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Impact of lactic acid bacteria and enzymes on the fermentation processes of sugarcane tops silage at a particular time interval

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Abstract
The objective of this study was to achieve the virtuous fermentation quality of the sugarcane tops silage by combining the most promising additives with the day of ensiling. Fresh sugarcane tops were ensiled in 3-L laboratory silos with enzymes (Cellulase+ Xylanase), enzymes plus *Lactobacillus plantarum* (C+X+LP), enzymes plus *Lactobacillus fermentum* (C+X+LF) and enzymes +*Lactobacillus plantarum* + *Lactobacillus fermentum* (C+X+LP+LF) for 15, 25, 35, and 45 days. Urea (0.5%) and molasses (1.5%) were added in all treatment groups. After storage, the silages were subjected to microbial and chemical analyses.

Results from the present investigation denoted that, fresh sugarcane tops had a low fermentation coefficient (<35) and Brix value. The sugarcane tops also had high structural carbohydrate content, with NDF and ADF accounting for about 77.10 percent and 42.01 percent of DM, respectively. Results indicated that the DM loss, significantly increases with increased the day of ensiling. However, the pH values of the additives silage decreased during the first 25 days of ensiling then tended to increase. The pH was significantly affected by additives, ensiling days but not by their interaction. All additives reduced pH and dry matter (DM) loss. DM loss was maximum in C+X treated silage and lowest in C+X+LP.

Consequently, among all treatments, exogenous enzymes and LAB treated silages (C+X+LF and C+X+LP+LF) were the best combination based on Flieg point and pH at day 25 of ensiling. Additives reduced the ensiling period and improve silage quality. Therefore, the present study revealed that at day 25 of ensiling, SCT silage achieved the virtuous fermentation by combining with exogenous enzymes plus LAB.

Keywords: Sugarcane tops silage, molasses, exogenous fibrolytic enzyme, lactic acid bacterial inoculant

1. Introduction
India is facing an acute shortage of animal feed. According to a study, there is a deficit of 23.4% dry fodder and 11.24 % green fodder to sustain 536 million population of livestock which remains a challenge (Roy *et al*., 2019) [21]. The root causes are limited grazing ground and underdeveloped production of forage. Sugarcane is the chief crop in the world by quantity of production and is mostly found in most tropical countries. Global production of sugar cane amounted to 1.91 billion tons in 2018, with Brazil accounts for 39 % of the total production worldwide, India generates 20 % of the total output, and China, Thailand contributes about 6 % of total output (FAOSTAT, 2019) [9]. According to ISMA (2019) [10] that India is a sugarcane growing country approximately 282 lakh tons of sugar cane are produced per year and tops of sugarcane comprise 15 to 25 percent of the plant's aerial component. According to the National policy for management of crop residues (NPMCR, 2019) [18], India produces 500 million tons of crop residues out of which 92 Mt is burned. The key adverse effects of crop residue burning include greenhouse gas emissions and global warming. Sugarcane tops are inexpensive and surplus material. However, its proper utilization is important. Ensiling is an auspicious technology is appropriate for tops preservation. Sugarcane tops are challenging to ensile due to its less palatability, deficient in true protein, various minerals, WSC, and a lesser amount of epiphytic lactic acid bacteria (LAB) (Singh, 1995) [22].

Thus, exogenous LAB, enzymes, and fermentable substrate are also widely used to upsurge the feeding value of these low-quality roughages. The aim of using silage additives in silage is to certify that the growth of lactic bacteria predominates during the fermentation cycle, as well as minimize losses and increase the quality of the silage to avoid other fermentation (*Clostridial* fermentation) products.

Adding *Lactobacillus plantarum* and *Lactobacillus fermentum* as an inoculant during ensiling ensures rapid and robust fermentation resulting in lower pH values at earlier stages of ensiling, and better-quality forage preservation. Fibrolytic enzymes degrade the cell wall at a faster rate, and additional WSC pooled to provide LAB growth fermentation substrate (Ebrahimi *et al*., 2014) [8]. In this context, the present study was undertaken to identify combinations of additives that are most promising for improving the quality of SCT silage and fermentation characteristics within a short ensiling time.

