fermentation time with a crude protein increase of 21.78%. At k7 (*Rhizopus oryzae*, *Aspergillus niger* and *Neurospora sitophila*) with an inoculum dose of 3 g and 96 hours fermentation time with a crude protein increase of 4.98%.

5. Discussion

5.1. Biodegradation of Phorbol esters

The measurements of phorbol ester levels were carried out to the highest crude protein yield from each treatment. Analysis of the phorbol ester level used a reverse phase column High Performance Liquid Chromatography (HPLC), the more polar compounds will be eluted first, while the phorbol ester is retained longer.

HPLC Analysis showed the presence of phorbol ester compounds in *Jatropha curcas* L. seed cake. It was detected at a retention time of 3.9 - 4 minutes. By using a PMA comparison, we know the levels of phorbol ester in the fermentation treatment.

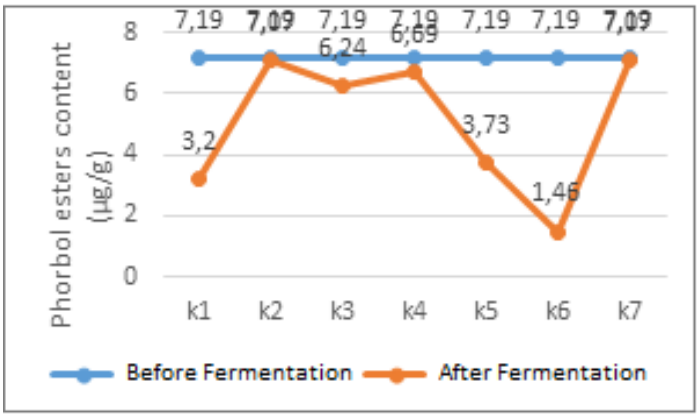

Figure 2. Graph of phorbol esters degradation of *Jatropha curcas* L. seed cake.

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As shown in figure 2, Duncan's multiple range test results on crude protein fermented by the *Aspergillus niger* mold consortium and *Neurospora sitophila* with 3g inoculum dose and 96 hours fermentation time has the lowest phorbol ester levels compared to other treatments. Phorbol esters level was decrease 79.69% , from 7.19 μg/g to 1.46 μg/g.

The reduction of phorbol esters level was caused by enzyme that produce *by Aspergillus niger* and *Neurospora sitophila* that can degrade the phorbol esters. *Aspergillus niger* which produces cellulose enzymes to break down crude fiber also produces lipase enzymes. This is in accordance with research conducted by Daryl and Vitale[23] the *Aspergillus niger* consortium and *Neurospora sitophila* are able to decompose esters from phorbol esters, because free phorbol become inactive in their toxicity.

5.2. Metabolic Energy

A biological test was performed on broiler chickens through determination of metabolic energy to determine the benefits of crude protein bioconversion.

Based on the tests carried out with 15 times replication, showed that the metabolic energy value of *Jatropha curcas* L. seed cake without bioconversion was 3885.7 kcal / kg while those converted were 3849 kcal / kg. The effect of bioconversion on the metabolic energy content of *Jatropha curcas* L. seed meal was determined by a statistical analysis of the "Student-t Test".

From the metabolic energy value of the experiments, there was a conversion of the gross energy of the feed into the metabolic energy of the feed. For feed without fermentation the energy conversion was 78.27% while for fermented feed was 77.53%.

6. Conclusions

Bioconversion and biodegradation by *Aspergillus niger* and *Neurospora sitophila* fermentation can increase crude protein content. *Aspergillus niger* produces cellulose enzymes that are able to simpler the structure of crude fibers to be easily digested. *Neurospora sitophila* produces high lipase enzymes that break down fats into digestible fatty acids. *Aspergillus niger* and *Neurospora sitophila* are able to decompose esters from phorbol esters, because phorbol esters without esters will become inactive.

Jatropha curcas L. have high energy use efficiency (above 70%) with or without bioconversion, it means that *Jatropha curcas* L. seed cake is a high energy source category. Thus, *Jatropha curcas* L. seed meal can be used as a new alternative ingredient for livestock feed.

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8. Conflict of Interest

The author declares no conflict of interest.

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