Bioprocessing of Crop Residues using Fibrolytic Enzymes and *Flavobacterium bolustinum* for Enriching Animal Feed

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Abstract: *Flavobacterium bolustinum* and its extracellular cellulase were tested for animal feed pretreatment. The fibrolytic enzymes, cellulase and pectinase were applied to various crop residues such as wheat straw, rice straw, corn seeds and sorghum for enriching animal feed. Different parameters like temperature, incubation time and enzyme dose had been optimized for maximum reducing sugar and protein release. The highest amount of reducing sugar obtained was 29.83 mg g^{-1} dry substrate and soluble protein was 27.34 mg g^{-1} dry substrate on single cellulase enzyme treatment at 50°C for 6 h. An increase in amount of released reducing sugar (39.5 mg g^{-1} dry substrate) and protein (33.88 mg g^{-1} dry substrate) was observed when enzyme cocktail (cellulose and pectinase) was used. Solid state fermentation using *F. bolustinum* had also been performed for all crop residues. It released higher amount of reducing sugar (41.36 mg g^{-1}) and protein (47.21 mg g^{-1}) as compared to enzymatic treatment. Different substrates resulted in appreciable weight loss by enzymatic treatment (15-35%) as well as fermentation using *F. bolustinum* (40%). Liquefaction of lignocellulosic rich crop residues, for better utilization of feed has never been reported earlier.

Keywords: Animal feed, Cellulase, Pectinase, *Flavobacterium bolustinum*, *Pseudozyma* sp. SPJ.

INTRODUCTION

The developing countries like India have majority of their living based on agriculture. People in villages use animals as field workers as well as a source of food products. But they cannot afford the special nutritious diet prescribed for animals by the specialists. The farm animals are fed on crop residues like wheat and rice straw, sorghum stem, green grass etc. These feed substrates are mainly composed of cellulose, hemicellulose, lignin, pectin and protein [1]. The polysaccharides are hydrolyzed in simple sugars to produce energy. They have great potential to be used as feed as well as biomass for production of biofuels and chemicals. But, the major obstacle in using these materials as animal feed is the low susceptibility of lignocellulose to hydrolysis due to crystalline structure of cellulosic fibrils [2,3]. This structural complexity limits the digestibility of conventional feed substrates in the animals' gut. That is why, majority of the farm animal population suffer from nutritional deficiencies and lay back in the context of good health and quality products. For the betterment of livestock, there is a need to upgrade the feed for proper nutrition of the animals.

The use of microorganisms and their fibrolytic enzymes (cellulases and pectinases) for biological pretreatment of the crop residues holds promise to improve its utilization in animals, increasing the nutritive and metabolizable energy value of these conventional forages. The use of cellulase is most promising in the degradation of lignocellulosic biomass into simple forms of nutrients [4]. Beauchemin and Rode [5] reported that the addition of commercial enzyme preparations containing cellulase and xylanase to hay diet increases the live weight gain of cattle by 35%. Similarly, a 5–25% increase in milk yield has been reported in the case of dairy cows when fed with forage treated with commercial fibrolytic enzymes [6]. Supplementing diets with cellulases can improve feed utilization and animal performance by enhancing fiber degradation *in vitro* [7-9]. Significant increases in the digestion of dry matter, organic matter and nitrogen have been reported using fibrolytic enzymes in ruminants [10,11]. More feed consumption and milk production have been reported in lactating dairy cows when cellulase enzyme preparations are used for their feed treatment [12,13]. Increased milk production in small ruminants has also been reported [8,9,13].

The cellulase and pectinase used in the study are produced by novel strains. The pectinase produced by *Pseudozyma* sp. SPJ has been produced using cost effective substrate [14,15] and also have been successfully studied for degumming of flax fibers [16].

The objective of this research was to study the pretreatment of four commonly used forage substrates (rice straw, wheat straw, corn seeds and green sorghum stem) by using *F. bolustinum* and fibrolytic enzymes (cellulase and pectinase), which have never been studied earlier. Different parameters (temperature, incubation time and enzyme dose) were also optimized for this treatment process under laboratory conditions. This study demonstrated the potential of the bacterium for feed pretreatment.

