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Evaluation of Exogenous Laccase Enzyme Treated Finger Millet Straw on Body Weight Gain, DM Intake and Nutrient Digestibility in Heifers

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ABSTRACT

Background: Fungal laccases have widespread application in a number of biotechnological processes including the biodegradation of lignin. Their low yield in the native state limits their practical use in the deconstruction of lignocellulosic biomass for feeding ruminants. Enzymes in bulk quantities are required to treat biomass which has got greater product consistency and less lot to lot variations. The present study was an attempt to evaluate the effect of feeding finger millet straw treated with exogenous laccases in heifers.

Methods: The effect of feeding exogenous laccases obtained from immobilized *Schizophyllum commune* (MTCC 11893) on body weight gain, dry matter intake and nutrient digestibility in heifers was evaluated in three groups of heifers (4-each) with body weights ranging between 275 ± 47.12 to 276.75 ± 64.48kg. The control group received *ad lib.* finger millet straw treated with only production media (GI). Test group 1 (GII) received *ad lib.* straw treated with laccase rich media in a 3:5 (w/v) ratio, while test group 2 (GIII) received *ad lib.* straw treated in a 4:5 (w/v) ratio.

Result: After 14- days of feeding, gain in body weights for GI, GII and GIII were 277.9 ± 68.47; 277.50 ± 46.43 and 278.85 ± 37.22 respectively with an overall increase of 1.15, 4.25 and 3.60 kgs. No significant variation (P>0.05) was observed with regard to DMD (%) between the groups which was 46 ± 5.8 for the control animals and 41±13.0 and 41± 3.4 in G2 and G3 groups. Though significant variations were observed digestibility studies proved inconclusive. Though preliminary results indicate that applying lignin degrading enzymes as feed supplements could enhance digestibility of crop residues in ruminants.

Key words: Digestibility, Exogenous enzyme, Heifers, Laccase, White rot fungi.

INTRODUCTION

Lignocellulosic biomass obtained from agricultural crops represents an enormous energy resource for feeding ruminants. But the presence of lignin in these residues decreases their digestibility. Of the wide range of pretreatments reported for deconstruction of lignin (Saritha *et al*., 2012); bioconversion with fungal enzymes *viz*. laccases, lignin peroxidases and manganese peroxidases, of the lignolytic, white rot fungi (WRF) is safe and with low environmental impact. Biological pretreatment however, is constrained by low enzyme production efficiency, long residence times and considerable loss of the carbohydrates. Hitherto, only exogenous amylolytic and fibrolytic enzymes have been used in studies to enhance animal efficiency (Beauchemin *et al*., 2003; Bowman, *et al*, 2003; Martins *et al*., 2006; Gencoglu *et al*., 2010). Direct application of lignin degrading enzymes to the lignocellulosic biomass enhances accessibility to the underlying cellulose rendering greater digestibility (Sridhar *et al*., 2015). An increase in average daily gain in body weight and dry matter digestibility (DMD) was reported in sheep after 40 days of feeding finger millet straw treated with exogenous lignolytic enzymes harvested from immobilized *Coriolus versicolor* and *Ganoderma lucidium* as compared to control sheep fed untreated straw (Sridhar *et al*., 2015). There is however,a dearth in literature on the effects of feeding any of the individual lignolytic enzymes in enhancing ruminant productivity.

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Laccases (benzenediol/oxygen oxidoreductases, EC.1.10.3.2) are enzymes that catalyze the one electron oxidation of a wide variety of organic and inorganic substrates including mono-, di- and polyphenols, amino phenols, methoxy phenols, aromatic amines and ascorbate with the concomitant four electron reduction of oxygen to water (Galhaup *et al*., 2002). On account of this exemplary capability to oxidize polyphenols, fungal laccases have widespread application in a number of biotechnological processes including the biodegradation of lignin. However, their low yield in the native state limits their practical use in the deconstruction of lignocellulosic biomass for feeding ruminants. Enzymes in bulk quantities are required to treat

biomass which has got greater product consistency and less lot-to-lot variations. A wild isolate of *Schizophyllum commune* NI-07, a potent laccase producer was isolated and its efficacy was tested in deconstruction of lignocelluloses by evaluating its lignin degradation capability and dry matter digestibility *in vitro* (Kumar *et al*., 2015). The present study was an attempt to evaluate the effect of feeding finger millet straw treated with exogenous laccases harvested from immobilized *S.commune on* body weight gain and *in vivo* digestibility in heifers. As far as our knowledge goes this is the first study with regard to feeding lignolytic enzymes harvested from WRF in general and exogenous laccases in particular in cattle.

