*Indian J. Anim. Nutr.* 2023. 40 (3): 288-295
doi:10.5958/2231-6744.2023.00036.1

Indian Journal of Animal Nutrition



# *In Vitro* Digestibility of Cotton Stalk Treated with Different Substrate Based Fiber Degrading Liquid Enzymes at Varying Duration

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## ABSTRACT

The present study was performed to evaluate the effect of fiber degrading liquid enzymes solution at different dilutions and duration on *in vitro* dry matter digestibility of cotton stalk. The cotton stalk was treated with rice straw, untreated cotton stalk and ozone treated cotton stalk substrate based liquid fibrolytic enzymes with 4 lit/kg(2 lit of extracted enzyme and 2 lit of water per kg substrate) for 24, 48, 72 and 96 hr soaking period. Liquid fibrolytic enzymes extraction, purification, characterization and standardization, enzyme activity, and application dosage optimization for substrate was also carried out. The substrates with different dilutions and durations were incubated to ascertain their effect on *in vitro* digestibility. The results revealed significant differences (P<0.01) in the *in vitro* of dry matter digestibility (%) of cotton stalk treated with ozone treatment followed by rice straw based liquid fibrolytic enzyme solution @ 4 lit/kg for 24 h soaking period and in untreated cotton stalk based liquid firbrolytic enzyme solution for 48 and 72 h soaking period. IVNDFD and IVADFD (%) higher in ozone treated cotton stalk based liquid fibrolytic enzyme treated cotton stalk as compare other substrate based enzymes. On conclusion, *in vitro* dry matter, neutral detergent fiber and acid detergent fiber digestibility (%) of cotton stalk treated with ozone treated cotton stalk based liquid fibrolytic enzymes were significantly higher (p<0.01) as compared to untreated cotton stalk and rice straw based treated cotton stalk.

**KEY WORDS:** Cotton stalk, Enzyme solution, *In vitro* dry matter digestibility, *In vitro* neutral detergent fiber digestibility, *In vitro* acid detergent fiber digestibility.

Article received: 04 January 2023; Article accepted: 03 July 2023

## INTRODUCTION

India produces over 501.73 million tonnes of crop residues annually. Every year, various crop residues are produced in India. Some of them are used as animal feed, and the majority are used as fuel or for composting and a significant amount of this material is burned causing environmental pollution (MNRE, 2009). Moreover, proper processing of crop residue can alleviate the feed scarcity in animals. Making feeds from a blend of crop residues and concentrates is the best approach to effective use of crop residues. Maize stover, sorghum straw, wheat straw, rice straw, and gram straw are among the major crop residues produced from various crops. The inclusion level of these crop residues in livestock feed varies from 17.5 to 75 percent.

Cotton produces over 40% of the world's fibre. Cotton is farmed in 80 countries throughout the world, with the United States, China, and India accounting for more than half of global cotton production. A large amount of cotton residue is thrown away every year since it is not utilised. It pollutes the environment because of its disposal difficulties and pests. It also presents lots of complications in agriculture due to its sluggish breakdown rate in soil. (Sharma and Chen, 2008).Cotton plant stalk production is predicted to be 43.5 million tonnes per year. Cotton stalks include approximately 46 percent alpha cellulose and approximately 26 percent lignin. The availability of animal feed has been hampered by drought. Cotton stalks are readily available in India. 22.33 million tonnes of cotton were grown on 9.175 million hectors, whereas 6.24 million tonnes of cotton stalk are produced on 3.124 million cultivated hectors in Maharashtra (Patil et al., 2007).

