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RESEARCH ARTICLE

LEMNA MINOR QUALITY IMPROVEMENT THROUGH FERMENTATION USING *TRICHODERMA HARZIANUM* AND *SACCHAROMYCES CEREVISIAE* ON WATER, LIPIDS, GROSS ENERGY, AND MINERAL CONTENT

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ABSTRACT

Lemna minor has been used as animal feed which contains high nutrients, i.e proteins and amino acids, however, water content is too high to be supplemented to animals. This research was conducted to lower water content, improve crude lipids, gross energy, mineral calcium and phosphor content by the approach of fermentation process. *Lemna minor* fermentation is divided into two stages, firstly, by using *Trichoderma harzianum* (Th) with the addition of $ZnHCO_3$ and dl-methionine, secondly, by using *Saccharomyces cerevisiae* (Sc). Completed randomized design (CRD) was used in experimental design with 20 experimental units, with five treatments of different incubation length between fermentation by Sc and Th with four replications. The results showed that fermentation using Th and Sc has a significant effect ($P<0.05$) on lower water content, higher crude lipid content, higher gross energy, higher mineral (calcium and phosphor) in the *Lemna minor*. The best duration of fermentation is Th for 3 days and Sc for 7 days (P2) that lowers water (7.29%), increasing crude lipids (2.37%), gross energy (3169 Kcal/kg), mineral calcium (0.93%), and phosphor (0.36%). *Lemna minor* fermentation with the combination of Th for 3 days and continued Sc for 7 days with added dl-methionine and Zn yield the highest dry matter, mineral calcium, and phosphor content, and lowest crude lipids contents. This study implied that the fermentation process by Th, Sc, dl-methionine, and Zn in the correct fermentation duration seems reliable to increase the quality of *Lemna minor* so that it more proper to be supplemented to animals.

KEYWORDS

Trichoderma harzianum, *Saccharomyces cerevisiae*, water content, crude lipids, gross energy.

1. INTRODUCTION

Lemna minor are small floating aquatic plants (Bokhari et al., 2016). A group researcher reported that the productivity of Lemna sp. planted in an effective planting system reach to 12–38 tons of dry weight/ha/year (Ansal et al., 2010). Lemna sp. has high protein content (38%), however it also rich in crude fiber content. A study reported that *Lemna minor* has a 6-7% lower ash content. *Lemna minor* has been used as animal feed (Nocera et al., 2005). The use of *Lemna minor* in poultry nutrition is limited to 5% level due to high water content, low ash and fat content. Fermentation is considered as one way to solve the limited use of *Lemna minor*. This is because fermentation can increase nutrient availability and reduce anti-nutrition (Haustetn et al., 1990). reported that fermentation process raised the content of minerals (Ghosh and Chattopadhyay, 2011).

Furthermore, some researchers found that fermentation can raise the content of crude lipids in fermented Lemna meal increased up to 5.76% after fermentation (Rostika et al., 2017). The use of *Trichoderma harzianum* for fermentation has been reported because of its positive effect on the increase of dry matter and ash due to the decrease in crude fiber. For example, indicated that the duckweed crude fiber content decreased by 76.06% after fermentation by *Trichoderma harzianum* for 6 - 9 days (Setiyatwan, 2007). *Saccharomyces cerevisiae* is a yeast that can produce heterologous proteins. The fermentation using *Saccharomyces cerevisiae* yeast can enrich the amino acid dl-methionine and inorganic Zinc (Zn) mineral because processing of vegetable-based feed usually results in a limiting number of amino acids and essential minerals, in turn such a process will increase the content of dry matter and *Lemna minor* ash. *Saccharomyces cerevisiae* is a yeast that can produce β-fructofuranoside fructohydrolase enzymes. Such enzymes are catalysts for converting sucrose to fructose and glucose (Lee et al., 2010).

Fermentation using *Saccharomyces cerevisiae* yeast can increase crude lipids and carbohydrate content, in turn such a process will increase the content of gross energy increases. *Lemna minor* quality can be improved by fermentation process using *Trichoderma harzianum* and *Saccharomyces cerevisiae*. The secondary metabolic pathway of *Trichoderma harzianum* and *Saccharomyces cerevisiae* are synergistic. *Trichoderma harzianum* can provide glucose, Ca, and P for the growth of *Saccharomyces cerevisiae* and subsequently used as raw material for

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energy formation. The activity of both microbes produces complementary enzymes that will result in better results. That way can be used to imitate a natural microbial growth place that is coexisting in a complex microbial community. *Lemna minor* fermentation process using two microbes (co-culture) is expected to produce functional complementary enzymes. This is due to the mutual expression of the metabolic pathways in the substrate utilization. However, the research on the quality improvement of *Lemna minor* through fermentation using *Trichoderma harzianum* and *Saccharomyces cerevisiae* has not been carried out. Therefore, the purpose of this research is to determine the best nutrient content of from the fermentation process based on two types of microbes.