MATERIALS AND METHODS

White rot fungi

The autochthonous strain isolated and designated as NI_07 was identified as *Schizophyllum commune* (Bank It1679236 *Schizophyllum* KF911323) and deposited in MTCC, Chandigarh, India (Accession No. MTCC 11893)) was used in the current study (Kumar *et al*., 2015). The stock culture was maintained on potato dextrose agar (PDA) media at 4°C and sub cultured every five days. Young cultures were grown on potato dextrose broth for 8 days at 39±2°C. This fungal biomass was used as the inoculum for immobilization.

Production and purification of laccase

Schizophyllum commune was used for production of laccase in submerged batch agitation culture in cotton-plugged 500 ml Erlenmeyer flasks containing 100 ml of production medium. The biomass after homogenization was used as the initial inoculum for immobilization. The production medium consisted of (g/100 ml of distilled water): 1.0 g glucose, 0.5 g yeast extract, 0.5 g urea (90 ml) and 10 ml of salt solution, pH 5.6. Immobilization was carried out on polyurethane foam (PUF) cubes of 1.5 cm × 1.5 cm ×1.5cm (Krishna Prasad *et al.,* 2005) with minor modifications. The enzyme rich media was harvested, accumulated and laccase was partially purified (Kumar *et al*., 2015) and used for treating the weighed quantity of finger millet straw of 2.3 cm length in ratios of 3:5 and 4:5 (w/v) by spraying. The straw was mixed manually for equal distribution and uniform action of the enzyme and kept at room temperature overnight before feeding to the heifers in the morning. This treatment step was regularly carried out so that sufficient amounts of treated straw was available for feeding heifers the next morning.

Experimental animals and feeding protocol

The experiments were carried out at the Experimental livestock unit of the Institute with prior approval of the Institutional Animal Ethics Committee (IAEC). Twelve heifers were divided into three groups of 4 animals each. The animals were healthy as verified by the clinical history and physical appearance. The control group received *ad lib.* finger millet straw treated with production media devoid of enzyme (GI). Test group 1 (GII) received *ad lib.* finger millet straw treated with *Schizophyllum commune* laccase rich media in a 3:5 (w/v) ratio, test group 2 (GIII) received *ad lib.* straw treated with *Schizophyllum commune* laccase rich media in a 4:5 (w/v) ratio. The treated and weighed straw was fed daily for a period of two weeks. Concentrate feeding met the deficiency which would have arisen out of feeding sole straw-based diets. The composition of the concentrate mixture (%) adopted for feeding heifers is given in Fig 1. Body weight change was recorded on a weekly basis and daily feed intake was monitored regularly The DMI was recorded during the digestibility trial in the following seven days and nutrient digestibility estimated. Proximate principles and detergent fibers were estimated in the control and treated straw.

Analytical methods and enzyme assays

Proximate principles (AOAC, 2016) and detergent fiber was estimated (Van Soest, 1991) in the straw and concentrate. *In vitro* dry matter digestibility (IVDMD) or 162 apparent digestibility was estimated as per the procedure of Tilley and Terry (1963). Laccase activity was determined by the oxidation of ABTS at 40C (Bourbonnais *et al*, 1998). Total protein was determined as per Lowry *et al.* (Lowry *et al*., 1951). All assays were performed in 3 replicates.

Statistical analysis

The data on various parameters was tabulated, mean values were calculated and deviations from the means were calculated according to Steel and Torrie, 1980 Analysis of Variance (ANOVA) and least square means was established using proc GLM procedure of SAS (Version 9.3) (SAS, 2009).

