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Ligninolytic Enzymes Produced from *Flammulina velutipes* Cultivation Residues as the Enhancer of *in vitro* True Digestibility in Ruminants

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Abstract. The major component that plays an important role in restricting ruminal degradation of structural polysaccharides such as cellulose and hemicelluloses is lignin. Mushroom cultivation residue (MCR) contains high proportion of lignin that no enzyme from domestic animals per se can digest them. MCR accompanying its ligninolytic enzymes including laccase (Lac), manganese peroxidase (MnP) and lignin peroxidase (LiP) for delignification has the potential to improve its value as ruminant feedstuff. The purpose of this study is to optimize the production of ligninolytic enzymes and the digestibility based on the *in vitro* true digestibility (IVTD) technique in MCR silage (MCRS), made from golden needle mushroom (*Flammulina velutipes*) ensilaged for 60 days. The activities of lignin peroxidase that produced in MCRS (1,866 U/mL) was higher ($P < 0.001$) than MCR (1,270 U/mL) after 60 days of fermentation. The contents of cell wall components such as neutral detergent fiber, acid detergent fiber and acid detergent lignin were significantly ($P < 0.001$) digested by IVTD technique along the duration (24 hours) of incubation. The result revealed that ensilaging *Flammulina velutipes* cultivation residue might enhance the activities of ligninolytic enzymes especially LiP, which could consequently improve the degradation of fiber contents in addition to the merit of being feedstuff for ruminants.

Key words: *Flammulina velutipes*, ligninolytic enzymes, mushroom cultivation residue, silage

1. Introduction

Agro-industrial byproducts are commonly used as feed resources. Mushroom cultivation residues (MCR) may be used as an alternative roughage source for ruminants. Lignin is the major component that plays an important role in restricting ruminal degradation of structural polysaccharides such as cellulose and hemicelluloses. MCR contains high proportion of lignin that no enzyme from domestic animals per se can digest it. Mushroom is grown on culture substrates usually comprised of ingredients such as straw, sawdust, wood shavings or crust, seed shells and those materials rich in cellulose and lignin [1]. Lignocellulose, a complex physically and chemically formed with cellulose, hemicelluloses and lignin, makes the polysaccharides less accessible to ruminal microbial digestion through blocking cellulose and hemicellulose in rumen bacteria [2]. White-rot fungi are able to colonize on different types of agriculture residues and able to decompose lignocelluloses without chemical or biological pretreatment because it possesses an enzyme to complete the system that includes phenol oxidases and peroxidases [3]. During the cultivation of mushroom, white-rot fungi produce some ligninolytic and cellulolytic enzymes such as laccase, manganese peroxidase and lignin peroxidase which are able to breakdown the structure of fiber fractions [4]. It has been reported that *Flammulina velutipes* had ability to degrade lignin by laccase produced in liquid growth medium [5]. In the current study we will further investigate the laccase produced during the ensilaging process of

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Flammulina velutipes cultivation residues, and evaluate the quality and digestibility of silage in the perspective of using it as alternative roughage for ruminants.

2. Materials and Methods

2.1. MCR silage making

MCR of golden needle mushroom (*Flammulina velutipes*) obtained from a mushroom factory in Wufeng district, Taichung province in Taiwan was used for making MCR silage (MCRS) ensiled for 60 days. Three hundred kilograms of MCR, randomly collected from mushroom factory, was contained in the sealed container for ensiling. MCRS samples were randomly collected after ensiled for 60 days. MCR and MCRS samples were used to analyze for ligninolytic enzymes activity and *in vitro* digestibility.