2. Materials and Methods

2.1 Fermentation Procedure

Substrate consisted of a mixture of *Lemna minor* and selective medium. The mixtures were boiled in water for 60 minutes at a temperature of 115°C and pressure of 1.1 atmosphere. *Trichoderma harzianum* microbes were incubated in each treatment of 3×10^7 spores/100 grams of the substrate for 1, 3, 5, 7, and 9 days at room temperature. After first stage fermentation, $ZnHCO_3$ (186 ppm) and dl-methionine (286 ppm) were added. Next, each substrate was fermented using *Saccharomyces cerevisiae* at 3×10^7 spores/100 grams substrate for 9, 7, 5, 3, and 1 day. After fermentation, the product was dried in the oven.

2.2 Experimental Design

The experimental design used Completely Randomized Design (CRD) for five treatments with a total of 20 experimental units. The treatments consisted of P1 (Fermentation using *Trichoderma harzianum* for 1 day continued with *Saccharomyces cerevisiae* for 9 days), P2 (*Trichoderma harzianum* for 3 days continued with *Saccharomyces cerevisiae* for 7 days), P3 (*Trichoderma harzianum* for 5 days continued with *Saccharomyces cerevisiae* for 5 days), P4 (*Trichoderma harzianum* for 7 days continued with *Saccharomyces cerevisiae* for 3 days), P5 (*Trichoderma harzianum* for 9 days continued with *Saccharomyces cerevisiae* for 1 day) with four replications.

2.3 Measurement Variables

The measured variables were the fermented *Lemna minor* nutritional contents, consisted of moisture content, lipid content, gross energy, calcium content, and phosphor content. The moisture content is determined by drying solid biomass samples at 105 °C in air atmosphere until constant mass is achieved and percentage moisture calculated from the loss in mass of the sample. The Methods used for quantitating total lipids in feedstuffs by Gas Chromatographic Analysis. The gross Energy value of a biomass is determined by a Bomb Calorimeter. Calcium and phosphor content were determined by the Atomic Absorption Spectrophotometry.

2.4 Statistical Analysis

Data collected were subjected to one-way analysis of variance (ANOVA) as per Steel and Torrie. If there are mean differences, they were compared using Duncan's multiple range test with 5% significant level (α=0.05). The data presented in this paper represent their least square means with their standard error of means (LSM±SEM).

3. Results and Discussion

3.1 Fermented *Lemna minor* Moisture Content

The fermentation using *Trichoderma harzianum* followed by *Saccharomyces cerevisiae* has a significant effect (P<0.05) to lower the *Lemna minor* moisture content. The rank of treatments effect is (higher to lower) P1 (9.295%), P5 (8.45%), P4 (7.51 %), P3 (7.05), and P2 (7.04 %; Table 1). The results of the present study are in-line with other study, such as which reported that in the fermentation process, dry matter weight loss was highly correlated with the biomass moisture content (Smits et al., 1996).

Table 1: Moisture, Lipids, Gross Energy, Mineral Calcium and Phosphor Content of *Lemna minor* Fermented with *Trichoderma harzianum* followed by *Saccharomyces cerevisiae*

Parameters	Treatments				
	P1	P2	P3	P4	P5
Moisture Content (%)	9.295 ± 0.57^{c}	7.04 ± 0.36^{a}	7.05 ± 0.38^{a}	7.51 ± 0.46^{ab}	8.45 ± 0.45^{b}
Lipids Content (%)	2.23 ± 0.13^{b}	2.57 ± 0.37^{bc}	2.37 ± 0.24^{bc}	1.82 ± 0.15^{a}	1.66 ± 0.19^{a}
Gross Energy (Kcal/kg)	3202 ± 82.0^{b}	3169 ± 86.16^{b}	3047 ± 43.28^{b}	2879 ± 83.2^{a}	2796 ± 83.31^{a}
Calcium (%)	0.89 ± 0.08^{b}	0.93 ± 0.02^{c}	0.84 ± 0.03^{a}	0.86 ± 0.01^{ab}	0.87 ± 0.09^{b}
Phosphor (%)	0.33 ± 0.03^{b}	0.36 ± 0.02^{c}	0.34 ± 0.03^{b}	0.26 ± 0.03^{a}	0.31 ± 0.01^{b}

Mean in the same column with different superscript differs significantly (P <0.05), P1: Fermentation using *Trichoderma harzianum* (Th) for 1 day continued with *Saccharomyces cerevisiae* (Sc) for 9 days, P2: Th 3 days continued with Sc 7 days, P3: Th 5 days continued with Sc 5 days, P4: Th 7 days continued with Sc 3 days, P5: Th 9 days continued with Sc 1 day.

