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Research Article

EFFECT OF FIBROLYTIC ENZYMES ON *IN VITRO* DIGESTIBILITIES OF COTTON STALK AND PADDY STRAW IN GOATS

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ABSTRACT

The present study was conducted to assess the effect of *in vitro* dry matter digestibility of cotton stalk and paddy straw treated with solid fibrolytic enzymes (dolomite, mono calcium phosphate, di calcium phosphate and lime stone powder based Vimozyme). The cotton stalk and paddy straw were treated with substrate based solid fibrolytic enzymes like dolomite, mono calcium phosphate, di-calcium phosphate and lime stone powder. Quantitative analysis revealed that the cellulase activity was highest (P<0.01) in dolomite based substrate with 156.83 IU/g followed by Mono calcium phosphate and Di calcium phosphate, while the xylanase activity was highest in dolomite 72.85 IU/g followed by MCP 50.41 IU/g and DCP 28.01 IU/g. The present study, could correlate the increased bioavailability of reducing sugars with increase in unfolding of complex lignocellulosic structures due to enzyme action, with overall fraction reaching up-to 94% increase in availability in a matter of 3-4 days. It was observed that the enzyme remained stable even at temperatures as high as 60° C. It was also observed that the degradation efficiency, owing to the reduced solvent content, has concentrated the enzyme. Different solid based substrates were incubated to ascertain their effect on in vitro dry matter digestibility. The results showed that the *in vitro* dry matter digestibility (%) of paddy straw was significantly higher (p<0.01) in dolomite based substrate 47.76±0.88) followed by Dicalcium phosphate (DCP) 45.35±0.92, Lime stone powder (LSP) 42.54±1.04 and mono calcium phosphate (MCP) 40.21±1.52. *In vitro* dry matter digestibility (%) of cotton stalk was found to be higher for dolomite 37.57±1.04 followed by mono calcium phosphate (MCP) and Lime stone powder (LSP) with 32.21±1.21 and 32.20±1.78, respectively and least for Di calcium phosphate (DCP) 29.21±0.90.

Keywords: Cellulase, Dolomite, *In vitro* dry matter digestibility, Lignocellulosic bond, Mono calcium phosphate base

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Among the eighty cotton producing countries, half of the world's cotton is produced by U.S, China, and India alone. India is one of the world's largest cotton producers, with approximately 26% of the world's cotton production (Huang *et al.*, 2015). India produces 26% of global cotton from 9.175 million hectares. Maharastra contributes around 6.24 million tones mostly in Vidarbha, their use as biomass and as application on animal feeds. The amelioration of cotton stalks as feed will improve animal nourishment (Vinjamur *et al.*, 2015). After harvesting the cotton crop, the remaining biomass is highly rich in cellulose, hemicelluloses which are the crucial source of nutrients for the ruminant animals (Patil *et al.*, 2007). The cotton plant residue (cotton straw) comprises of stalks and estimated production nearly about 2.5 to 3.5 tons of cotton stalks per acre (Travaini *et al.*, 2016). Such harvested cotton crop by-products can be useful as new non-conventional roughage resource in animal feeding (Sharma and Shivappa, 2008). Cotton stalks are low in protein and energy hence efforts must be made to improve their quality as feed for ruminants.

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Paddy (rice) is the most important staple food crop cultivated across the country and accounts for more than 40 per cent of the country's food grain production with the estimated annual yield of 112 MMT (Santosh, 2019). Paddy and wheat straw are by-products available after harvesting the grains, form the main bulk of roughages in tropical region, including India. They form the staple feed for cattle and buffaloes throughout the region. Though they are poor in nutritive value, containing about 3% protein and 40-45% TDN, these straws can maintain adult non-producing cattle as a sole feed along with small quantities of protein supplements (Roupesh *et al.*, 2023). However, certain factors like high lignin content, reduced palatability, dustiness, high oxalic acid content in straw which limits calcium absorption, limit their extensive use as cattle feed. On milling, Oryzasativa, commonly known as Asian rice or paddy rice, produces around 50% whole rice then approximately 16% broken rice, 20% husk, 14% bran.