Cotton stalks have heavily lignified cell walls which cause them to be deficient in protein, energy, with poor digestibility. Necessary improvements have to be made to improve the roughage quality. The nutritional value of cotton stalks was attempted to be increased using a variety of procedures, including grinding (Reddy et al., 1992), pelleting (Reddy and Reddy, 1985), treatment with chemicals including $NH_3$ (Reddy and Reddy, 1986), ozone, and sodium hydroxide (Ben-Ghedalia et al., 1982). Microbial conversion of cellulosic/lignocellulosic biomass into useful products is complex processes involving combined action of three enzymes namely endogluconase, exogluconase and beta-glucosidase (Lynd et al., 2002; Zhang and Lynd, 2004). By destroying lignocellulose components, cotton stalks' nutritional value can be increased by using yeast and an NSP-degrading multienzymes as cellulase, xylanase, pectinase etc. Therefore, present study was undertaken to evaluate the effect of liquid enzyme treatment on cotton stalk *in vitro* digestibility.

## MATERIALS AND METHODS

### Preparation of liquid enzymes

Enzyme extraction and purification, characterization and standardization, effect of enzyme activity on different substrates, development of substrate (rice straw base) specific enzymes and activity optimization and application dosage optimization for substrate was done in Department of Chemical Engineering, Visvesvaraya National Institute of Technology, Nagpur.

For preparation of liquid enzyme, obtained biomass was shredded into particle size of 1 mm x 2 mm. Then took the Biomass : Water: Culture at 1:1:0.5 ratio in container and set for fermentation for 14-15 days (residence time) in nearly anaerobic condition. Buffer used for extraction is Phosphate buffer ($Na_2HPO_4$ and $NaH_2PO_4$), pH 6, soaked overnight and solid to liquid ratio is 1:10 because result indicate the most efficient extraction volume and possible reason is solvent inhibition. Then centrifuged at 10000 rpm for 15 min at < $10^0$C. Centrifuged 4Lextract per cycle was obtained. Purpose of centrifuge is to lyse remaining microbial cells in buffer extract. Separated by membrane separator MWCO- 75k Da. Operated under 2 bar pressure to prevent damage to membrane. Backwashing was done to prevent membrane clogging after every extraction process. Filtration rate was0.756 L/min and column life up to 6 kL.

Quantitative analysis of enzymes (total cellulose assay) was carried out as per standard procedure and enzyme activity of cellulase and xylanase were observed ranged between 339 to 408 U/L and 70.27µmole/min/g. Cellulase and xylanase activity were carried out as per filter paper assay (FPA) and DNS method (Adney and Baker, 2008; Millar, 1959) as recommended by the International Union of Pure and Applied Chemistry (IUPAC).

### Treatment of liquid fibrolytic enzymes on cotton stalk

As per optimization study, extracted and purified rice straw, untreated cotton stalk and ozone treated cotton stalk substrate based liquid enzymes were prepared separately and each enzyme were mixed separately with equal quantity of water to form enzyme solution**.** The enzyme solution so prepared was sprayed on the shredded cotton stalk at different dilution rate and for different period of soaking. The experiment was conducted with enzyme solution treatment rate of4 lit/Kg (2 lit extracted enzymes + 2 lit water) allowed for 24, 48, 72 and 96 h duration. The pre-digestion composition and fibre fraction of enzyme treated cotton stalk was also studied (AOAC, 2005). For, *in-vitro* digestibility study, three types of treated cotton stalk used which treated with three types of substrate-based enzymes. The details of different experimental treatment groups are mentioned below.

Treatment-1 (T1): Rice straw based enzyme solution @ 4 lit/kg of Cotton Stalk (2 lit extracted enzymes + 2 lit water); Treatment-2 (T2): Untreated cotton stalk based enzyme solution @ 4 lit/kg of Cotton Stalk (2 lit extracted enzymes + 2 lit water) ; Treatment-3 (T3): Ozone treated cotton stalk based enzyme solution @ 4 lit/kg of Cotton Stalk (2 lit extracted enzymes + 2 lit water)

