Jurnal_PJN_2016_Febrina et al

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Submission date: 30-May-2023 07:04AM (UTC+0800)
Submission ID: 2104831733
File name: Artikel_Effects_of_Calcium_Ca_and_Manganese_Mn_S.docx (48.72K)
Word count: 4191
Character count: 23985

PJN
ISSN 1680-5194

PAKISTAN JOURNAL OF NUTRITION

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Pakistan Journal of Nutrition 15 (4): 352-358, 2016 ISSN 1680-5194

Effects of Calcium (Ca) and Manganese (Mn) Supplementation During Oil Palm Frond Fermentation by *Phanerochaete chrysosporium* on *In vitro* Digestibility and Rumen Fluid Characteristics

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Abstract: The objectives of the study were to evaluate the effects of calcium and manganese supplementation during oil palm frond fermentation by *Phanerochaete chrysosporium* on *in vitro* digestibility and rumen fluid characteristics. This research used a Completely Randomized Design comprising two factors as treatments and each treatment was repeated three times. The factors were Ca dose (1.000, 2.000 and 3.000 ppm) and Mn dose (50, 100 and 150 ppm). The data were analyzed using analysis of variance to measure the differences among treatments; Duncan's Multiple Range Test was used to determine the significance of differences. The parameters measured were as follows: digestibility of dry matter (%), organic matter (%), Neutral Detergent Fibre (NDF) (%), Acid Detergent Fibre (ADF) (%), cellulose (%) and hemicellulose (%). The rumen fluid characteristics considered included the following: pH and the concentrations of NHa (mM) and Volatile Fatty Acid (VFA) (mM). The results indicated interactions between Ca and Mn in relation to the digestibility of dry matter, organic matter, NDF, ADF, cellulose, pH, NHa and VFA concentration of rumen fluid. The optimal combination of the minerals used were 2.000 ppm of Mn and 150 ppm of Ca; these values yielded the highest values of digestibility of dry matter (41.914%), organic matter (40.990%), NDF (66.429%), ADF (64.396%) and cellulose (68.524%) and the highest concentration of VFA (117.302 mM).

Key words: Oil palm frond, *Phanerochaete chrysosporium,* **Ca,** Mn

INTRODUCTION

Indonesia is the largest palm oil producer in the world and 10.956.231 hectares were planted with oil palms in 2014 (Ministry of Agriculture, 2014). Palm trees produce various agricultural by-products, such as the leaves and petiole, which are also termed fronds. Use of oil palm frond as an animal feed is very limited due to its high lignin content (30.18%) (Febrina *et a/.*, 2014; Zain *et a/.*, 2014), low dry matter digestibility (40%) (Kawamoto *et al.,* 2001) and low energy value (4.9 and 5.6 MJ metabolizable energy (ME)/kg DM (Alimon, 2005; Zahari and Alimon, 2005).

Lignocellulose is a macromolecular complex comprising lignin, cellulose and hemicellulose. The degradation of lignin is the key step in lignocellulose transformation (Zeng *et al.,* 2013). *Phanerochaete chrysosporium* is white-rot fungus that can degrade all major components of plant cell walls, including cellulose, hemicellulose and lignin (Aitken *et al.,* 1989; Gusse *et al.,* 2006) and is the most active ligninolytic organism described to date. Its ability to degrade lignin and a wide variety of aromatic compounds is due to a non-specific extracellular enzyme system including manganese peroxidase (MnP), lignin peroxidase (LiP) and laccases (Lac) (Lopez *et al.,* 2007; Tang *et al.,* 2006). *Phanerochaete chrysosporium* is the most intensively studied white-rot fungus due to its ability to degrade a wide range of organic substrates (Fragoeiro and Magan, 2005; Wen *et al.,* 2009; Huang *et al.,* 2010). Ligninolitic enzyme production can be increased by adding Ca and Mn salts (Brown *et al.,* 1990). Extracellular enzymes can be added directly to the media or extracellular enzymes can be produced by altering the levels of transcription and translation (Baldrin, 2003). Wuyep *et al.* (2003) reported that Mn^{2+} and Ca^{2+} ions can support the growth and extension of 2 *Basidiomycetes* mycelia: *Lentinus squarrolusus* and *Psathyrella atroumbonata.,* the addition of 2.400-3.200 ppm of Ca stimulated growth and the optimal activity was obtained using *Lentinus squarrolusus* dan *Psathyrella atroumbonata[1],* addition of Mn salts into the substrate improved the degradation of lignin and dry matter digestibility.