RESULTS AND DISCUSSION

Rumen microbes lack the enzyme machinery capable of decomposing lignocellulosic material. Studies (Hristov *et al*., 2000; Nsereko *et al*., 2002) conducted in cattle showed that enzyme supplementation can influence ruminal variables,

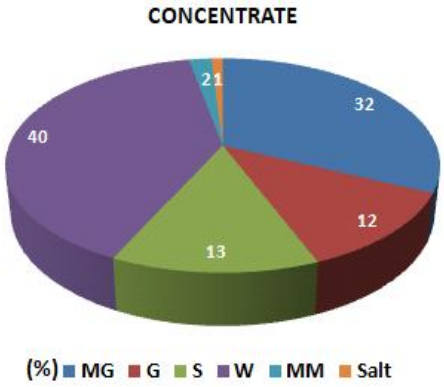


Fig 1: Pie Chart showing the composition of the concentrate mixture (%) adopted for feeding heifers.
MG-Maize Grain, G-Ground nut cake, S-Soybean, W-Wheat bran, MM-Mineral Mixture

fibrolytic activity of ruminal fluid and microbial populations, but the effects seem to be dependent on the type of supplemented enzyme.The white rot fungi (WRF) are the most efficient to degrade lignin on account of their ligninolytic enzymes and predigestion of ligno-cellulosic materials with these enzymes enhances their digestibility for ruminants (Sridhar *et al*., 2014; Arora *et al*., 2002). The procedure of treating the crop residues with lignolytic enzymes and laccase in particular appears to be a safe cost effective strategy to make the cellulose easily available for the ruminants thereby increasing the digestibility which in turn impacts animal performance (Kumar *et al*., 2015).

Earlier, we reported an increase in ADG (gd-1) and dry matter digestibility (DMD) in Bannur sheep,fed finger millet straw treated with a mixture of exogenous lignolytic enzymes harvested from immobilized *Coriolus versicolor* and *Ganoderma lucidium* for 40 days compared to those fed untreated straw (Sridhar *et al*., 2015). In the quest to effectively elucidate the effect of the laccase in bio delignification of crop residues, in this study, we evaluated the effects of feeding finger millet straw treated with exogenous laccase enzyme obtained from *Schizophyllum commune* on body weight changes, DM intake and nutrient digestibility in cattle.

In the present study, two groups of heifers were fed with enzyme treated straw (Group II-40% enzyme and Group III-20% enzyme and Group I served as control where no enzyme was used) for a period of three weeks (14 days pre trial and 5 days trial period) and the effect on proximate composition analysis, lignin degradation and dry matter digestibility was analyzed. The heifers were fed with 70% treated and untreated straw along with 30% concentrate (Appendix I). Analysis of the body weights after 14- days of feeding showed marginal gain in body weights for GI, GII and GIII groups of 277.9 ± 68.47; 277.50 ± 46.43 and 278.85 ± 37.22 kgs respectively with an overall increase of 1.15, 4.25 and 3.60 kgs (Fig 2). The increase in Group II was better compared to other groups. Difference in weights however was not statistically significant in all the three groups with no significant variation in LS-means data as the period of study was only for three weeks.

Diet chart for heifers (Appendix 1).

Feed component (%)	**Proportion**
Finger millet straw	70
Concentrate mixture	30
Total	**100**
Concentrate ingredients (%)	**Proportion**
Maize grain	32
Ground nut cake	12
Soybean	13
Wheat bran	40
Mineral mixture	2
Salt	1
Total	**100**

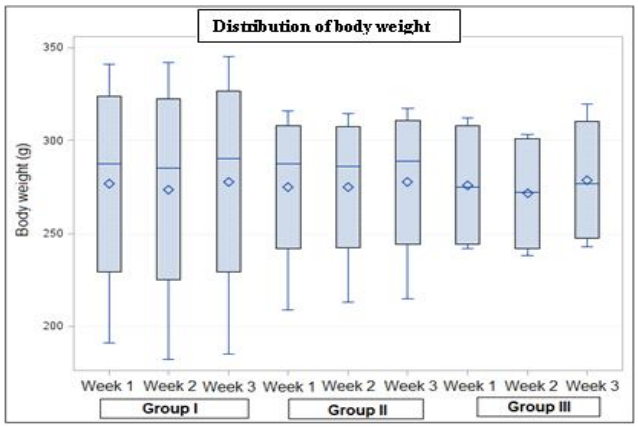


Fig 2: Box plot showing computed least squares means (LS-means) to monitor changes in the body weights in different groups of experimental heifers.

Group I- control group where straw treated with production media alone was fed to heifers; Group II- straw treated with laccase enzyme in the ratio of 3:5 (w/v); Group III- straw treated with laccase enzyme in the ratio of 4:5 (w/v).