Enzymes are proteins which consists of a chain of amino acids. They catalyze reactions by binding to substrates and stabilize the entire reaction process up to

product formation (Sheppy, 2001). Activity of enzyme depends on its type, the species of animal used, pH, temperature and gastro-intestinal conditions, dosage, substrate, degradation of the exogenous enzyme along the tract, practical management conditions and other factors (Rojo *et al.*, 2007). The liquid enzymes are generally less stable than solids although they may have greater activity than their solid counterparts. In general practice, it is assumed that the loss in activity of liquid enzymes is due to thermal and mechanical treatments that can be minimized once incorporated into the feed, as their hydric content is decreased.

Enzyme immobilization is an attractive strategy for improving biocatalyst properties such as chemical stability, catalytic activity and making enzyme insoluble thereby enabling its reuse in consecutive reaction cycles, consequently reducing costs (Sheldon and Brady, 2021). In the enzyme immobilization procedure, it is essential to consider the support properties (Zdarta *et al.*, 2018). Different materials have been used for the immobilization of phytase, involving interactions like adsorption on allophanic synthetic compounds and mont morillonite, covalent linkage on graphene oxide (Dutta *et al.*, 2017), entrapment in calcium alginate, and coordination bonds with the calcium ion in hydroxyapatite. Perhaps, treatment with immobilization of solid enzyme based substrate has been identified as a promising alternative to improve the energy availability and thereby increasing the hydrolytic capacity for ruminants (Sheldon and Brady, 2021) within the rumen environment. The present study was designed to study Enzymatic activity in cotton stalk and paddy straw treated with various substrate based solid fibrolytic enzymes in goats.

MATERIALS AND METHODS

The trial was performed in VNIT, Nagpur and Department of Animal Nutrition, CVSc, Nagpur. Enzyme extraction, purification, enzyme quantification/assay, enzymes thermal stability and enzymes degree of degradation were studied at Department of Chemical Engineering, Visvesvaraya National Institute of Technology, Nagpur. The trial was approved by IAEC with ref no of NVC/IAEC/05/2024. Enzyme preparation was performed in 5 stages. Shredding: Obtained biomass (rice straw) is shredded into a particle size of 1 mm × 2 mm followed by Solid State Fermentation: Biomass: Water: Culture = 1:1:0.5, set for fermentation for 14-15 days (residence time). Buffer Extraction: The buffer used for extraction was the Phosphate buffer (Na_2HPO_4 and NaH_2PO_4) of pH 6, soaked overnight and solid to liquid ratio is 1:10 because the result indicated the most efficient extraction volume and possible reason is solvent inhibition. Centrifuging: Carried out the at 10000 rpm for 15 min at < 10° C. Centrifuged 4 litre extract per cycle. The purpose of the centrifuge was to lyse the remaining microbial cells in buffer extract. Membrane Separation/Filtration: Filtration was done by membrane separator MWCO- 75k Da. Operated under 2 bar pressure to prevent damage to the membrane. Backwashing is done to prevent membrane clogging after every extraction process.

Endoglucanases, exoglucanases and β-d-glucosidases were the three enzymatic activities that made up a whole cellulase system. Insoluble substrates, such as pure cellulosic substrates like cotton linter, Whatman No. 1 filter paper, microcrystalline cellulose, bacterial cellulose and algal cellulose, as well as cellulose-containing substrates like dyed cellulose, a-cellulose, and pretreated lignocellulose, are always used to measure total cellulase activities. Using the Filter Paper Assay technique, a cellulose analysis was conducted. Baker and Adney (2008). The xylanase assay was designed using the DNS method (Miller, 1959) and involved generating reducing sugar from a xylan solution, which was subsequently reacted with 3,5 Dinitrosalicyclic Acid (DNS). The said assay was outsourced from Cargill Premix & Nutrition- India. A 100 ml of dilute enzyme was taken in a wide petri plate and was subjected to drying under vacuum at different temperatures, ranging from 40° C, 50° C and 60° C. Treatment duration was fixed at 6 hours and samples were collected at the end of the drying cycle. The concentrated samples thus obtained were then applied on to virgin rice straw and incubated for a period of 4 days. Degree of degradation was then determined using the Anthrone method for measurement of cellulose and hemicellulose content. Similar experiment was done with the dilute enzyme cocktail being subjected to a rotary evaporator. The vacuum was intermittently applied while the temperature was maintained constant at 40° C. The treatment was carried out until the final volume reached one tenth of the initial value. This was done as an observation experiment to evaluate the extent upto which concentration of enzyme is possible.