Supplementation with Ca salts (Febrina, 2014) during palm frond biodelignification using *Phanerochaete chrysosporium* decreased lignin content by up to 27.34% and by 29.89% when Mn salts were added (Febrina *et al.,* 2014). The addition of 2.000 ppm Ca and 150 Mn salts to palm frond fermentation using Phanerochaete *chrysosporium* resulted in low lignin contents of 22.4% and 25.77%, respectively (a decrease in lignin content from 30.18 to 22.4%) (Febrina, 2015). The purpose of this study was to evaluate the effects of calcium and manganese supplementation during oil palm frond fermentation using *Phanerochaete chrysosporium in vitro*.

MATERIALS AND METHODS

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Oil palm frond (OPF) fermentation: The OPF substrate was

cut, dried and finely milled. Ca was used as CaCl and Mn was used as MnSO4.H2O. *Phanerochaete chrysosporium* was maintained on Potato Dextrose Agar (PDA), slanted at 4°C and transferred to PDA plates at 37°C for 6 days; the fungus was subsequently grown on rice bran. The fermentation process was initiated by adding water to the OPF to a water content of 70%; Ca and Mn were then added according to the various treatments. After 10 days, all oil palm fronds were covered by *white rot fungus* and samples were taken for proximate analysis and fibre fraction determination. The proximate components were determined as described by AOAC (1995) using Foss equipment. The predominantly fibre residues (hemicellulose, cellulose and lignin) were determined according to the method of Van Soest *et al.* (1991) using Foss Fibretec.

***In vitro* digestibility assay:** *In vitro* digestibility was analyzed as described by Tilley and Terry (1969). Buffalo rumen fluid was diluted using McDougal buffer (1:2) and dispensed into a 1 g substrate-prepared incubation tube, which was purged with CO_2 to maintain anaerobic conditions. The tubes were incubated in a water bath at 39°C for 24 h. After fermentation, the Erlenmeyer tube containing the sample was inserted into ice water to stop the fermentation. All samples were then centrifuged at 1.200 rpm for 15 min. The pH, NH3-N and total VFA of the supernatants were then recorded. NH3-N concentration was determined using microdiffusion Conway method and the total VFA concentration was measured using the steam distillation method. The previously incubated samples were vacuum-filtered (Whatman no. 41) and dried at 60°C in an oven. The dried samples were used to analyze the dry matter, organic matter, NDF, ADF, hemicellulose and cellulose in an *in vitro* digestibility assay.

Experimental design and statistical analysis: The study implemented a Completely Randomized Design using a 3 x 3 factorial pattern and 3 repetitions. The first factor was the level of Ca (1.000, 2.000 and 3.000 ppm) and the second factor was the level of Mn (50, 100 and 150 ppm). The significance of differences among the treatments were then analyzed using Duncan's Multiple Range Test.

RESULTS AND DISCUSSION

In this study, the interaction between Ca and Mn affected ($p<0.05$) the *in vitro* digestibility of dry matter, organic matter, NDF, ADF and cellulose. Supplementation with Mn significantly ($p<0.05$) affected the digestibility of hemicellulose (Table 1).

The lowest dry matter digestibility (34.038%) appeared in experiment A1B2 (1.000 ppm Ca and 100 ppm Mn). This indicated that the combination of Ca and Mn was not optimal for supporting the growth and mycelial increase of the fungus. The fungus did not produce optimal amounts of enzyme to degrade lignin; therefore, large amounts of cellulose and hemicellulose remained attached to the lignin. Lignin degrades with difficulty into fractions (Lynch, 1993); lignin bonds to cellulose and hemicellulose, forming lignocellulose and lignohemicellulose, which rumen microbes find difficult to degrade. Cleavage of the lignocellulosic bonds facilitates access to the substrate by hydrolytic enzymes such that microbes can degrade and thus decrease lignin contents; according to Sun and Cheng (2002), breakdown of the crystal structure of cellulose facilitates access to the substrate by hydrolytic enzymes.

Increasing the concentration of Ca from 1.000 ppm to 2.000 ppm and the concentration of Mn from 100 ppm to 150 ppm (A2B3) stimulated the growth of fungal mycelia, indicating that the production of ligninase was increased. Ligninase belongs to a group of isoenzymes, oxidases and peroxidases that depolymerize lignin (Chahal and Chahal, 1998). When more enzymes are produced, lignocellulose and lignohemicellulose become more stretchable. The cellulose and hemicellulose can then be separated and utilized by rumen microbes as a source of energy. Optimal microbe growth, as achieved through appropriate nutrient supplementation, will produce the highest dry matter digestibility.