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MATERIALS AND METHODS

Substrates

The agro-residues conventionally used in northern India as feed substrates were selected for the present study. They were named as **S1** wheat straw (*Triticum aestivum*); **S2** rice straw (*Oryza sativa*); **S3** corn seeds (*Zea mays*) and **S4** green sorghum stem (*Sorghum* sp.). They were washed, chopped and dried in the hot air oven overnight at 50ºC.

Microorganisms

An alkaline cellulase producing bacteria was isolated from soil sample collected from local sugar mill and identified as *F. bolustinum* by The Microbial Type Culture Collection & Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, and given accession no. 10203. The identification was done on the basis of phenotypic characterization. The culture was maintained on nutrient agar medium (NAM comprising: 0.5% (mass/volume) Peptone, 0.3% Yeast extract, 0.5% NaCl and 1.5% Agar in distilled water) slants and stored at 4℃ (sub-cultured every 3 months).

An alkaline pectinase producing yeast was isolated from fruit waste disposal site and identified as *Pseudozyma* sp. SPJ by MTCC, IMTECH, Chandigarh, and given accession No. 9842. The identification was done on the basis of phenotypic characterization and results were further confirmed by 26S rRNA sequencing method. The culture was maintained on yeast extract peptone dextrose (YEPD comprising: 0.3% Yeast extract, 1% Peptone, 1% Dextrose and 2% Agar in 1 L distilled water) medium slants and stored at 4℃ (sub-cultured every 3 months).

Enzymes

The crude cellulase was produced by *F. bolustinum* using pineapple peel as the solid substrate with moistening agent (g l^{-1}: $MgSO_4.7H_2O$, 1.0; KH_2PO_4, 4.0; $(NH_4)_2SO_4$, 10.0; NH_4Cl, 2.5) at pH 9.0. Similarly, pectinase was produced by *Pseudozyma* sp. SPJ on an inexpensive substrate citrus peel wetted with a moistening agent (g l^{-1}: $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 1.0; $(NH_4)_2SO_4$, 2.0) at pH 7.0 under solid state cultivation [14].

Pretreatment of Feed Substrates with Enzymes

Each substrate (1g) placed in 100 ml flasks containing 20 ml buffer (Glycine-NaOH pH 9.0) was treated with cellulase and cocktail of cellulase and pectinase in a shaking water bath at 100 rev/m. Different parameters like temperature (35-60ºC), incubation time (2-10 h) and enzyme dose (20-100 IU for cellulase and 20-50 IU for pectinase in combination with optimum dose of cellulase) were optimized for maximum release of sugar and protein content. The amount of total reducing sugars and proteins released in the reaction filtrate were determined after every 2 h of incubation by Miller's DNS method [17] and Lowry's method [18], respectively. After enzyme treatment, the feed substrates were thoroughly washed with distilled water and dried in oven overnight at 50 ℃ to determine the weight loss.

Pretreatment of Substrates with F. bolustinum under Solid State Fermentation

Dried substrates (5g) were placed in 250 ml flask containing 15 ml buffer (Glycine-NaOH pH 9) and inoculated with 20 h old culture of *F. bolustinum*. The flasks were incubated at 40ºC for 96 h and the extracts were taken out after every 24 h for analyzing the production of cellulase and amount of sugar and protein, released by the substrate.

RESULTS AND DISCUSSION

Treatment of Forages with Enzymes

Effect of Incubation Temperature

All substrates released highest amount of reducing sugar and protein at 50˚C, when treated with the enzymes (Table **1**). The substrates when treated with a cocktail of cellulase and pectinase resulted in 1.6 fold increase in reducing sugar and 1.3 fold increase in protein content released. This may be due to the fact that after digestion of cellulose by cellulase; pectin becomes easily accessible to pectinase for release of more reducing sugar and protein. Results showed that pretreatment of forage prior to feeding can make significant difference. While, from the reports of Howes *et al.* [19] it was not clear whether the major benefit of enzyme application occur in prefeeding treatment or after the feed enters the rumen.