2. Materials Methods

2.1 Forage harvesting for silage preparation

The study was conducted at the National Dairy Research Institute, Karnal, Haryana, India. NDRI, Karnal is situated at an altitude of 250 meters above mean sea level, latitude, and longitude position being $29^0$42**"** N and $79^0$54**"** E, respectively. Sugarcane tops were procured after cutting stem during the harvesting period. Whole sugarcane tops were chaffed into 2-4 cm particle length using an electrical chaff cutter. At the time of ensiling DM content was 27.80. Urea (0.5%)and molasses (1.5%) were added in all treatment groups. Before this, molasses was treated with dilute sulphuric acid @2% FM basis which hydrolyses large sucrose molecule to glucose and fructose for better utilization by lactobacillus. Thermal treatment was also given and autoclaved at 120°C for 20 minutes to prevent fungal infection.

2.2 Preparation of Silage additives

In biological additives treatment i.e. bacterial inoculants namely *Lactobacillus plantarum* ($2x10^6$ cfu/g) (NCDC-344) Homolactic acid fermenter and *Lactobacillus fermentum* ($1x10^6$cfu/g) (NCDC-412) a heterotactic acid fermenter bacterial inoculants ampules were subcultured in 10 ml of sterile De Man, Rogosa and Sharpe (MRS) broth (Himedia Laboratories Pvt. Ltd, Mumbai, India); broth pH was 6.5±0.2 at 25°C. Serially 2% of LAB inoculum was inoculated on MRS broth and incubated at 37°C for 24 h when growth reached 10^6-10^8 LAB cfu/ml, inoculants were added to the treatments with exogenous fibrolytic enzyme cellulase (OM Biosciences, Pvt.limited, Ahmedabad) with a dose of 6000 NCU/Kg and xylanase (1500 IU/Kg) based on FM. The activities of enzymes were 60,000 Novo cellulose units (NCU) per g and 10,000 IU per gram, respectively.

2.3 Schedule of Ensiling Experiment

The chopped sugarcane tops were mixed and divided into an equal portion for four treatments: C+X, C+X+LP, C+X+LF, and C+X+LP+LF. Plastic jar silos were prepared in plastic vacuum-sealed containers of 3-3.5 kg capacity (CELLO, Packing Co. Ltd., India) the materials were supplemented with the additives and packed. The additives were sprayed evenly into 3-3.5 kg of chopped material with two replicates of each treatment. A total of 32 jars (1 materials×4 ensiling days× 4 treatment ×2 repeat) were made and kept in the laboratory at ambient temperature (at 21±^{0}C), for different time interval. Two jars for each treatment were opened for analysing DM loss and pH after 15,25,35 and 45 days of ensiling respectively.

2.4 Estimation of silage characteristics

Organoleptic criteria are the most practical way of judging the silage quality. The silage sample was analysed for colour, texture, and smell. The texture was observed by pressing the silage between two fingers. Colour was observed visually. Mildly, pleasantly acidic, and natural yogurt smell was preferred (Breirem and Ulvesli, 1960) [3].

2.5 Proximate and cell wall constituents Analysis

DM contents were determined by oven drying at 65℃ for 72h and ground to pass a 1-mm screen. Dry matter loss was determined by ashing of fresh fodder and silage sample. (Dickerson *et al*., 1991 [4]; Ashbell and Weinberg, 1992) [1]

$$\text{DM loss (\%)} = [1- (\text{ash}_{\text{fresh}}/\text{ash}_{\text{silage}})] *100$$

Crude protein (CP) and ether extract (EE), were analysed according to standard procedures detailed by the association of official analytical chemists. The neutral detergent fibre (NDF) and ADF were analysed by the method of Van Soest *et al*. (1991) [23]

2.6 pH, Buffering capacity, Flieg point and water-soluble carbohydrates (WSC) analysis

The pH was measured by using a Eutech pH meter (Oakton Instruments, IL USA) and the buffering capacity of the sample was done as per the method of Playne and McDonald (1996) [20]. Water-soluble carbohydrates were determined by using the Yemm and Willis (1954) [26] method.