### *1. In vitro* digestibility determination

**A. Preparation of phosphate carbonated buffer:** The Phosphate Carbonate Buffer solution (artificial saliva) was prepared according to McDougall (1948) as

| | | |
|---|---|---|
| a. | $NaHCO_3$ | 9.80g |
| b. | $Na_2HPO_4 2H_2O$ | 7.00g |
| c. | KCL | 0.57g |
| d. | NaCl | 0.47g |
| e. | $MgSO_4 2H_2O$ | 0.12g |
| f. | $CaCl_2$ | 0.04g |

Mixing above chemical except $CaCl_2$, just before use, add $CaCl_2$, keep at 39$^0$C and pass $CO_2$ through solution

**B. Collection of rumen liquor**

Rumen liquor was collected from the rumen of goat. About 200-250 ml of rumen liquor was collected, and then filtered by four layers of muslin cloth and incubated in a water bath at 39$^0$C.

***C. In vitro* digestibility**

Dried and ground (particle size< 1mm) components of each treated rice straw, untreated cotton stalk and ozone treated cotton stalk based fiber degrading liquid enzymes solution with 4 lit/kg substrate (CS) for 24, 48, 72 and 96hrs used as for first stage *in vitro* method (Tilley and Terry, 1963). Rumen liquor was collected from goat3-4 h after feeding and strained through four layers of muslin cloth. Incubations were carried out with 40 ml phosphate carbonated buffer (McDougall, 1948), 10 ml of strained rumen liquor (SRL) and 0.5 g substrate and passing $CO_2$ gas for each of ten replicates for 48 hr incubation with periodically shaking at 39$^0$C and parallel blank with phosphate carbonated buffer and rumen liquor without substrate. Anaerobic conditions were created in the system by bubbling $CO_2$ gas and maintaining pH to 6.8 and filter the contents through Sintered Glass Crucible (G1). The residue is dried at 100$^0$C overnight and used for estimation of % IVDMD. Along with NDF and ADF carried out of feed residues of IVDMD samples and IVNDF and IVADFD calculated.

Calculations were made using the following equation:

DM disappearance= Wt. of sample – (wt. of residue of test – wt. of residue of blank)

$$\text{DM digestibility (\%)} = \frac{\text{DM disappearance x 100}}{\text{Wt. of sample (DM basis)}}$$

### 2. Statistical analysis

The entire experiment was conducted using three factorial experimental design and the data were statistically analyzed as per Snedecor and Cochran (1994).

## RESULTS AND DISCUSSION

The pre-digestion composition and cell wall constituents of enzyme treated and untreated cotton stalk with different duration are presented in Table 1. The pre-digestion composition and fibre fraction of enzyme treated cotton stalk revealed greater DM% and CF % of untreated cotton stalk than the various treatment groups while, NFE% was less in cotton stalk treated with ozone treated cotton stalk based enzyme solution than the other treated groups. The NDF, ADF and ADL percent of untreated cotton stalk was observed to be higher than the pre-treated groups whereas, the cellulose was highest for ozone treated cotton stalk at 72 h interval. The hemicellulose percent greater in untreated cotton stalk at 96 hrs intervals when compared with other groups. The proximate composition was found to be less in enzyme treated cotton stalk due to release of non-structural carbohydrates or soluble sugars in leachate which leads to less percent of DM when compared to untreated cotton stalk. The observations recorded in present study are in accordance with Jadhav (2019) and Burghate (2021), who reported analogous finding with regard to proximate and fiber composition of untreated cotton stalk. However, study of nutrient composition and fibre fraction of cotton stalk made by Grevel et al. (2003), Nagalakshmi and Reddy (2011) and Hashim et al.

(2017) deviate from the present findings, which may be attributed to the difference in variations in chemical composition, variety of cotton crop, thrashing methods, maturity of plant at the time of harvesting, soil and other factors affecting plant composition.

