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Evaluation of rubber seed meal (*Hevea brasiliensis*) by fermentation method using *Rhizopus oligosporus* and *Neurospora sitophila* fungi

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Abstract. Rubber seeds are plantation waste that can be used because they contain important nutrients for the body. However, rubber seeds also contain anti-nutritional substances in the form of cyanide acid (HCN). This study aims to evaluate the content of rubber seeds through the fermentation method with yeast based on the fungi *Rhizopus oligosporus* (tempeh yeast) and *Neurospora sitophila* (red oncom yeast) so that they are safe for consumption. The types of fungi came from the Feed technology Laboratory, Jember State Polytechnic. The dose of yeast was adjusted according to the treatment. The curing time is 48 hours at 30 °C. This study used a completely randomized design (CRD) with 3x5 factorial pattern. The factor I was the type of microbes used. Factor II was the dose of yeast (0%;0.8%;1.6%;3.2%;6.4%). Each treatment was repeated 2 times. The different results were continued with Duncan's multiple distance test to determine the differences between treatments. All of the fungi had the ability to degrade rubber seed HCN at yeast doses up to 6.4% (concentration 10^9 CFU/g). However, fermentation with *Neurospora sitophila* resulted in a higher protein content of rubber seeds, namely 20.64% when compared to using *Rhizopus oligosporus*, which was 19.93% (incubation time of 48 hours).

1. Introduction

Rubber plant (*Hevea brasillensis*) is a tropical plantation plant that is widely cultivated for its sap. This plant produces waste in the form of rubber seeds that have not been utilized optimally. Rubber seed flesh contains dry matter (DM) 92.22%; crude protein (CP) 19.20%, crude fat 47.20%, and crude fiber 6% [1]. This shows that rubber seeds can be used as a source of feed ingredients. Rubber seeds contain amino acids that are needed by poultry, such as: glutamic acid, aspartic acid, and leucine, as well as methionine and cysteine. Each tree produces about 5,000 seeds per seed per year or one hectare of land can produce 2,253 to 3 million seeds per year [2]. Indonesia occupies the second position as the second largest rubber producer after Thailand in the Asian Region. Statistical data shows that in 2019 the area of rubber plantations in Indonesia was 3,676,035 Ha, of which East Java Province had a rubber plantation area of 24,241 Ha. This shows that rubber seed waste is very potential as a cheap poultry feed ingredient. The problem is that rubber seeds contain cyanide (HCN) poison [3], for this reason, efforts need to be made to eliminate the poison so that it is safe for consumption by livestock, especially native chickens. Microbes commonly used for grain fermentation and proven safe for consumption such as *Rhizopus oligosporus* (tempeh yeast) and *Neurospora sitophila* (red oncom yeast). It is hoped that through the fermentation process the cyanogenic glycogen derivatives in the form of linamarins (which will produce HCN) will be damaged so as to prevent the formation of HCN. The heat generated from the fermentation process can also evaporate the HCN formed, because HCN is volatile. These microbes are also a source of protein, so it is expected to increase the nutritional content of rubber seeds as feed ingredients for native chickens. This study aims to evaluate the content of rubber seeds through the fermentation method by yeast made from the fungi *Rhizopus oligosporus* and *Neurospora sitophila* so that they are safe for consumption

2. Methodology

2.1. Fungi Cultivation substrate

The Feed technology laboratory at Politeknik Negeri Jember is where the species Rhizopus oligosporus and Neurospora sitophila came from.The cultivated medium in which the fungi were grown included:ammonium sulfate (Sigma Aldrich, Germany), molasse 360 g/L, peptone 50 g/L, Potato Dextrose Broth (PDB 50 g/L), 1.5% palm oil, and distilled water [4]. An autoclave at 121 °C was used to sterilize the media, which was then cooled for two hours.After that, the media in the test tube was planted with fungi from Neurospora sitophila and Rhizopus oligosporus, and it was incubated in a shaker incubator at 37 °C and 200 x g for 24 hours.After 24 hours, the medium was transferred to an Erlenmeyer volume of 1000 milliliters and shaken as before.For 10 minutes, cell biomass was centrifuged at 4 °C, 9000 x g. After being harvested, the fungi were counted and dried in an oven at 40 °C for 48 hours, resulting in a concentration of 10^9 CFU/g.Using a sample mill, the yeast was ground into a powder and was prepared for use in the fermentation of rubber seeds.