Effect of Incubation Time

Table **2** showed accumulation of reducing sugar and protein at different incubation time by all the substrates. For all substrates, the amount of reducing sugar and protein was found highest after 6 h of incubation. There was 1.35 fold increase in reducing sugar and 1.1 fold increases in protein when cocktail of

Table 1: Effect of Temperature on the Enzymatic Treatment (Pectinase and Pectinase-Cellulase Combination) of Four Feed Substrates in Terms of Reducing Sugar And Soluble Protein Released

Substrates	Temperature											
	35 ºC		40 ºC		45 ºC		50 ºC		55 ºC		60 ºC	
	S	P	S	P	S	P	S	P	S	P	S	P
S1[a]	8.22	13.28	9.14	15.4	9.99	15.61	10.56	22.82	9.18	12.7	9.06	7.6
S2[a]	8.05	10.01	8.1	10.44	8.41	11.31	9.98	15.86	8.62	13.2	7.94	11.5
S3[a]	8.06	6.4	8.45	9.79	9.09	10.33	10.02	11.31	9.94	9.8	8.74	6.2
S4[a]	8.19	12.38	8.48	13.36	8.77	15.49	9.39	16.9	8.59	13.79	7.96	12.6
S1[b]	10.2	20.4	13.73	24.4	16.16	25.8	17.6	31.9	12.56	16.8	9.85	12.6
S2[b]	9.34	12.1	10.86	13.5	14.78	15.2	16.65	18.4	12.62	10.4	10.93	9.2
S3[b]	8.53	9.29	11.09	9.85	12.48	10.7	14.26	14.2	11.65	11.0	9.03	8.9
S4[b]	8.08	12.2	9.03	16.04	9.66	16.4	12.72	17.6	11.7	13.7	10.79	10.4

S1– Wheat straw, S2 – Rice straw, S3 – Corn seeds, S4 – Green Sorghum stem
a – treated with cellulose, b – treated with Cellulase + Pectinase.
S – Reducing sugar released (mg/g dry substrate), P – Soluble Protein released (mg/g dry substrate).

Table 2: Effect of Incubation Time on the Enzymatic Treatment (Pectinase and Pectinase-Cellulase Combination) of Four Feed Substrates in Terms of Reducing Sugar and Soluble Protein Released

Substrates	Time (h)									
	2		4		6		8		10	
	S	P	S	P	S	P	S	P	S	P
S1[a]	8.85	20	10.3	22.18	11.85	24.74	8.99	22.62	7.73	18.34
S2[a]	7.69	12.37	8.35	13.21	9.69	16.1	8.98	14.63	7.85	12.54
S3[a]	7.71	11.02	7.79	13.62	10.35	15.65	9.06	13.81	8.11	11.8
S4[a]	8.72	16.4	9.47	18.07	10.62	19.71	10.31	17.66	8.11	15.97
S1[b]	11.6	18.61	12.41	21.01	14.74	25.75	11.69	22.9	9.96	17.34
S2[b]	8.93	13.11	9.93	13.98	13.16	17.02	12.83	14.03	10.07	11.77
S3[b]	7.91	6.36	9.37	12.39	12.08	15.99	11.38	14.39	8.79	9.73
S4[b]	9.98	17.56	11.06	17.86	14.03	21.77	11.2	16.87	9.26	15.64

S1– Wheat straw, S2 – Rice straw, S3 – Corn seeds, S4 – Green Sorghum stem.
a – treated with cellulase.
b – treated with Cellulase + Pectinase.
S – Reducing sugar released (mg/g dry substrate).
P – Soluble Protein released (mg/g dry substrate).

cellulase and pectinase were used. Temperature and time are two crucial factors for effective enzyme activity. The release of reducing sugar and protein decreases after 6 h which may be due to degradation of sugar and protein at high temperature (50ºC) for longer duration.

Effect of Enzyme Dose

Table **3** showed accumulation of reducing sugar and protein at different cellulase dose by wheat straw, rice straw, corn seeds and sorghum. In every substrate except corn seeds 60 IU of cellulose dose resulted in maximum release of sugar and protein with weight loss of 20% in wheat straw, 12% in rice straw and 25% in sorghum. In case of corn seeds maximum sugar (11.44 mg g^{-1} dry substrate) and protein (15.8 mg g^{-1} dry substrate) released with an enzyme dose of 40 IU (cellulase) with 30% weight loss. Table **4** showed the amount of sugar and protein released with varying dose of pectinase (20-50 IU) is applied with optimum dose of cellulose.