Table 1. Pre-digestion composition and cell wall constituents of enzyme treated cotton stalk

| Nutrients% | Cotton stalk | Rice straw based enzyme solution @ 4 lit/kg Cotton Stalk (T1) | | | | Untreated cotton stalk based enzyme solution @ 4 lit/kg Cotton Stalk (T2) | | | | Ozone treated cotton stalk based enzyme solution @ 4 lit/kg Cotton Stalk (T3) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Soaking period (h) | | | | | | | | | | | |
| | | 24 | 48 | 72 | 96 | 24 | 48 | 72 | 96 | 24 | 48 | 72 | 96 |
| Dry matter | 93.6 | 90.2 | 90.3 | 90.6 | 90.5 | 91.4 | 90.2 | 91.2 | 90.7 | 92.0 | 90.6 | 91.4 | 91.2 |
| Organic matter | 92.3 | 91.8 | 91.2 | 91.4 | 91.2 | 89.9 | 88.4 | 88.7 | 88.8 | 89.4 | 88.4 | 89.7 | 89.4 |
| Crude protein | 3.10 | 3.02 | 3.22 | 3.18 | 3.10 | 3.33 | 3.01 | 3.10 | 3.02 | 3.11 | 3.25 | 3.31 | 3.21 |
| Ether extract | 1.06 | 1.38 | 1.82 | 1.64 | 1.48 | 1.05 | 1.06 | 1.11 | 1.09 | 1.02 | 1.05 | 1.10 | 1.04 |
| Crude fibre | 52.4 | 44.2 | 43.2 | 44.8 | 45.0 | 48.1 | 46.5 | 49.1 | 51.0 | 47.5 | 45.1 | 49.5 | 50.8 |
| Nitrogen free extract | 35.7 | 43.2 | 42.9 | 41.8 | 41.6 | 37.3 | 37.8 | 35.4 | 33.7 | 37.7 | 39.0 | 35.8 | 34.4 |
| Total ash | 7.66 | 8.20 | 8.77 | 8.55 | 8.80 | 10.12 | 11.58 | 11.25 | 11.15 | 10.56 | 11.58 | 10.26 | 10.58 |
| NDF | 81.3 | 78.0 | 76.2 | 76.4 | 75.1 | 76.6 | 73.2 | 76.3 | 74.8 | 77.6 | 72.3 | 77.2 | 75.3 |
| ADF | 68.5 | 67.5 | 66.6 | 67.3 | 66.0 | 62.5 | 59.3 | 60.5 | 58.2 | 62.5 | 58.3 | 61.6 | 60.5 |
| ADL | 30.7 | 30.6 | 49.6 | 48.2 | 48.2 | 45.2 | 39.6 | 39.7 | 38.6 | 44.5 | 38.1 | 39.1 | 40.4 |
| Hemicellulose | 12.8 | 11.3 | 9.56 | 9.26 | 9.49 | 14.0 | 13.9 | 14.7 | 16.6 | 15.0 | 14.0 | 15.6 | 14.8 |
| Cellulose | 17.8 | 16.9 | 17.0 | 19.0 | 17.8 | 17.3 | 19.7 | 20.9 | 19.6 | 17.9 | 20.2 | 22.4 | 20.1 |

The *in vitro* DM, NDF and ADF digestibility(%) of cotton stalk treated with liquid fibrolytic enzyme solution with different duration are presented in Table 2. In order to distinguish the effect of treatment with enzyme solution on *in vitro* digestibility of cotton stalk with similar quantity and period of soaking. Accordingly, trial was conducted for 3 different groups viz. T1, T2 and T3 for 24, 48, 72 and 96 h of soaking period. The average *in vitro* dry matter, neutral detergent and acid detergent fiber digestibility % of untreated cotton stalk was found to be 42.1±0.41, 60.5±0.52 and 55.1±0.64 respectively.