To assess the quality of the silage, Flieg points from the pH value and DM of silage were measured at the end of the fermentation period with the following equation (Moselhy *et al*. 2015) [16]

$$\text{Flieg points} = 220 + [(2*\text{DM}-15)] -40*\text{pH}$$

And suggested a score, very bad for < 20, bad with a score between 21 to 40; to be medium with a score between 41 to 60; to be good between 61 to 80, and to be very good when it had a score between 81 to 100.

2.7 Experimental design and statistical analysis

The Data was subjected to two -way analysis of variance (ANOVA) with the fixed effects of additives and ensiling period using the general linear model procedure of SPSS (20.0). Data related to LAB, yeast, and mould were transformed by log10. Pairwise comparisons of the mean values were tested by Duncan multiple range test (Duncan 1955) [7] and the Hypothesis testing was done at a 5% significance level.

3. Results

3.1 The chemical composition and microbial population of fresh sugarcane tops

The chemical composition and pH, buffering capacity, WSC, microbial counts of sugarcane tops have been presented in Table 1. Results from the present investigation denoted that, fresh sugarcane tops had a low fermentation coefficient (<35) and Brix value. However, the epiphytic LAB on sugarcane tops was too low (<$1*10^6$g/Kg FM) to dominate fermentation (MacDonald *et al*., 1991) [15]. The sugarcane tops also had high structural carbohydrate content, with NDF and ADF accounting for about 77.10 percent and 42.01 percent of DM, respectively. Consequently, the sugarcane tops present long-term storage difficulties through natural fermentation.

Table 1: Chemical and microbial compositions of silage material

Parameters	Mean(±Standard error)
DM (%)	27.80±0.93
Crude protein (g/100g DM)	6.30±0.48
EE (g/100g DM)	2.48±0.13
Organic matter (g/100g DM)	93.41±0.02
Total ash (g/100 g DM)	7.59±0.07
NDF (g/100g DM)	77.10±0.30
ADF (g/100g DM)	42.01±1.90
Hemicellulose	35.09±0.02
Brix value(°Brix)	6.21±0.04
Ph	6.69±0.02
WSC (g/100 g DM)	13.14±0.55
Fermentation coefficient	31.28±0.03
NDICP(g/100g DM)	1.62±0.05
ADICP(g/100g DM)	0.81±0.60
Lactic acid bacteria (log_{10}cfu/g)	5.23±0.05
Yeast and moulds (log_{10}cfu/g)	5.01±0.21

DM, dry matter; mEq, milli equivalent; cfu, colony forming units; NDF, Neutral detergent fiber; ADF, Acid detergent fiber; NDICP, Neutral detergent fiber insoluble protein; ADICP, Acid detergent insoluble protein; Fermentation coefficient =DM (%)+ 8WSC/BC calculated from Weissbach and Honig formula (1996) [24].

3.2 Physical Assessment and Chemical Composition of the SCT silage at different days of ensiling

In all the LAB plus enzymes and enzymes(C+X) treated silages, the colour of sugarcane tops silage was ranged from olive-green to light amber brown at 15, 25, 35, and 45 days of ensiling. For all LAB plus enzyme-treated silages, the smell of 15 days' silage was a mild fruity smell to a strong fruity smell. For the C+X silage, mild vinegar smell to strong vinegar smell perceived. The smell was heavy vinegar after 45 days of ensiling. For all the silage the frameworks of the sugarcane top silage were solid, clear, and non-sticky throughout the entire ensiling period in the overall silage. The DM, DM loss and pH, of sugarcane tops silages, are enumerated in Table 2. The DM and DM loss were significantly affected by additives, ensiling days, and their interaction (p<0.05). The DM was significantly reduced (p< 0.05) along the ensiling period accompanied by a continuous increase of DM loss. Enzymes plus *L. plantarum* treated silage showed relatively high (p<0.05) DM contents (26.26g/100g DM) at day 15 of ensiling, while enzymes(C+X) treated silage preserved less (p<0.05) DM contents at the end (day 45) as well as at day 15 of ensiling (25.50 g/100 g DM).