The various substrate based solid fibrolytic enzymes mainly dolomite, mono calcium phosphate, di-calcium phosphate and lime stone powder were treated with cotton stalk and paddy straw at the dose rate of 0.3 gm/kg. In vitro digestibility was determined, taking 0.5 g dried and ground (particle size < 1mm) shredded cotton stalk of each treated with enzymes solution in 100ml conical flask for the first stage in vitro method (Tilley and Terry, 1963) along with 40 ml phosphate carbonated buffer (McDougall, 1948), 10

Table 1. Enzymatic Assay of various substrates used in trail and *in-vitro* dry matter digestibility of treated and untreated cotton stalk and paddy straw

1. Enzymatic Activity

a) Cellulase activity

S.No.	Immobilization	Activity	P Value
1.	MCP	72.82^{C}±5.23	0.001
2.	Dolomite	156.83^{D}±9.75	
3.	DCP	39.21^{A}±7.64	
4.	LSP	58.21^{B}± 4.28	
b)	Xylanase activity		
1.	Dolomite	72.85^{D}±6.87	0.009
2.	MCP	50.41^{C}±10.51	
3.	DCP	28.01^{A}±4.55	
4.	LSP	40.21^{B}±6.65	

2. *In vitro* dry matter digestibility

a) Paddy straw

Sr. No.	Substrate	IVDMD %	P- value
1.	Untreated	35.76^{A}±2.11	0.045
2.	MCP	40.21^{B}±1.52	
3.	Dolomite	47.76^{E}±0.88	
4.	DCP	45.35^{D}±0.92	
5.	LSP	42.54^{C}±1.04	

b) Cotton stalk

Sr. No.	Substrate	IVDMD %	P value
1.	Untreated	22.36^{A}±2.98	0.021
2.	MCP	29.21^{B}±0.90	
3.	Dolomite	37.57^{D}±1.04	
4.	DCP	32.21^{C}±0.92	
5.	LSP	32.20^{C}±1.78	

ml of strained rumen liquor (SRL) with flushing of CO_2 gas and incubated for 48 h with periodically shaking at 39° C and parallel blank (without sample) with phosphate carbonated buffer and rumen liquor without substrate. Anaerobic conditions were created in the system by bubbling CO_2 gas, maintaining pH to 6.8, and after 48 hr incubation, filter the contents through Sintered Glass Crucible (G1). The residue is dried at 100° C overnight and used to estimate % IVDMD. The data obtainedwere subjected to analysis as per Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

Cellulase activity was highest ($P<0.01$) in dolomite based substrate with 156.83 IU/g followed by MCP (72.82±5.23), LSP (58.21± 4.28) and DCP (39.21±7.64). The xylanase activity was highest in MCP 72.85 IU/g followed by dolomite 50.41 IU/g , LSP (40.21±6.65) and DCP 28.01 IU/g. Use of Solid Substrate Fermentation (SSF) for enzyme production has been studied extensively by Delabona *et al.* (2012) who also reported activities of three types of cellulase enzymes, up to 160.1 IU/g for CMCase (endo-glucanase), 5.0 FPU/g for FPase (exo-glucanase), 105.82 IU/g for β-glucosidase and 1055.62 IU/g for xylanase by using Amazonian wood fungi over common agricultural residues like sugarcane bagasse, orange peels and wheat brans.

Enzyme production and catalytic efficiency are governed by multiple parameters including substrate composition, microbial strain specificity and environmental factors such as moisture content and temperature (Biswal *et al.*, 2024) The activity profile of the enzyme cocktail produced in this experiment shows greater xylanase activity over cellulase, as reported by Delabona *et al.* (2012). Cellulase activities in the range of 5.69 U/g to 106 U/g have been reported by Salihu *et al.* (2015), Kholif *et al.* (2017)a, Ijoma *et al.* (2017) and Astolfi *et al.* (2019).