The Duncan's multiple-range test showed that the fermentation using *Trichoderma harzianum* for one day followed by *Saccharomyces cerevisiae* for nine days (P1) had the highest moisture content was significantly (P<0.05) compared with other treatments (P2, P3, P4, and P5). Fermentation with *Trichoderma harzianum* for one day followed by with *Saccharomyces cerevisiae* for nine days (P1) and fermentation with *Trichoderma harzianum* for nine days followed by *Saccharomyces cerevisiae* one day (P5), has the highest substrate moisture content. It seems that the metabolic activities carried out by each fungus for nine days has freed moisture. It was reported that the fermentation process decreases the moisture content (Flodman and Noureddini, 2013). The high moisture content in P1 and P5 products seem suggesting that the fermentation process does not worked out optimally, possibly due to improper incubation length that caused irregular development of microbial communities across the substrate (Gharechahi et al., 2020).

Three days of fermentation with *Trichoderma harzianum*, followed by *Saccharomyces cerevisiae* seven days (P2), and *Trichoderma harzianum* five days were continued by *Saccharomyces cerevisiae* five days (P3) had the lowest moisture content of the substrate. The decrease in moisture content that occurs during the fermentation process is a result of a two-stage fermentation process. This finding is in line with the study by which reported that fermentation process decreases substrate moisture content (Flodman and Noureddini, 2013). P2 had significantly (P<0.05) lowered the moisture content (7.04%) compared to other treatments (P1, P3, P4, and P5). This result might suggest that the combination of *Trichoderma harzianum* and *Saccharomyces cerevisiae* can be used as a *Lemna minor* modification agent to reduce moisture content.

3.2 Fermented *Lemna minor* Lipids Content

Lemna minor fermented in *Trichoderma harzianum* followed by *Saccharomyces cerevisiae* was significant (P<0.05) to increase the content of lipids. The highest to the lowest mean scores in sequence are P2 (2.57%), P3 (2.37%), P1 (2.23 %), P4 (1.82%) and P5 (1.66%). The Duncan's multi-range test showed that the P2 had the highest lipid content significantly (P <0.05) compared to other treatments (P1, P3, P4, and P5). Lipid content is part of organic matter, which value depends heavily on the nutrients of other organic ingredients such as carbohydrates and crude proteins (De Beni Arrigoni et al., 2016; Neely and Morgan, 1974; Owens and Basalan, 2016; Trichopoulou et al., 2002). This dependence seems demonstrated in the finding in this study that along with carbohydrates and crude protein increases, lipids content is also increasing. In rumen, fermentation process by ruminal microorganisms dynamically transforms carbohydrate fraction and protein fraction into lipids when needed (De Beni Arrigoni et al., 2016). A group researcher reported that amount of crude fat in fermented *Lemna minor* increased up after fermentation, seems caused by the microorganism's capability to produce lipids during fermentation process (Rostika et al., 2017).

3.3 Fermented *Lemna minor* Gross Energy Content

The fermentation using *Trichoderma harzianum* followed by *Saccharomyces cerevisiae* has a significant effect (P<0.05) to lower the *Lemna minor* Gross Energy content. The rank of treatment effects is (higher to lower) P1 (3202 Kcal/kg), P2 (3169 Kcal/kg), P3 (3047 Kcal/kg), P4 (2879 Kcal/kg), and P5 (2796 Kcal/kg) (Table 1). The Duncan's multiple-range test showed that P1, P2 and P3 had higher Gross energy content significantly (P<0.05) compared to other treatments (P4, and P5). While P1 gross energy content is not significantly differ (P>0.05) compared with other treatments (P2, and P3). Energy is the largest part supplied by all feed ingredients which can be converted into heat. At the seven and nine days, the rate of fermentation decreased due to the low availability of nutrients as a result of more nutrient breakdown.