During the ensiling process DM loss constantly increases with increased days of ensiling. All additives, especially C+X+LP treatment, significantly decreased DM loss of sugarcane tops silages. The silage treated with enzymes plus *L. plantratum* had the lowest (11.34%) DM loss as compared to other treatments at day 45 of ensiling. The highest DM loss was observed in the case of enzymes(C+X) and enzymes plus L. *fermentum* (C+X+LF) treated silage. C+X and C+X+LF did not show any significant difference (p<0.05) but C+X+LF (13.39%) treatment show higher mean value, as compared to C+X (12.99%) at day 45 of ensiling.

The pH was significantly affected by additives, ensiling days, and but not by their interaction (p<0.05). Before the ensiling pH value of fresh sugarcane tops was 6.69, which was reduced in all treatment after ensiling. Among all the treatments silage pH ranged from 4.10 to 4.33 (Table 2). The pH values of the additives silage decreased during the first 25 days of ensiling then tended to increase. Meanwhile, C+X+LF and C+X+LP+LF silages always maintained a lower pH value below 4.22 during ensiling. In the present study, it found that LAB treated silages always maintained a lower pH value below 4.19 during the ensiling (up to day 45) as compared to C+X (4.23) treated silage. Among the treatments, minimum pH values were observed in combinations where *L. fermentum* inoculant was used with xylanase plus cellulase it was 4.14 at 45 days of ensiling. The drop in pH values mainly occurred in the first 15 or 25 days of ensiling. After that, there was no further huge alteration in pH reduction with prolonged ensiling days (day 25 to day 45 of ensiling).

A significant difference (p<0.05) was observed in the Flieg point values in all the days of ensiling (p<0.05). The Flieg point is a collective record, used to assess the silage quality. The calculated values of flieg point ranged from 82.80 to 92.00 (Figure 1). In the contemporaneous study, it found that the highest flieg point was at day 25 of ensiling after it significantly decreased with increased SCT silage ensiling day. C+X+LF (92.00) was found to have the highest mean value of Flieg point at day 25 of ensiling but there was no substantial difference in C+X+LF and C+X+LP+LF treatments (p<0.05). Lowest Flieg point (82.80) was reported after the first 15 days of ensiling in enzymes treated silage(C+X).

Table 2: Effect of additives and ensiling days on DM, DM loss and pH of SCT silage

Parameters	Day 15	Day 25	Day 35	Day 45	SEM	D	T	D×T
DM(g/100gDM								
C+X	25.50^{dA}	25.43^{cA}	25.00^{bA}	24.10^{aA}				
C+X+LP	26.26^{dD}	25.70^{cD}	25.35^{bD}	24.60^{aD}				
C+X+LF	25.80^{dB}	25.50^{cB}	25.03^{bB}	24.05^{aB}	0.65	*		*
C+X+LP+LF	26.16^{dC}	25.6^{cC}	25.15^{bC}	24.50^{aC}			*	
DM loss (%)								
C+X	7.34^{aC}	8.45^{bC}	9.94^{cC}	12.99^{dC}				
C+X+LP	5.54^{aA}	7.44^{bA}	8.67^{cA}	11.34^{dA}				
C+X+LF	7.14^{aC}	8.16^{bC}	9.87^{cC}	13.39^{dC}	0.41	*		*
C+X+LP+LF	5.89^{aB}	7.79^{bB}	9.29^{cB}	11.73^{dB}			*	
pH								
C+X	4.33^{cC}	4.19^{aC}	4.18^{abC}	4.23^{bC}				
C+X+LP	4.26^{cB}	4.17^{aB}	4.18^{abB}	4.19^{bB}				
C+X+LF	4.21^{cA}	4.10^{aA}	4.13^{abA}	4.14^{bA}	0.01	*		NS
C+X+LP+LF	4.22^{cA}	4.13^{aA}	4.14^{abA}	4.16^{bA}			*	