The degradation experiments were carried out for a period of six days. 5 g of biomass (rice straw) was mixed with 40 mL of dilute enzyme solution in six identical flasks. Whole flask cultures were considered for evaluation of degradation efficiency. Flasks were opened per day to depict the degradation pattern for each day. Control flask was added with 40 ml of distilled water instead of enzyme solution. It was observed that the release of sugar flat lined after day 4, indicating a cessation in enzymatic action over cellulosic and hemicellulosic fibres. The present study could correlate the increased bio-availability of reducing sugars with increase in unfolding of complex lignocellulosic structures due to enzyme action, with overall fraction reaching up-to 94% increase in availability in a matter of 3-4 days. These findings are in acceptance with findings of Yoshida *et al.* (2008) and Choi *et al.* (2010) who reported about the increased availability of reducing sugars as a result of degradation of complex lignocellulosic structures due to enzyme action.

Thermal stability was estimated by, diluting enzyme and subjected to vacuum drying for 6 hours at three different temperatures- 40, 50 and 60° C. The biomass obtained after the end of incubation was dried and estimated for cellulose and hemicellulose. It was observed that the enzyme remained stable even at temperatures as high as 60° C. It was also observed that there was degradation efficiency due to the reduced solvent content, thus concentrated the enzyme. The present observations are in complete agreement with Delabona *et al.* (2012) who reported that the enzymes produced in the process tend to be thermostable, up to 75° C, although maximum activity was observed at incubation temperature of 65° C. Further, present findings are also in acceptnce with Astolfi *et al.* (2019) who reported that the enzyme stability was at 40° C at an acidic range of pH (4.5-5.5) in soybean husk when

used as substrate as agro-residues for the production of enzymes for application in biofuel production with Trichodermareesi.

Different solid based substrates were incubated to ascertain their effect on in vitro dry matter digestibility. The results indicated that paddy straw treated with dolomite based substrate exhibited significantly higher digestibility ($P<0.01$) than the other enzymatic bases. In vitro dry matter digestibility (%) of cotton stalk was found to be higher for dolomite (37.57±1.04) followed by MCP and LSP which were comparable. Lunagariya *et al.* (2017), who reported higher IVDMD with higher level of incorporation of exogenous fibrolytic enzymes. Similarly, Sheikh *et al.* (2017) reported that significant improvement in vitro digestibility of DM was observed due to probiotics mix and fibrolytic enzyme mixture supplementation in paddy straw with maximum values at 3g and 9g/ kg DM, respectively. Reddy *et al.* (2016) observed the % IVDMD result of total mixed rations supplemented with exogenous fibrolytic enzyme and/or live yeast culture was contradictory in present studies when cotton stalk treated with liquid fibrolytic enzymes. The results in the present study of treated cotton stalk with different substrate based liquid fibrolytic enzymes solution nearly similar with the findings of Rajamma *et al.* (2015) in total mixed ration containing different roughages–concentrate ration with or without fibrolytic enzymes. The present findings concur with the Research finding of *in vitro* dry matter digestibility (%) as reported by Bhaskar *et al.* (2012) and Ganai *et al.* (2011). Cotton stalk treated with enzymes might have caused fiber hydrolysis or solubilisation which might have increased digestion rate (Nipane *et al.*, 2023). Thus the enzyme treatment enhances the attachment of rumen micro organisms to the feed particles, thereby increasing the hydrolytic capacity (Wang *et al.*, 2012). From the observation we draw that dolomite treated substrate has shown best results.

CONCLUSION

Dolomite based substrate treated with cotton and paddy straw was found to be superior than those of calcium based enzyme substrate sources. Enzymatic activity was found to be same to those of liquid fibrolytic enzyme solution without changing the structural integrity of the substrate which is similar to the liquid based enzymes. Due to the best enzymatic activity there was better enzyme degradation in crop residues as well as in staple crop.

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