2.2. Ligninolytic enzymes assay

Laccase was measured by assaying the oxidation of 1 mM 2,2'-azino-bis(3-ethlbenzthaizoline)-6-sulphonate (ABTS) in 0.1 M sodium acetate buffer (pH 5). Laccase activity was evaluated by spectrometry at 436 nm and using an extinction coefficient of 29300 M^{-1} cm^{-1} as a study of Pant and Adholeya [6]. Manganese peroxidase (MnP) was measured by assaying the oxidation of 5 mM 2,6- dimethoxyphenol (2,6 DMP) in 1 M sodium acetate buffer (pH 4.5). MnP activity was evaluated by spectrometry at 469 nm and using an extinction coefficient of 27500 M^{-1} cm^{-1} follows Leontievsky et al. [7]. Lignin peroxidase (LiP) was measured by assaying the oxidation of 10 mM veratryl alcohol in 0.25 M *D*-tartaric acid buffer (pH 3). LiP activity was evaluated by UV spectrometry at 310 nm and using an extinction coefficient of 9300 M^{-1} cm^{-1} as a study of Tychanowicz et al. [8]. For all enzymes under evaluation, one activity unit was defined as the amount of enzyme necessary to oxidize 1 μM of substrate per minute. All activities determined in this study were expressed as U/L as a study of Pant and Adholeya [6], Leontievsky et al. [7], and Tychanowicz et al. [8].

2.3. *In vitro* true digestibility

Ruminal fluid was collected 4 h post feeding from a lactating Holstein cow for using in this study. The sample of MCR and MCRS were grinded and contained into F57 filter bag (ANKOM TECHNOLOGY). Each F57 filter bag contained 0.5 g of sample. The comparison of *in vitro* true digestibility (IVTD) between MCR and MCRS was estimated according to the IVTD using the Daisy II Incubator ANKOM TECHNOLOGY for 24 hours, following the daisy manual [9].

2.4. Proximate and cell wall composition of samples

The samples of MCR and MCRS (before and after incubations in the Daisy II Incubator ANKOM TECHNOLOGY) were analyzed for proximate principles following AOAC [10] and cell wall components [11].

2.5. Statistical analyses

Analysis of variance was used to test the significance of the treatment effect. Tukey's range test was used to rank treatment means using GLM procedure [12].

3. Results

Ligninolytic enzymes produced and chemical compositions changed along with the fermentation duration (60 days), the results are shown in Table 1. The activity of lignin peroxides was maximum produced 1,867 U/mL in MCRS throughout the fermentation period followed by 1,270 U/mL in MCR. MnP and Lac were produced in MCRS (69.33 and 7.06 U/mL, respectively) and MCR (32.68 and 3.63 U/mL, respectively) measured on 60th day. The chemical compositions of MCR and MCRS are presented in Table 2. The results show that the nutrient contents (DM, OM, CP, and EE) of MCRS increased and also the fiber contents (CF, ADF, NDF, ADL, hemicelluloses, and cellulose) decreased after ensiling for 60 days.

Table 1: The activities of ligninolytic enzymes produced by MCR and MCRS

Terms	MCR	MCRS	SEM	P-value
LiP (U/ml)	1270.11	1867.08	114.10	< 0.001*
MnP (U/ml)	32.77	69.48	4.84	< 0.001*
Lac (U/ml)	3.70	7.07	0.18	< 0.001*

*Means (n = 12) in the same row are significantly different (P < 001).

MCR = Mushroom cultivation residue. MCRS = Mushroom cultivation residue silage. Lac = Laccase. MnP = Manganese peroxides. LiP = Lignin peroxides.

Table 2: Chemical compositions of MCR and MCRS (% on DM basis)

Chemical compositions (% on DM basis)	MCR	MCRS	SEM	P-value
DM	52.35	48.03	0.58	0.0213*
OM	89.48	92.20	0.46	0.1223
CP	7.76	8.43	0.06	0.0555
EE	3.70	4.21	0.23	0.0174*
CF	49.48	45.62	0.01	0.0417*
NDF	79.42	74.83	1.37	0.0508
ADF	63.55	57.37	0.28	0.0073*
ADL	27.11	24.84	0.18	0.0363*
HEM	15.87	17.46	2.85	0.5420
CEL	36.44	32.53	0.92	0.0520

*Means (n=2) in the same row are significantly different (P < 0.05).