The changes in proximate compositions of the feed after various treatments and the digestibility studies observed in different groups of heifers after feeding enzyme treated straw when compared to control group is tabulated in Table 1. The decrease obtained in NDF, ADF and ADL suggests that vegetal cell wall components of the straws were degraded, on account of laccase treatment. Values in NDF showed very marginal decrease in treatment groups when compared to control. However, decrease was more pronounced in ADF and ADL of Group II heifers compared to control, encouraging use of enzyme treatment.

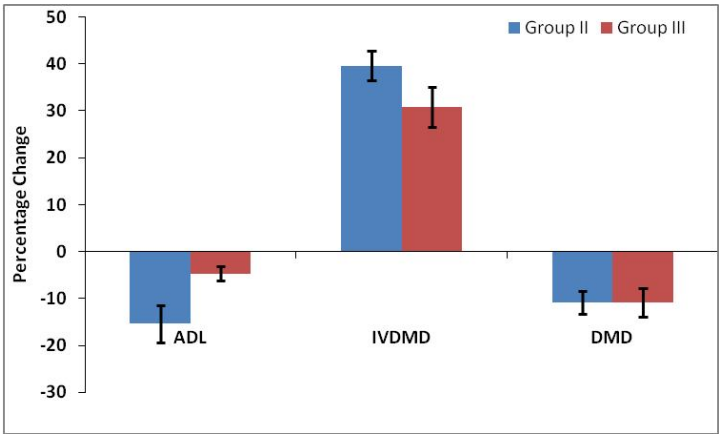
Comparative study of lignin degradation and dry matter digestibility (Fig 3) in enzyme treated groups shows 15.40±4% decrease in lignin content in Group II and 4.67±1.50% in Group III owing to the fact that Group II heifers were fed with 40% enzyme treated straw and Group III heifers with 20% enzyme treated straw clearly indicating the positive effect of laccases on lignin degradation. In vitro dry matter digestibility studies showed an enormous increase of 39.65±3.20% digestibility in Group II and 30.81±4.30% digestibility in Group III experimental heifers again indicating the positive effect of laccases on digestibility with Group II heifers showing 10% greater digestibility than Group III. In both cases strong negative correlation was observed between lignin degradation and increase in *in vitro* digestibility in both the treated groups compared to control. Treatment for *in vitro* and *in vivo* DMD did not cause any significant loss in DM when compared to control sample.

In the present study, no significant increase in the *in vivo* dry matter digestibility was observed in the test groups fed with enzyme treated straw when compared to the control group which fed on untreated straw giving contrasting results compared to *in vitro* dry matter studies. The reduced efficacy of exogenous enzymes applied to ensiled feeds may be due

Table 1: Changes in proximate compositions of feed after various treatments and the dry matter digestibility observed in different groups of heifers after feeding enzyme treated straw.

Parameter	DM	OM	CP	NDF	ADF	ADL	Ash	IVDMD	DMI	DMD
GI (C)	91.8±0.21^{a}	84±0.46^{a}	3.26±0.95^{a}	79.49±0.11^{a}	50.89±1.02^{a}	5.78±0.08^{a}	7.38±0.11^{a}	39.67±1.09	4±0.6	46±5.8
GII (3:5)	93.4±0.19^{b}	86±0.29^{b}	4.32±0.03^{b}	78.28±0.1^{b}	46.91±0.01^{b}	4.89±0.01^{b}	7.56±0.01^{a}	51.82±1.89	3±0.4	41±13.0
GIII (4:5)	93.3±0.20^{b}	83.8±0.08^{a}	4.09±0.32^{b}	78.5±0.51^{b}	47.94±0.64^{b}	5.51±0.33^{ab}	7.21±0.05^{a}	55.33±3.09	3±0.4	41±3.4

GI - control group where straw treated with production media alone was fed to heifers; GII- straw treated with laccase enzyme in the ratio of 3:5 (w/v); GIII- straw treated with laccase enzyme in the ratio of 4:5 (w/v); NDF-neutral detergent fiber; ADFacid detergent fiber; ADL-acid detergent lignin; DM-dry matter; OM-organic matter. Values are means±SE; a, b, means in the same column with different superscripts are significantly different ($p<0.05$).



