Fibrolytic enzyme activities in axenic cultures of *Piromyces sp.* from ruminants and non ruminants

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Abstract - In the present study, twenty-three strains of Piromyces sp. obtained from ruminants and non-ruminants were grown in Medium 10 in the presence of paddy straw as the sole source of fermentable carbon. The activity of extra-cellular fibrolytic enzymes such as avicelase, filter paper cellulase, β 1, 4 endoglucanase, β glucosidase and acetyl esterase were estimated in the various axenic cultures. The activity of the exoglucanase on avicel (avicelase activity) and filter paper was found to be significantly higher ($P<0.05$) in the Piromyces strains isolated from Nilghai, the Indian elephant and the camel. In the four strains of the Piromyces isolated from the domestic ruminants, the activity of the exoglucanase on avicel (avicelase activity) and filter paper was found to be the lowest as compared to all other strains of Piromyces isolated from the wild herbivores, camel/horse (< 4.0 µmoles/mg protein).

Index Terms- gut fungi, xylanase, endo glucanase, acetyl esterase

1. INTRODUCTION

Under Indian conditions the domestic ruminants are fed on low quality roughages like straws and stovers wherein the main component is lignocellulose. These roughages are poorly digested in the rumen leading to low production efficiency. The efficient breakdown of lignocellulose in the rumen usually requires a battery of glycosyl hydrolases (GH) including endoglucanases (endo 1,4-β-D glucan hydrolases EC 3.2.1.4), which cleave *β*-1,4-glycosidic bonds randomly within the cellulose chain; cellobiohydrolases or exoglucanases (exo 1,4-β-D glucan cellobiohydrolase EC 3.2.1.91), which cleave cellobiose from the ends of the cellulose chain; and *β*-glucosidases (β-D glucosidase EC 3.2.1. 21), which convert cellobiose and other low molecular mass cellodextrins into glucose. The cleaved cellobiose and short chain cellodextrins are then converted to glucose by β glucosidases to stop end product inhibition. All the three types of GH have isolated from different rumen cellulolytic microorganisms. The anaerobic fungi have the potential to contribute substantially to fibre degradation in the alimentary tract of host animals due to their ability to produce a wide range of enzymes with hydrolytic capacity. Molecular evidence shows that the hydrolytic enzymes of anaerobic fungi are either associated with fungal cellulosome attached to the fungal cell wall or secreted outside the mycelia as individual free enzymes. In order to improve the digestibility of low quality roughages several methodologies have been adopted. One of the approaches is obtaining a superior fiber degrading consortia of microbes and dosing them into the ruminants. In this study we have isolated *Piromyces* spp. of the gut fungi from faeces of various animals and evaluated their enzyme profile *in vitro* in axenic cultures.

2. OBJECTIVES

To study the activity of various carbohydrate active enzymes secreted by axenic cultures of *Piromyces* spp. isolated from various animals

3. METHODOLOGY

Organisms

Piromyces strains were isolated from the domestic and captive zoo ruminants and non ruminant animals include: BBP1(from buffalo), BTP1 (from cattle), LCP1 (from African elephant), ACP1and ACP2 (from Black buck), CDP1 (from camel), GCP1 (from giraffe), CHP1(from goat), HAP1 and HAP2 (from hippopotamus), ECP1 (from horse), EMP1and EMP2 (from Indian elephant), BGP1 and BGP2 (from Mithun), BTrP1 and BTrP2 (from Nilgai or blue bull), RUP1, (from rhinoceros), OAP1 (from sheep), CAP1, CAP2 and CAP3 (from spotted deer) and ZEP1 (from Zebra) were identified based on the monocentric growth pattern, large globose sporangia and monoflagellated zoospores. These characteristics of *Piromyces* strains corroborated with the strains described by

Medium

Cultures of the various fungi were grown in tubes containing modified medium 10 of Caldwell and Bryant (1985). The Hungate tubes containing 9mL of medium wherein cellobiose and glucose was replaced with paddy straw cut to 2-3mm length. The paddy straw was used as the sole energy source for the growth of the fungi. All incubations were carried out at 39ºC without shaking and all experiments were performed in triplicate.