The highest dry matter digestibility was found in treatment A2B3 and was associated with the optimal degradation of lignin by *Phanerochaete chrysosporium* (this treatment resulted in the lowest lignin content of 22.40%). Low lignin content (indicating greater lignin degradation) leads to the digestion of more nutrients and produces the highest dry matter digestibility. Lignin degradation causes the stretching of lignocellulose and lignohemicellulose such that the cellulose and hemicellulose can be utilized by fungi mycelia for growth. Suparjo (2010) suggested that mycelial multiplication is an indicator of growth and shows an increase in the of dry matter content. Ballgees *et al.* (2009) also described the increase of dry matter digestibility of bagasse by fermentation using white rot fungi. The addition of Mn to the substrate increases the degradation of lignin (Kerem and Hadar, 1997) and the dry matter digestibility of substrates (Kerem and Hadar, 1995). Dry matter with high digestibility is considered appropriate for feeding to livestock (Alwi *et al.,* 2013). Decreased lignin content was found to be positively correlated with the *in vitro* dry matter digestibility of bagasse when fermented using L. edodes and C. subvermispora (Okano *et al.,* 2006).

The organic matter digestibility of oil palm frond was 24.34% before fermentation and increased to 24.81- 40.61% after fermentation, ranging from 34.396- 40.990%. This result indicates that the addition of Ca and Mn affected the organic matter digestibility of the fermented oil palm fronds. Muhktarudin and Liman (2006) found that supplementing with organic macro minerals in feed rations increased the organic digestibility. Organic matter digestibility follows the trends seen with dry matter because low dry matter digestibility causes low organic matter digestibility. Muhktarudin and Liman (2006) reported that increased dry matter digestibility results in increased organic matter digestibility. Elihasridas (2012) reported that increased dry matter digestibility, which was caused by increased supplementation of Zn salts in rations of ammoniated corn cobs, was associated with increased dry matter digestibility.

The lowest digestibilities of NDF, ADF and cellulose were observed in treatment A1B2 (57.29, 50.86 and 50.04%, respectively). The low digestibilities of NDF, ADF and cellulose resulted from the high contents of NDF and ADF,

indicating that the lignin was still bound to cellulose and hemicellulose. According to Moore and Jung (2001), the lignin in plant cell walls reduces the digestibility of cellulose and hemicellulose by rumen microbes; lignification process limits the ability of microbes to break down cellulose and hemicellulose as an energy source for growth (Tripathi *et al.,* 2008). The increased NDF digestibility observed for treatment A2B3 indicates that the additions of Ca and Mn were able to increase the amount of fungal mycelium and the production of lignolytic enzymes, thereby cleaving the bonds within lignocellulose and lignohemicellulose. Hammel (1997) suggested that Phanerochaete chrysosporium can degrade lignin; Ke rem and Hadar (1997) suggested that the addition of Mn to the substrate would increase lignin degradation. Lignin biodegradation is a key process involved in lignocellulosic waste composting; the increased use of carbon will increase the activity of microorganisms that increase the activity of lignolytic enzymes, thus increasing lignin degradation (Feng *et al.,* 2011).

Hemicellulose digestibility increased as the Mn concentration increased from 100 to 150 ppm. Hemicellulose digestibility increased as the addition of Mn increased, indicating that Mn affected lignin degradation by affecting the production of ligninolytic enzymes. Baldrin (2003) suggested that Mn plays a role in the Manganese Peroxidase (MnP) reaction cycle. The fragmentation of lignocellulosic bonds and lignohemicellulose caused hemicellulose to become an energy source; therefore, the digestibility of hemicellulose by rumen microbes was increased. MnP activity and stability are influenced by the concentration of Mn^{2+} (Urek and Pazarlioglu, 2005); Mn^{2+} is a specific effector that encourages the production of MnP (Zhao *et al.,* 1996).

Characteristics of rumen fluid: pH affects the activity of microorganisms in the rumen and greatly affects the growth and activity of rumen microorganisms. Rumen pH is altered by the production of acid by microorganisms during fermentation in the rumen. Table 2 shows the effects of Ca and Mn on pH and the concentrations of NH3 (mM) and VFA (mM) in fermented oil palm fronds.