The cellulase enzyme produced can reduce crude fiber levels from 44.99% to 33.15%. The second stage of fermentation using *Saccharomyces cerevisiae* for nine, seven and five days increases crude protein content (Setiyatwan et al., 2018). The highest gross energy content in the *Lemna minor* is found in P1 (3202 Kcal/kg). From this result, it seems that fermentation process of P1 able to maintain gross energy content of *Lemna minor*. However, previous study reported that fermentation process decreased the energy content in *Lemna minor* (Bairagi et al., 2002). Therefore, it is tempting to conclude if the fermentation process increases gross energy content in fermented product, at least, further study needs to confirm if this is plausible.

3.4 Fermented *Lemna minor* Calcium Content

The fermentation using *Trichoderma harzianum* followed by *Saccharomyces cerevisiae* has a significant effect (P<0.05) to increase *Lemna minor* calcium content. The rank of treatments effect is (higher to lower) P2 (0.93%), P1 (0.89%), P5 (0.87 %), P4 (0.86), and P3 (0.84 %) (Table 1). The Duncan's multiple-range test showed that the fermentation using *Trichoderma harzianum* for three days followed by *Saccharomyces cerevisiae* for seven days (P2) had the highest calcium content was significantly (P<0.05) compared with other treatments (P1, P3, P4, and P5). A group researcher reported that fermentation process increases minerals bioavailability and accessibility in some grains through phytate degradation mechanism. Furthermore, some researchers found an increase in mineral content in lentil substrate fermented by Aspergillus sp., suggesting releases of minerals from food matrix by fermentation (Castro-Alba et al., 2019; Dhull et al., 2020). This increase of mineral content (Ca and P) in forage fermentation was also reported, in which positive correlation was reported in mineral contents with in vitro digestibility level (Khan and Chaudhry, 2010).

A study stated that fermentation of cocoa beans pod using *Trichoderma harzianum* increase calcium yield as much as 9.7863% (Nocera et al., 2005). P3 had the lowest calcium content of the substrate. The decrease in calcium content that occurs during the fermentation process is a result of a two-stage fermentation process. While P1 and P5 significantly (P<0.05) reduce calcium, levels compared to other treatments (P2). This shows that the time combination of fermentation of *Trichoderma harzianum* and *Saccharomyces cerevisiae* influences calcium content. The mineral content is directly proportional to the crude fiber content and nutrient content of the substrate. Substrate fermentation with *Trichoderma harzianum* for more than three days caused the content of nutrients other than crude fiber to be degradation and fermentation time of less than three days crude fiber has not been completely degradation so that the Calcium content is low. Timing of the combination can be used as a small glue modification agent to increase calcium content. Three days of fermentation with *Trichoderma harzianum* followed by *Saccharomyces cerevisiae* seven days (P2) was the best treatment.

3.5 Fermented *Lemna minor* Phosphor Content

The fermentation using *Trichoderma harzianum* followed by *Saccharomyces cerevisiae* has a significant effect (P<0.05) to lower the *Lemna minor* phosphor content. The rank of treatments effect is (higher to lower) P2 (0.36%), P3 (0.34%), P1 (0.33%), P5 (0.31), and P4 (0.26 %) (Table 1). The Duncan's multiple-range test showed that the P2 had the highest phosphor content was significantly (P<0.05) compared with other treatments (P1, P3, P4, and P5). P3, P1, and P5 significantly reduce phosphor levels (P <0.05) compared to other treatments (P2). This might suggest that the time combination of fermentation of *Trichoderma harzianum* and *Saccharomyces cerevisiae* influences phosphor content.

The mineral content is directly proportional to the crude fiber content and nutrient content of the substrate. Substrate fermentation with *Trichoderma harzianum* for more than three days caused the content of nutrients other than crude fiber to be degraded in less than three days crude fiber has not been completely degraded so that the phosphor content is low. Timing of the combination can be used as a small glue modification agent to increase phosphor content. Three days of fermentation with *Trichoderma harzianum* followed by *Saccharomyces cerevisiae* seven days (P2) was the best treatment in terms of phosphor content.

4. Conclusion

Fermentation with the combination of *Trichoderma harzianum* for three days and continued with *Saccharomyces cerevisiae* for seven days that are supplemented with amino acid dl-methionine and Zn is the best treatment to improve *Lemna minor* quality. It can increase the content of moisture (7.04%), lipids (2.57%), gross energy (3169 Kcal/kg), calcium (0.93%) and phosphor (0.36%).

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