Table 2. *In vitro* DM, NDF and ADF digestibility (%) of cotton stalk treated with rice straw, untreated cotton stalk and ozone treated cotton stalk based liquid enzymes with different duration at 48 h of incubation

| Particulars | Soaking period(h) | Rice straw based enzyme solution @ 4 lit/kg Cotton Stalk (T1) | Untreated cotton stalk based enzyme solution @ 4 lit/kg Cotton Stalk (T2) | Ozone treated cotton stalk based enzyme solution @ 4 lit/kg Cotton Stalk (T3) |
|---|---|---|---|---|
| IVDMD % | 24 | $48.4^{Bc}$±0.64 | $44.1^{Aab}$±0.85 | $54.6^{Cb}$±2.02 |
| | 48 | $43.3^{Abc}$±0.69 | $46.8^{Bb}$±1.62 | $45.3^{Ba}$±0.68 |
| | 72 | $34.6^{Aab}$±1.50 | $46.3^{Bb}$±0.72 | $47.7^{Bab}$±1.60 |
| | 96 | $30.1^{Aa}$±2.20 | $40.1^{Ba}$±1.22 | $43.6^{Ca}$±0.81 |
| | Mean | $36.0^{A}$ | $44.3^{B}$ | $47.8^{C}$ |
| | SE | 2.74 | 1.32 | 2.10 |
| IVNDFD % | 24 | $62.0^{Ac}$±0.66 | $63.5^{Abc}$±0.42 | $67.0^{Bc}$±0.64 |
| | 48 | $57.0^{Aab}$±1.00 | $61.8^{Bb}$±0.46 | $65.0^{Cb}$±1.20 |
| | 72 | $60.4^{Ab}$±0.90 | $61.5^{Ab}$±0.83 | $64.4^{Bb}$±0.70 |
| | 96 | $53.0^{Aa}$±1.05 | $56.2^{Ba}$±1.20 | $61.5^{Ca}$±1.05 |
| | Mean | $58.1^{A}$ | $60.8^{B}$ | $64.5^{C}$ |
| | SE | 1.99 | 1.59 | 1.13 |
| IVADFD % | 24 | $57.1^{Ac}$±0.41 | $62.1^{BCc}$±0.49 | $63.1^{Cc}$±0.41 |
| | 48 | $53.7^{Aab}$ ±0.91 | $57.3^{Bb}$±0.56 | $60.7^{Cbc}$±0.91 |
| | 72 | $55.8^{Ab}$±0.57 | $58.2^{Bb}$±1.07 | $62.8^{Cc}$±0.57 |
| | 96 | $48.2^{Aa}$±0.55 | $51.2^{Ba}$±1.65 | $56.8^{Ca}$±0.84 |
| | Mean | $53.7^{A}$ | $57.2^{B}$ | $60.9^{C}$ |
| | SE | 1.96 | 2.26 | 1.45 |

Mean within the same row (ABC) and column (abc) with different superscripts differ significantly (P<0.01)

The overall IVDMD % of cotton stalk was significantly higher (p<0.01) in T3 (47.83±2.10) followed by T2 (44.3±1.32) and T1 (36.0±2.74). With respect to the soaking period at 24 h IVDMD% was highest for T3 followed by T1 and in T2 whereas, for 48 and 72 h comparable for T3 and T2 but, was lower in T1. At 96 h interval IVDMD% was significantly differed in T3. At 24 h rice straw based cotton stalk and ozone treated cotton stalk showed highest value when compared with other interval similarly, at 48 hrs untreated cotton stalk was highest. This might be due to more escape of reducing sugars in leachate (water extract) due to rapidly action of rice straw based enzyme solution on cell wall constituents of cotton stalk to break down lignocellulosic bond and IVDMD % goes on decreasing as soaking period increases as compare to ozone treated and untreated cotton stalk based enzyme solution.