2.2. Fermentation of rubber seeds (Hevea brasillensis)

Rubber seeds were shelled for their flesh. The rubber seed flesh was washed and then soaked overnight, and drained. The rubber seed flesh was boiled for 60 minutes from the time the water boils or until the rubber seed flesh was cooked. The next process was steaming for 60 minutes, after that washing until clean while removing the epidermis and draining. Rubber seeds were ready to be inoculated.

The concentration of yeast (microbial) was 10^9 CFU/g. The dose of the selected yeast was adjusted according to the treatment. The curing time was 48 hours with the best curing time prelium being done previously. Incubation was carried out at a temperature of 30 °C by placing the inoculated rubber seeds in a sterile tray covered with plastic. The plastic was perforated by pricking it with a needle and incubated using an incubator. Before further analysis, the samples were dried using an oven at 60^0C to dry with a moisture content of about 15%.

2.3. Data analysis

This study used a completely randomized design (CRD) with a 3x5 factorial pattern. Factor I was the type of microbes used (*Rhizopus oligosporus* and *Neurospora sitophila*). Factor II was yeast dose (0%, 0.8%, 1.6%, 3.2%, and 6.4%). Each treatment was repeated 2 times. Significantly different results were continued with Duncan's multiple distance test to determine the differences between treatments.

2.4. Observed parameters

Parameters observed were dry matter content (DM), crude protein (CP) according to AOAC (2005), and anti-nutrient cyanide (HCN) according to Arianto et al. [5].

3. Results and Discussion

3.1. Dry matter (DM) content of fermented rubber seed

The results of the analysis showed that there was no interaction between the effect of the type of fungi and the dose given, but the use of the dose of fungi for fermentation had a significant effect on the dry matter content of fermented rubber seeds. The data are presented in **Table 1**.

Table 1. Dry matter (DM) content of fermented rubber seeds (%)

Fungi type	Dose (%)					Mean
	0	0.8	1.6	3.2	6.4	
Rhizopus oligosporus	98.36	98.26	98.10	97.50	96.93	97.83
Neurospora sitophila	98.36	98.33	97.80	97.36	97.11	97.79
Mean	98.36[c]	98.30[c]	97.95[bc]	97.43[ab]	97.02[a]	

[abc] different superscript at the same row indicated significant differences ($p<0.05$)

The larger the dose of yeast *Rhizopus oligosporus* and *Neurospora sitophila*, the tendency to decrease the dry matter content of rubber seeds. The decrease in DM content was due to a reshuffle of the rubber seed structure by enzymatic activity during the processing (soaking, boiling, and steaming) and the rubber seed fermentation process, where some of the results of the reform were volatile, such as CO_2 and HCN. According to Mushollaeni [6] that the CO_2 produced during fermentation comes from the transformation of sugar into ethanol caused by fungi cells. The increase in CO_2 also can be increased the water content which functions the decompose DM from rubber seeds. Dry matter content after fermentation decreased because was decomposed by microorganisms. As stated by Malianti [7] the fermentation with *Rhizopus oligosporus* showed a decrease in DM content due to the decomposition of DM which was used as an energy source by the microorganisms contained. Malianti [8] also stated that the increased water content is an indicator of the success of fermentation.

3.2. The content of cyanide acid (HCN) fermented rubber seeds

The effect of rubber seed fermentation with different fungi (*Rhizopus oligosporus* and *Neurospora sitophila*) and different yeast doses is presented in **Table 2.**

Table 2. HCN content of fermented rubber seeds (mg/100g)

Fungi type	Dose (%)					Mean
	0	0.8	1.6	3.2	6.4	
Rhizopus oligosporus	35.45	19.95	18.15	15.25	11.88	20.14
Neurospora sitophila	35.45	21.95	20.45	17.55	14.00	21.88
Mean	35.45[c]	20.95[b]	19.30[b]	16.40[ab]	12.94[a]	

[abc] different superscript at the same row indicated significant differences ($p<0.05$)

The results of the analysis showed that *Rhizopus oligosporus* and *Neurospora sitophila* both had the potential to reduce HCN content. The more doses of yeast added, the lower the HCN content, where the HCN content with the use of yeast doses was 0%; 0.8%; 1.6%, 3.2%, and 6.4% produced HCN of 35.45%, 20.95%, 19.30%, 16.40%, and 12.94% (Table 3.2).