MCR = Mushroom cultivation residue. MCRS = Mushroom cultivation residue silage. DM = Dry matter. OM = Organic matter. CP = Crude protein. CF = Crude fiber. EE = Ether extract. NDF = Neutral detergent fiber. ADF = Acid detergent fiber. ADL = Acid detergent lignin. HEM = Hemi cellulose. CEL = Cellulose.

Table 3: IVTD results of MCR and MCRS measured by Daisy II Incubator

In vitro digestibility (% on DM basis)	MCR	MCRS	SEM	P-value
DM	17.28	21.59	3.56	<0.0001*
OM	16.55	22.18	3.59	<0.0001*
CP	39.03	52.47	1.74	<0.0001*
EE	39.64	61.08	1.57	<0.0001*
CF	40.46	46.84	1.78	<0.0001*
NDF	39.84	46.79	1.80	<0.0001*
ADF	32.87	40.60	2.25	<0.0001*
ADL	28.67	40.94	2.44	<0.0001*
HEM	67.78	67.66	0.56	0.692

CEL	35.99	40.06	2.11	<0.0001*

*Means (n = 12) in the same row are significantly different (P < 0001).

MCR = Mushroom cultivation residue. MCRS = Mushroom cultivation residue silage. DM = Dry matter. OM = Organic matter. CP = Crude protein. CF = Crude fiber. EE = Ether extract. NDF = Neutral detergent fiber. ADF = Acid detergent fiber. ADL = Acid detergent lignin. HEM = Hemi cellulose. CEL = Cellulose.

Table 4: Effect of ligninolytic enzymes production on correlation coefficients of IVTD

	DM	CF	NDF	ADF	ADL	CEL	HEM
DM	1	0.9933	0.9966	0.9962	0.9940	0.9756	-0.1088
		<.0001	<.0001	<.0001	<.0001	0.0009	0.8375
CF		1	0.9959	0.9941	0.9925	0.9463	-0.0673
			<.0001	<.0001	<.0001	0.0042	0.8991
NDF			1	0.9998	0.9993	0.9643	-0.1478
				<.0001	<.0001	0.0019	0.7799
ADF				1	0.9996	0.9670	-0.1652
					<.0001	0.0016	0.7545
ADL					1	0.9646	-0.1792
						0.0019	0.7340
CEL						1	-0.1088
							0.7117
HEM							1

MCR = Mushroom cultivation residue. MCRS = Mushroom cultivation residue silage. DM = Dry matter. OM = Organic matter. CP = Crude protein. CF = Crude fiber. EE = Ether extract. NDF = Neutral detergent fiber. ADF = Acid detergent fiber. ADL = Acid detergent lignin. HEM = Hemi cellulose. CEL = Cellulose.

The digestibility measured by IVTD technique of fiber contents (i.e. in terms of CF, NDF, ADF, ADL, hemicellulose and cellulose) increased throughout the incubation period along with the ligninolytic enzymes production increased (Table 3). There were significant improvements (P < 0.0001) in the digestibility of DM, OM, CP, EE, CF, NDF, ADF, ADL, and cellulose contents.

In vitro digestibility of dry matter and cell wall contents especially of CF, NDF, ADF, and ADL were highly correlated (P < 0.0001) under the ligninolytic enzymes production (Table 4). Ligninolytic enzymes can break down fiber especially of lignin which is part of the cell wall of lignocellulosis biomass and liberates simpler molecules freely available to the in vitro digestibility. These enzymes are important for fiber such as hemicellulose, cellulose, and lignin degrading enzymes that can have significant impact on enhancing feed utilization and animal performance [13].

4. Conclusions

It is possible the activities of ligninolytic enzymes, especially lignin peroxides, were improved with the ensilaging golden needle mushroom (*Flammulina velutipes*) cultivation residue, and improve the degradation of fiber content in the silage. Ensiling MCR may improve the nutrient in the feedstuff such as increasing protein content and decreasing fiber content. The silage thus made from MCR tended to be used as an ideal alternative feedstuff for ruminants.

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