D, Ensiling days; T, Treatment; D×T, Interaction;.*p<0.05,significant; NS, not significant; SCT, sugarcane tops silage; SEM, standard error means; LP, Lactobacillus *plantarum*; LF, Lactobacillus *fermentum*; C, Cellulase; X, Xylanase

$^{a-d}$ values with different small letters show significant differences among ensiling days in the same treatment (p<0.05)

$^{A-D}$ values with different capital letters show significant differences among treatments in the same ensiling days (p<0.05)

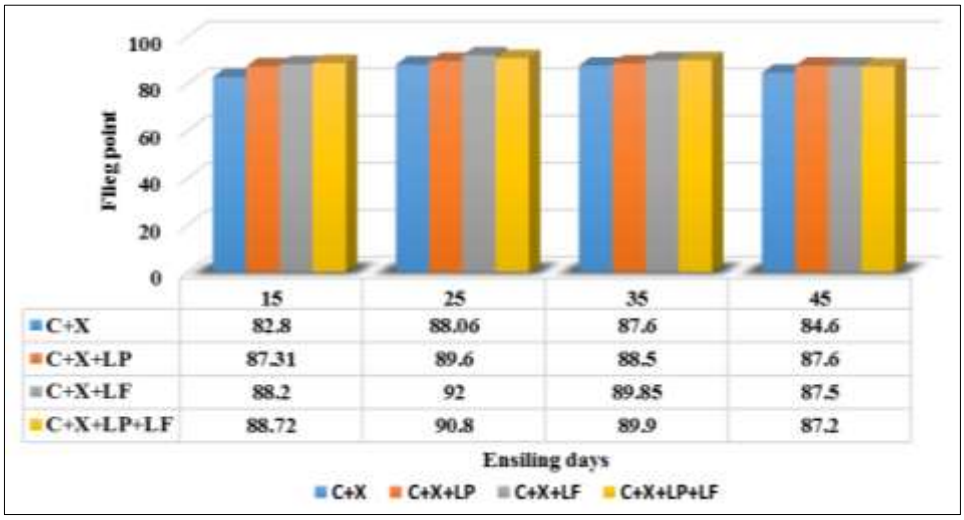

Fig 1: Flieg point of silage at different days of ensiling. LP, *Lactobacillus Plantarum*; LF, *Lactobacillus fermentum*; C, Cellulase; X, Xylanase; bars indicate standard error of the means

4. Discussion

4.1 Analysis of raw materials

The sugarcane tops used in this study contained low Brix value (6.20 ºBrix), low epiphytic LAB it was >10^6 cfu/ g FM (McDonald, 1991) [14], and FC (31.28), which practically are not suitable for natural fermentation. According to Lemus and white., (2014) [13] if the Brix value is 4-7 percent, then the forage comes into a bad to moderate type category. The higher Brix value indicates a greater concentration of sugar, protein, and mineral. Results from the present investigation, fresh sugarcane tops had a fermentation coefficient of less than 35. According to Weissbach and Honig (1996) [24] If FC < 35 = bad ensilable and considered as a low quality which is hard to ensile and should be subjected to reasonable additives application. Consequently, in the present, to improve the FC and Brix value of sugarcane tops molasses applied on tops. Molasses directly provide soluble sugar during the initial stage of fermentation and reduced the alkalization effect of urea which is a barrier for pH drop (Kebede *et al*., 2018 [11]). The extensive amount of energy incorporated in molasses provides extra fuel for lactic acid production. According to McDonald (1991) [15], for best ensiling fresh material should have Dry matter, 25 -30 g/100 g DM, Water-soluble carbohydrates 6-7g/100g DM, and probable number of LAB (>10^6 cfu/g FM).