Rumen pH ranges from 6.667 to 6.867. This range is suitable for the growth of rumen microbes. This pH value indicates a balance between buffering capacity and the acid or alkaline fermentation products, VFA and N-NH3 (Arora, 1995). Erdman (1988) found that the optimal pH range for cellulose digestion in the rumen is 6.4 to 6.8; when the rumen pH falls below 6.2, cellulolytic bacteria are disrupted, causing decreased fibre digestibility. The highest rumen pH (6.867) in this study was found in treatment A1B1 (1.000 ppm Ca and 50 ppm Mn). Increased doses of Ca and Mn significantly ($p<0.05$) lowered rumen pH. Supplementation of 3.000 ppm Ca and 150 ppm Mn (A3B3) resulted in the lowest rumen pH (6.667). Rumen pH decreased as the Ca and Mn doses increased. Similar results were reported by Suyitman *et al.* (2013), who noted that the effects of various treatments (steam, ammoniation and ensilage) were highly significant ($p<0.01$) on rumen fluid pH.

The concentration of NH3 in this study ranged from 5.523 mM to 8.365 mM. This value is sufficient for the optimal growth of rumen microbes. The concentration of ammonia required to support microbial growth is 6-21 mM (McDonald *et al.,* 2010). The concentration of NH3-N depends on protein intake. Higher protein intake will stimulate higher microbial activity, which increases NH3- N concentration. The lowest NH3, which occurred in response to the addition of 3.000 ppm Ca and 100 ppm Mn (treatment A3B2), caused the sub optimal production of ligninases. Therefore, lignin degradation was also suboptimal. The addition of 3.000 ppm Ca and 100 ppm Mn (treatment A3B2) resulted in lower crude protein digestibility. The low concentrations of NH3 found in this treatment were associated with low nutrient digestibility. Low digestibility also produced the lowest concentration of NH3 in this study. Low concentrations of NH3 in the rumen limit the activity of rumen fermentation (Fariani *et al.,* 2015). The highest NH3-N concentration found in this study was observed for treatment A2B1 (2.000 ppm Ca and 50 ppm Mn) and was associated with higher protein digestibility. Proteins degraded in the rumen are used to form NH3 and N, which are used by rumen microbes to

support their growth. The more proteins are degraded, the 150 ppm Mn produced the highest concentration of VFA

Table 1: Nutrient Digestibility resulting from oil palm frond fermentation by *Phanerochaete chrysosporium* while supplemented with Ca and Mn (%)

Ca (ppm)	B1 (50)	B2(100)	B3(150)	Average
Dry matter				
A1 (1.000)	37.151±0.842^{c}	34.038±0.712^{d}	38.643±0.191^{b}	36.610^{c}
A2 (2.000)	38.928±0.244^{b}	36.016±0.631^{c}	41.914±1.031^{a}	38.953^{a}
A3 (3.000)	38.653±0.911^{b}	36.408±0.940^{c}	38.920±0.191^{b}	37.994^{b}
Average	38.244^{b}	35.487^{c}	39.826^{a}	
Organic matter				
A1 (1.000)	35.727±1.521^{cd}	32.375±0.512^{e}	37.395±0.626^{bc}	35.166^{b}
A2 (2.000)	37.296±0.931^{bc}	34.396±1.041^{d}	40.990±1.581^{a}	37.560^{a}
A3 (3.000)	37.787±0.559^{b}	34.798±1.037^{d}	37.461 ±0.690^{bc}	36.682^{a}
Average	36.937^{b}	33.856^{c}	38.616^{a}	
NDF				
A1 (1.000)	58.332±0.84^{cd}	57.293±0.55^{d}	64.225±1.78^{ab}	59.946
A2 (2.000)	61.803±4.40^{abcd}	61.018±3.98^{abcd}	66.429±0.71^{a}	63.083
A3 (3.000)	60.402±4.88^{bcd}	63.378±4.20^{abc}	64.303±1.57^{ab}	62.695
Average	60.176^{b}	60.563^{b}	64.986^{a}	
ADF				
A1 (1.000)	56.535±0.92^{b}	50.863±5.79^{c}	60.131±2.37^{ab}	55.843^{b}
A2 (2.000)	63.822±0.94^{a}	57.553±0.37^{b}	64.396±0.72^{a}	61.924^{a}
A3 (3.000)	59.836±2.24^{ab}	60.513±3.60^{ab}	61.257±2.94^{ab}	60.535^{a}
Average	60.065^{a}	56.31^{b}	61.928^{a}	
Cellulose				
A1 (1.000)	56.849±0.922^{ab}	50.044±9.046^{b}	66.153±3.477^{a}	57.682
A2 (2.000)	65.780±5.003^{a}	62.281 ±9.603^{ab}	68.524±3.777^{a}	65.528
A3 (3.000)	56.705±1.792^{ab}	53.152±17.832^{ab}	63.715±6.303^{ab}	57.857
Average	59.778^{ab}	55.159^{b}	66.131^{a}	
Hemicellulose				
A1 (1.000)	65.25±6.172	75.87±19.554	83.62±18.314	74.915
A2 (2.000)	55.13±16.459	71.53±14.334	73.89±1.744	66.851
A3 (3.000)	60.06±26.956	73.51±8.158	76.74±14.317	70.102
Average	60.148^{b}	73.638^{ab}	78.082^{a}	