For ADL and DMD- Group II and Group III means straw treated with enzymes fed to heifers (GII- straw treated with laccase enzyme in the ratio of 3:5 (w/v); GIII- straw treated with laccase enzyme in the ratio of 4:5 (w/v)); For IVDMD-Group II and Group III means straw treated with enzymes was subjected to *in vitro* digestibility as per the procedure of Tilley and Terry 1963. ADL-Acid Detergent Lignin, IVDMD-*In vitro* dry matter digestibility, DMD-Dry matter digestibility.

Fig 3: Bar graph showing relationship between lignin degradation and dry matter digestibility.

to inhibitory compounds in fermented feeds. Nsereko *et al*. (2002) reported the presence of compounds in whole-crop barley silage that inhibited endo-1,4-β-xylanase activity of an enzyme product from *T. longibrachiatum* by 23 to 50%, although there was no effect on cellulase activity. Applying enzymes to feed also provides a slow-release mechanism for enzymes in the rumen (Beauchemin *et al*., 2003). Thus, the greater the proportion of the diet treated with enzymes, the greater the chances that enzymes endure in the rumen. This is also consistent with our earlier studies where different types of dry pastures were treated with 60% enzyme (1:25 w/v) (Kumar *et al*., 2015) unlike the present scenario where Group II and Group III heifers are fed with enzyme treated straw at the rate of 40% and 20% enzyme respectively. Adding exogenous fibrolytic enzymes to dairy cow and feedlot cattle diets can potentially improve cell wall digestion and the efficiency of feed utilization by ruminants. Positive responses in milk production and growth rate have been observed for cattle fed with some enzyme products, although results have been inconsistent.

The enzymes from white rot fungi *Trametes versicolor* (TV1, TV2), *Bjerkandera adusta* (BA) and *Fomes fomentarius* (FF), that are responsible for breaking down the bonds in lignin and within the matrix of cell wall carbohydrates were extracted, but without also extracting enzymes affecting hemicellulose and cellulose. Using these enzymes there was an increase in IVNDFD ($P<0.05$), resulting from treatment of wheat straw with enzyme extracts from BA, TV1 and TV2, reaching a difference of 13% for TV2 ($P<0.05$), *versus* the non-treated straw control. The study indicated that enzyme extracts from white-rot fungi could be used to develop new approaches to overcome low digestibility of some plant cell walls and utilization of different substrates to produce enzyme extracts could lead to production of viable ligninolytic complexes which could improve the nutritive value of fibrous feeds (Rodrigues *et al*., 2008).

The performance of confined beef cattle supplemented with amylolytic enzyme complex produced by fungus *Aspergillus awamori* and a commercial product containing multienzyme complex, yeast and MOS was evaluated (Oliveira *et al*., 2015). The addition of products did not significantly increase daily weight gain, intake, feed conversion and carcass yield of cattle. There was no difference between *in vitro* digestibility of dry matter (IVDMD) in the diets. The percentage of residual fecal starch was not influenced by exogenous amylolytic enzymes of amylase and compound treatments. The tested products were not able to improve animal performance.

Some of these variations in results of various studies can be attributed to under- or over-supplementation of enzyme, treatment time required for proper action on the feed and the level of productivity of the test animal. There is a need to understand the mode of action on the feed to improve on farm efficacy. With increase in demand for more productive ruminant animals, there is an urgent need for improving the digestibility of the feeds using lignolytic enzymes before feeding it to the ruminants making the trapped energy in the form of cellulose more available for digestion. Coming up with a proper enzyme to feed ratio considering all the intricate factors would definitely play a prominent role in future ruminant production systems.

CONCLUSION

There is a dearth of studies on effect of feeding lignolytic enzyme treated straw in ruminants. In this study, treatment of coarse roughages such as finger millet straw with laccase enzyme rich media from white rot fungi failed to influence performance of heifers. This may be on account of the short feeding duration of two weeks adopted on account of dearth of the large volume of enzyme required to treat more quantity of straw required for longer duration of feeding in case of large animals. Also, the maturity stage of the animals used in the experiments influences the obtained results. Longer feeding periods in calves/younger animals will definitely accord better results in terms of growth. Further research on form and dosage of the enzyme rich supplements for effectively treating crop residues are also warranted. However, treatment of finger millet straw with exogenous laccase enzyme harvested from the white rot fungi *Schizophyllum commune* could help in enhancing the digestibility of straw and also body weight gain in heifers in younger animals adopting longer feeding durations.

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