Abid et al. (2022) reported that DMD (%) 58.3±2.5, 58.6±2.5, 66.1±2.5 and 54.6±2.5 of olive leaves treated different level of exogenous fibrolytic enzymes in 0µL(control), 1µL, 2µL and 4µL/g of dry matter respectively because of synergy effect between EFE and rumen flora, these results are agreement with present study. Muruz et al. *(*2019) stated that *in vitro* DM digestibility of corn stover treated with enzymes and inoculant correlated with cotton stalk treated with untreated cotton stalk and ozone treated cotton stalk based enzyme solution. Findings of present study on cotton stalk treated with ozone treated cotton stalk based enzyme solution for 24 h was found to be comparable with *in vitro* digestibility of DM (63.03%) reported by Lunagariya et al.(2017)as incorporation level of exogenous fibrolytic enzymes higher. 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 not agreement with treated cotton stalk with liquid fibrolytic enzymes.The values 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. Research finding of *in vitro* dry matter digestibility (%) reported by Bhaskar et al. (2012) and Ganai et al. (2011) are in agreement with the present findings.

Average IVNDFD (%) of cotton stalk significant (P<0.01) greater inT3 (64.5±1.13) as compare to T2 (60.8±1.59) and T1 (58.1±1.99). As observed that IVDMD (%) increased in present study that associated with higher NDF digestibility. IVNDFD (%) increased significantly (p<0.01) in T1 (62.0±0.66), T2 (63.5±0.42) and T3 (67.0±0.64) for 24 h due to short soaking period which lead to less escape of reducing sugar in leachate and reduced the action of enzymes on cell wall constituents then decreased for 48 (61.8±0.46, 65.0±1.20), 72 (61.5±0.83, 64.4±0.70) and 96 h (56.2±1.20,61.2±1.05) in T2 and T3 respectively. Sheikh et al. (2017) reported that significant improvement in NDF digestibility due to supplementation dose (3 g, 6 g and 9 g/kg DM) respectively. Present findings are similar with Rajamma et al. (2015) was found significantly higher (P<0.001) IVNDFD (%) in supplemented exogenous fibrolytic enzymes (EFE) in TMR-1 (60R:40C) and TMR-2 (70R:30C) than without EFE supplementation. Also Zhao et al. (2015) concluded that fibrolytic enzyme supplement potentially improved fiber digestion (NDFD) of corn stover in this *in vitro* experiment. Reddy et al. (2016) reported that *in vitro* digestibility (%) of NDF increased linearly in treatment group supplemented with EFE and/or live yeast culture in TMR (70R:30) than control TMR (70R:30C) group that similar with present findings. This finding confirms with previous studies have also shown increase in NDF digestibility with use of fibrolytic enzymes (Ganai et al.2011; Thakur and Shelke,2011; Shojaeian and Thakur, 2007; Wang et al. 2004).

IVADFD (%) significantly (p<0.01) differed in all groups and periods being highest in ozone treated cotton stalk (60.9±1.45) followed by untreated cotton stalk (57.2±2.26) and lowest in rice straw based enzymes solution (53.7±1.96). At 24 h IVADFD values were highest in all groups later on the values decreased this might be due to shorten soaking period and reduced stability of enzymes. At 48, 72 and 96 h of soaking interval IVADFD % was highest for ozone treated cotton stalk based enzyme solution. Cotton stalk treated with enzyme solution might have caused fiber hydrolysis and/or solubilisation of ADF which might have increase digestion rate. Result of present findings are correlated with finding of Rajamma et al. (2015) that IVADF (%) was significantly higher (p<0.001) supplemented with exogenous fibrolytic enzymes (EFE) in TMR-1 (60R:40C) and TMR-2 (70R:30C) than without EFE supplementation. These results are consistent with Reddy et al. 2016; Thakur and Shelke, 2011, who have reported.

## CONCLUSION

Based on the results found in the present study it is inferred that *in vitro* dry matter, neutral detergent fiber and acid detergent fiber digestibility (%) of cotton stalk treated with ozone treated cotton stalk based fiber degrading liquid enzyme solution was proved to be superior than the rice straw and untreated cotton stalk based liquid enzyme solution for 4 lit/kg dilution.

## ACKNOWLEDEGEMENT

The authors are thankful to Maharashtra Animal and Fishery Science University and Visvesvaraya National Institute of Technology, Nagpur for providing facilities to carry out work.

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