Wheat straw released highest amount of reducing sugars (39.5 mg/g dry substrates) and soluble proteins (31.43 mg g^{-1} dry substrates) and weight loss of 27% when treated with a combination of cellulase and pectinase (30 IU) under same conditions. Rice straw

Table 3: Effect of Cellulase Dose on the Four Feed Substrates in Terms of Reducing Sugar and Soluble Protein Released

Substrates	Cellulase dose (IU/ml)									
	20		40		60		80		100	
	S	P	S	P	S	P	S	P	S	P
S1	18.39	20.95	18.81	24.22	29.83	27.34	19.84	24.44	17.72	22.80
S2	18.43	12.63	18.62	13.02	24.82	16.23	17.20	16.00	13.89	14.83
S3	7.99	12.48	11.44	15.80	10.80	10.30	8.33	11.83	8.14	10.98
S4	9.49	15.53	11.72	17.91	12.84	21.28	10.54	15.04	9.08	13.97

S1– Wheat straw, S2 – Rice straw, S3 – Corn seeds, S4 – Green Sorghum stem.
S – Reducing sugar released (mg/g dry substrate).
P – Soluble Protein released (mg/g dry substrate).

Table 4: Effect of Different Doses of Pectinase in Combination with Optimum Dose of Cellulase in Terms of Reducing Sugar and Soluble Protein Released

Substrates	Pectinase dose (IU/ml)							
	20		30		40		50	
	S	P	S	P	S	P	S	P
S1	28.42	29.75	39.5	31.43	39.06	29.59	37.6	28.86
S2	18.18	12.82	23.69	14.6	29.43	16.07	33.88	17.48
S3	9.59	12.89	13.93	14.25	14.92	16.64	15.75	17.25
S4	12.08	21.08	12.45	23.94	13.76	24.31	11.46	21.7

S1– Wheat straw, S2 – Rice straw, S3 – Corn seeds, S4 – Green Sorghum stem.
S – Reducing sugar released (mg/g dry substrate).
P – Soluble Protein released (mg/g dry substrate).

released highest amount of reducing sugars (33.88 mg g^{-1} dry substrates) and soluble proteins (17.48 mg g^{-1} dry substrates) and weight loss of 15%, when treated with a combination of cellulase and pectinase (50 IU) under same conditions. Corn seeds released highest amount of reducing sugars (15.75 mg g^{-1} dry substrates) and soluble proteins (17.25 mg g^{-1} dry substrates) and weight loss of 35%, when treated with a combination of cellulase and pectinase (50 IU) under same conditions. Sorghum released highest amount of reducing sugars (13.76 mg/g dry substrates) and soluble proteins (24.31 mg g^{-1} dry substrates) and weight loss of 29% when treated with a combination of cellulase and pectinase (40 IU) under same conditions. The variation in results obtained for different substrates can be attributed to the cell wall composition of the substrates which might not be same.

The significant amount of reducing sugars found in the reaction filtrates after enzymatic treatment proved the liquefaction of feed substrates. The amount of soluble proteins released in the reaction mixture was also appreciable. The proteins are present in the cell walls bound by the strong and fibrous pectins [20,21] thus are not available as nutrients. By the action of pectinase and cellulase these proteins became available and these soluble proteins can be easily estimated in the reaction mixture. In terms of weight loss by combination of both enzymes, the results were commendable but reducing sugar do not showed such improvement in case of rice straw, corn seeds and sorghum. This can be explained as incomplete degradation of cellulose resulted in release of large amount of proteins but less sugar. Insoluble cellulose was acted upon by cellobiohydrolases and endoglucanases to release cellobiose which was further hydrolyzed to glucose β–glucosidase [22]. But in some cases, this β-glucosidase was inhibited by reducing sugars present in the reaction mixture by competitive inhibition [23].