The reduction of rubber seed HCN through fermentation which began with boiling. The increased temperature due to boiling could detoxify toxins, thereby reducing HCN and enriching nutrients. Decreased cyanide levels were also caused by fermentation [6]. Cyanogenic glycosides are compounds found in plant foods that can potentially be toxic because they can decompose and release hydrogen cyanide (HCN). Through the fermentation process, the cyanogenic glycogen derivative in the form of linamarin (2-β-D-glucopyranosyloxy-2-methylpropanenitrile) which is the precursor for the formation of HCN will be damaged so as to prevent the formation of HCN. The HCN that has been formed will also evaporate due to the heat generated by fermentation. This is because HCN is volatile [6]

The ability of these two fungi to degrade cell walls are due to the presence of cellulolulas and hemicellulase enzymes. Even according to Li [9] that *Neurospora sitophila* also has lignocellulolytic enzymes capable of degrading lignin that is difficult to degrade. Kusnanto [10] further stated that the texture of fermented rubber seeds will be softer due to a decrease in cellulose to a simpler form.

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According to Kusnanto [10] that the dose of HCN rubber seeds without processing is quite high, namely 330 mg/100 g, while the fermentation results in this study were the lowest at 12.94 mg/100g, resulting in a significant decrease with fermentation using fungi at a dose of 6.4 % for 2 days (the yeast dose used was 10^9 CFU/g). However, the HCN dose 12.94% (or equivalent to 12.94 g/kg rubber seeds) in this study was still not at a safe level for consumption. The safe limit that is recommended by the Codex Alimentarius Commission of Food and Agricultural Organization (FAO) and the World Health Organization (WHO) of the United Nations for human consumption of 10 ppm or 10 mg HCN equivalent/kg dry weight, while according to Ihsan[2] the safe dose for consumption is HCN <50 ppm. At a concentration of 50 mg/kg will cause poisoning. The high HCN is due to the fact that the HCN content of rubber seeds which are processed before being fermented still has a high HCN content of 35.45 (P0). Research results from Rahmawati [11] that rubber seeds soaked for 24 hours have an HCN content of 1,426 ppm. This is possible after steaming, no further soaking was carried out accompanied by periodic water changes. According to Mushollaeni [6] that the reduction of HCN by immersion aims to cause an enzymatic hydrolysis process to occur in cyanide bonds because one of the properties of HCN is that it is easily soluble in water.

3.3 *Crude protein (CP) content of fermented rubber seeds*
Crude protein content (CP) of rubber seeds by fermentation of different types of fungi (*Rhizopus oligosporus* and *Neurospora sitophila*) and different doses of yeast are presented in **Table 3.**

Table 3. Protein content of fermented rubber seeds (%)

Fungi type	Dose (%)					Mean
	0	0.8	1.6	3.2	6.4	
Rhizopus oligosporus	20.70	19.87	19.52	19.98	19.59	19.93^a
Neurospora sitophila	20.70	20.90	20.88	20.78	19.93	20.64^b
Mean	20.70	20.38	20.20	20.38	19.76	

abc different superscript at the same row indicated significant differences (p<0.05)

The results of the factorial analysis showed that there was no interaction between the different types of fungi factors with increasing the yeast dose up to 6.4% (yeast concentration 10^9 CFU/g). However, the type of fungi affects the fermentation results. The CP content of rubber seeds fermented using *Neurospora sitophila* was higher than the CP content of rubber seeds fermented using *Rhizopus oligosporus*, although overall there was no decrease in the CP content of fermented products. The CP content of rubber seeds based on SNI 01-39[illegible]-2006 is >19% with a moisture content of <14%.

This increment can be attributed to the degradation of complex proteins to amino acids through proteolysis as well as the production of additional amino acids during fermentation. Accumulation of microbial cells of the fermenting organisms can also contribute to the protein increment so that there is no significant decrease in protein levels [12]. The results of rubber seeds fermentation using *Neurospora sitophila* which had a higher protein content than using *Rhizopus oligosporus* was possible because According to Syaruddin [13], differences in the growth of this fungi will influence the protein content of the product, increasing the protein due to the contribution of protein from the mycelium of the fungi.

4. Conclusion
Neurospora sitophila and *Rhizopus oligosporus* both had the ability to degrade rubber seed HCN at yeast doses up to 6.4% (concentration 10^9 CFU/g). However, fermentation with *Neurospora sitophila* resulted in a higher crude protein of rubber seed content of 20.64% when compared to using *Rhizopus oligosporus* which was 19.93% (incubation time of 48 hours).

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