4.2 Impact of exogenous enzymes and LAB on the DM loss, pH, and Flieg point of SCT silage

The greenish colour of the silage is sustained in all the silage and the smell and texture of the silage are preserved. All the silage formed in this experiment has been silage of good quality from appearance and odour. DM loss, pH, and Flieg point were significantly affected by additives, ensiling days, and their interaction (p<0.05). During the ensiling process DM loss constantly increases with increased days of ensiling could be attributed to nutrient breakdown and fermentation shifted from homofermentive to heterofermentive direction due to shortage of substrate (Nishino *et al*., 2004 [17]). The highest DM loss was observed in the case of enzymes(C+X) and enzymes plus *L. fermentum* (C+X+LF) treated silage might be due to L. *fermentum* produced gas, CO_2, and acetic acid. The homofermentative LAB was the most effective at minimizing carbon dioxide losses during the initial ensiling fermentation. DM loss could be supported by the higher unwanted microbes like yeast, mould, and enterobacteria, which degraded the lactic acid into CO_2 and water (Wilkinson and Davies, 2013) [25]. Before the ensiling pH value of fresh sugarcane, tops were 6.69, which was reduced in all treatments after ensiling. The pH in this experiment always remained just above 4 possibly due to the alkalinisation effect of urea later it converts into ammonia (Kebede *et al*., 2018 [11]). Correspondingly, Kung *et al*., (2018) [12] found the same results that silage treated with ammonia had higher pH (~4.0) than the control.

The decline in silage pH was significantly influenced by treatment. The fermentation time of silages was regularly and significantly shorter by inoculation. The drop in pH values mainly occurred in the first 15 or 25 days of ensiling after that, there was no further huge alteration in pH reduction with prolonged ensiling time (day 25 to day 45 of ensiling) might be associated with degradation of the acidic product (Lactic acid; pKa=3.9) into less-acidic metabolites (mainly acetic acid; pKa= 4.8) (Driehuis *et al*., 2001) [5]. Meanwhile, the synergistic effect of LAB inoculants and the exogenous fibrolytic enzyme could result in C+X+LF and C+X+LP+LF silages always maintaining a lower pH value below 4.22 throughout the ensiling. (Zhang *et al*., 2011) [27]. Among the treatments, minimum pH values were observed in combinations where *L. fermentum* inoculant was used with xylanase plus cellulase it was 4.14 at 45 days of ensiling could be supported by the fibre hydrolysis and significantly increased LA concentrations with a concomitant decrease in pH. Enzymes(C+X) treated silage had a higher pH than others due to low epiphytic bacteria. Low bacterial numbers extended the time so pH decreased by the very slow rate (Pitt *et al*., 1985) [19]. Consequently, both bacterial inoculants and enzyme additions are of interest for improving sugarcane tops silage. The Flieg point of all the silages was above 80 and had very good quality when analysed according to the reference values. It means the additives had improved the SCT silage quality significantly (p<0.05). In the present analysis it was found that the highest flieg point was at day 25 of ensiling after that it significantly declined with increased the day of ensiling because intensification of pH values and reduced the DM content of the silage. Lowest Flieg point was reported in enzymes treated silage(C+X) after the first 15 days of ensiling (82.80) due to higher pH value compared to LAB treated silages (p<0.05).

5. Conclusions

Preservation practices like silage making can be the most viable way of utilizing agriculture wastage in livestock feeding. Sugarcane tops would be well preserved by ensiling with additives, providing a continuous roughage source for ruminant livestock in sugarcane tops production area. The results confirmed that using exogenous enzymes with LAB additive somehow would be one of the ways to improve the fermentative quality of silage in the tropical area. Consequently, among all treatments, exogenous enzymes and LAB treated silages (C+X+LF and C+X+LP+LF) were the best combination based on Flieg point pH at day 25 of ensiling. Additives reduced the ensiling period and improve silage quality. Hence, the present study revealed that at day 25 of ensiling, SCT silage achieved the virtuous fermentation by combining with enzymes plus LAB.

6. Conflict of interest statement

None of the major conflicts of interest has reported by authors and all authors are agreed for the final statement.

7. Acknowledgments

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