Treatment of Animal Feed by *F. bolustinum*

In Indian villages, rice straw, wheat straw and sorghum are mainly used as feed for cattle and corn

seeds for feed of poultry. These lignocellulosic materials are not accessible to the animals due to their compositional heterogeneity and structural complexity. Cellulose and pectin are hydrolyzed and depolymerized into fermentable sugar by the enzymes cellulase and pectinase. To extract the whole nutritive values of these substrates, pretreatment is necessary. Use of enzymes for the pretreatment of feed substrates has its advantages and disadvantages. We have to provide optimum conditions for the maximum activity of enzymes. Moreover, enzyme preparations are a bit costly. Thus, solid state fermentation using microbial culture on the feed substrates is comparatively simple and cost-effective. *F. bolustinum* has proved its potential as it is able to grow appreciably on all the substrates without using any other nutrient supplement. Figures **1** and **2** showed maximum production of reducing sugar and protein after 72 h of incubation producing maximum amount of enzyme activity 4445.7 IU g^{-1} for all substrates except rice straw. Maximum weight loss of all four substrates after 72 h of incubation is shown in Table **5**. Though, pretreatment

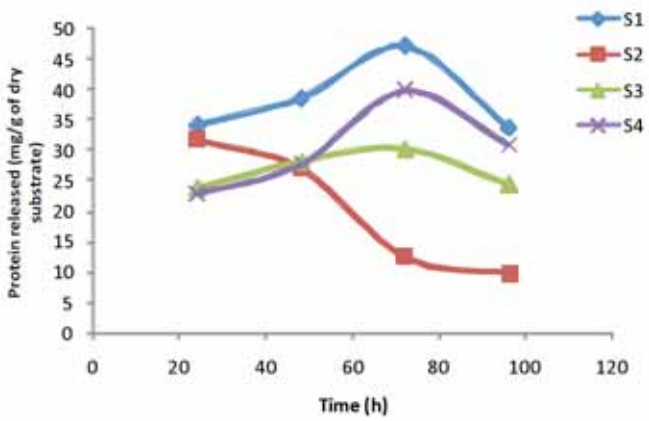

Figure 1: Total protein released during growth of *F. bolustinum* on different feed substrates (S1– Wheat straw, S2 – Rice straw, S3 – Corn seeds, S4 – Green Sorghum stem).

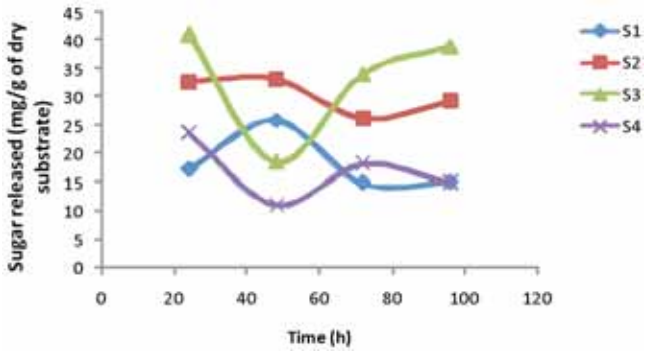

Figure 2: Total sugar released during growth of *F. bolustinum* on different feed substrates (S1– Wheat straw, S2 – Rice straw, S3 – Corn seeds, S4 – Green Sorghum stem).

of feed with culture was easier and economical but it involves repeated revival of the culture and cannot withstand the inhibitory microorganisms. In contrast to this, enzyme treatment was advantageous in terms of handling and storage as enzyme can be stored at low temperature easily and sprayed to feed when required. Kung *et al.* [24] reported that combing both microbial and enzymatic treatment was more significant as compared to the single one. By using *Pleurotus sajor-caju* culture maximum release of sugar from pulse husk and sugarcane bagasses was obtained after 6 week [25].

Table 5: Dry Weight Loss (%) of Feed Substrates during Enzymatic Pre-Treatment and Fermentation with *F. bolustinum*

Substrate	Treatment		
	Cellulase	Cellulase + Pectinase	*F. bolustinum*
Wheat straw	20	27	35
Rice straw	12	15	22
Corn seeds	30	35	40
Sorghum stem	25	29	36

CONCLUSION

Forage pretreatment either by enzyme or with microorganism, both are effective. Using cellulase from *F. bolustinum* and pectinase from *Pseudozyma* sp. SPJ and fermentation with *F. bolustinum* showed promising results with all the commonly used feeds (rice straw, wheat straw, corn seeds and sorghum) for their upgradation. This work is novel as no research on these conventional feed substrates has been reported earlier.

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