Description: Different superscripts in the same row and column indicate significant differences ($p<0.05$)

Table 2: Characteristics of rumen fluid in response to oil palm fronds that were fermented by *Phanerochaete chrysosporium* in the presence of Ca and Mn supplementation

Ca (ppm)	B1 (50)	B2(100)	B3(150)	Average
pH				
A1 (1.000)	6.867±0.057^{a}	6.833±0.057^{ab}	6.866±0.057^{a}	6.856^{a}
A2 (2.000)	6.833±0.057^{ab}	6.833±0.057^{ab}	6.800±0.100^{ab}	6.822^{a}
A3 (3.000)	6.700±0.100^{ab}	6.700±0.173^{ab}	6.667±0.152^{b}	6.689^{b}
Average	6.800	6.789	6.778	
NH_3 (mM)				
A1 (1.000)	7.964±0.113^{a}	5.557±0.212^{c}	8.342±0.178^{a}	6.856^{a}
A2 (2.000)	8.365±0.252^{a}	5.528±0.131^{c}	8.266±0.273^{a}	6.822^{a}
A3 (3.000)	6.674±0.287^{b}	5.348±0.253^{c}	6.669±0.276^{b}	6.689^{b}
Average	6.800	6.789	6.778	
VFA (mM)				
A1 (1.000)	113.556±16.730^{a}	71.873±4.486^{b}	111.971±3.041^{a}	99.134
A2 (2.000)	113.928±0.422^{a}	67.119±10.230^{b}	117.302±5.120^{a}	99.450
A3 (3.000)	107.064±12.990^{a}	63.067±5.864^{b}	111.178±6.505^{a}	93.769
Average	111.516^{a}	67.353^{b}	113.484^{a}	

Description: Different superscripts in the same row and column indicate significant differences ($p<0.05$)

more NH3 is formed and the greater the growth of the microbes. Ali *et al.* (2015) reported that an increase in the concentration of NH3 was associated with the degradation of a protein-rich substrate.

The ability of rumen microbes to degrade feed affects the concentration of VFA. The concentration of VFA was 63.067 and 117.302 mM. According to the recommendations of Waldron *et al.* (2002), the total VFA concentration in the rumen was 60-120 mM. The interaction of 2.000 ppm Ca and (117.302 mM). This value was similar to that obtained using some other treatments but was significantly different ($p<0.05$) and higher compared to treatments A2B2 and A2B3. The highest VFA found for treatment A2B3 (2.000 ppm Ca and 150 ppm Mn) suggests that using the appropriate combination of Ca and Mn can optimize the production of ligninolytic enzyme by *Phanerochaete chrysosporium. Phanerochaete chrysosporium* produces haeme peroxidase, lignin peroxidase (LiP) and manganese peroxidase (MnP),which

degrade the lignin that binds cellulose and hemicellulose together. LiP is a major catalyst in lignolysis by the fungus because it can degrade non-phenolic compounds that comprise 90% of the lignin structure (Srebotnik *et al.,* 1994). The lowest VFA was found for treatment A3B2 (3.000 ppm Ca and 100 ppm Mn), which is assumed to represent the lowest nutrient digestibility. Organic matter digestibility and dry matter correlate with the production of VFA (Liu *et al.,* 2002); high *In vitro* digestibility of organic matter and crude fibre increase the total production of VFA in the rumen (Sudekum *et al.,* 2006).

Conclusion: Supplementation of 2.000 ppm Ca and 150 ppm Mn provided a better result than the other tested treatments. The *in-vitro* digestibilities of the nutrients were as follows: dry matter, 41.914%; organic matter, 40.990%; NDF, 66.429%; ADF, 64.396% and cellulose, 68.524%. The highest value of VFA obtained was 117.302 mM.

ACKNOWLEDGMENTS

This study was partially supported by the DP2M Directorate General of Higher Education, Republic of Indonesia. The authors are very grateful to the Directorate General of the Department of Higher Education, National Education of the Republic of Indonesia, which funded this experiment through the “Hibah Pasca” grant program in 2015.

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