Cellulolytic activity of anaerobic fungi

Paddy straw was used as growth substrates for the production of cellulolytic enzymes. The 100 mL syringes containing M10 medium (Caldwell and Bryant), were incubated

with the axenic cultures at 39°C for 24 h. Enzyme activities were measured at the end of 24 h. Five uninoculated syringes were used as negative controls. After incubation for 24 h the medium was centrifuged at 15000 g for 15 min, and the supernatant was tested for the presence and the activity of the various enzymes. The reaction mixture used for the estimation of carboxymethyl cellulase (CMCase) and xylanase contained:1.0 ml phosphate buffer (pH 6.8, 0.1 M), 0.5 ml substrate (1% carboxymethyl cellulose and 0.25% xylan from oat spelt for carboxylmethylcelluase and xylanase respectively) and 0.5 ml enzyme; incubated at 39°C for 1 h for CMCase and 30 min for xylanase. Glucose and xylose released during incubation were estimated (Miller. 1959). The activities of carboxy methyl cellulase and xylanase were expressed as µmole of glucose and xylose released per min under the assay conditions. The optical density (OD) was recorded at 575nm using a spectrophotometer (model UVS-2700, Labomed INC, USA) with glucose as standard.

The activity of microcrystalline cellulase (MCCase) was assayed with Avicel (Sigma Aldrich Chemical Co., Bangalore, India) and filter paper as substrate. The substrate Avicel (1.0g) was suspended in 100 mL of 0.1mol L^{-1} phosphate buffer, pH 6.8, and kept at 4°C for 48 h for swelling. The assay mixture comprised of 1 mL of enzyme, and 1mL of substrate that was incubated for 1 h at 39° C with continuous shaking. The reaction was terminated by the addition of 3.0 mL of dinitrosalicylic acid reagent and the residual avicel was removed by centrifugation. A measured quantity of the reaction mixture was boiled for 10 min in a water bath. After cooling to room temperature, 1 mL of sodium potassium tartarate was added and the optical density (OD) was recorded at 575 nm using a spectrophotometer (model UVS-2700, Labomed INC, USA) with glucose as standard.

The activity of β-Glucosidase was determined by measuring the absorbance at 410 nm of the p-nitrophenol released from the substrate p-nitrophenyl glucopyranoside (Himedia Ltd, Mumbai, India). The assay mixture contained 0.1 mL of enzyme, 0.9 mL of substrate (1 g L^{-1} p-nitro phenylglucopyranoside dissolved in 0.1 mol L^{-1} phosphate buffer, pH 6.8) and 1 mL of 0.1 mol L^{-1} phosphate buffer (pH 6.8). After incubation for 10 min at 39° C the reaction was terminated by cooling the mixture on ice and the OD was measured at 410 nm using a spectrophotometer (Shewale and Sadana, 1978).

The activities of CMCase and MCCase were expressed as µmol glucose released min−1 under the assay conditions. The activity of xylanase was expressed as µmol xylose released min−1 under the assay conditions. The activities of β-glucosidase were expressed as µmol p-nitrophenol released min−1 under the assay conditions. One international unit (1 IU) of enzyme activity was defined as the amount of enzyme that released 1 µmol reducing sugar (glucose or xylose) or 1 µmol p-nitrophenol min−1. The protein content of enzyme samples was estimated by the method of Lowry *et al* (1951).

Statistical Analysis

All the data were analyzed by one-way ANOVA using SPSS version 11 and the difference between the means were compared by Duncan's Multiple range tests. Pearson correlation coefficients among the various parameters were computed and tested for significance to detect bivariate correlation between the various variables.

4. RESULTS AND DISCUSSION

Piromyces spp. could be isolated from of domestic ruminants and identified based on their morphological and molecular characteristics. These genera of fungus could be isolated from the faeces of zoo ruminants, wild herbivores and pseudoruminants. The dominant fungi isolated from faeces of the zoo and wild herbivores from different locations in India were found to be affiliated to the genera P*iromyces.* Our results are in accordance with those of Liggenstoffer *et al*., 2010 who found that *Piromyces spp.* were the most abundant fungi. The P*iromyces* isolates from the various sources of animals were capable of utilizing paddy straw as a sole source of fermentable carbon. All the isolates produced an array of enzymes that allowed them to hydrolyze plant cell walls. The enzymatic activity paralleled the growth of the fungi and this is in corroboration with the results obtained by Paul *et al.,* 2006 and Tripathy *et al.,* 2007.

The data on the specific activities of avicelase, filter paper cellulase, β 1, 4 endoglucanase, xylanase, β glycosidase and acetyl esterase in axenic cultures of gut fungi belonging to the genus *Piromyces* is given in Table 1.

The activity of the microcrystalline cellulase on avicel (avicelase) and filter paper was found to be significantly higher ($P<0.05$) in the *Piromyces* strains isolated from nilghai (5.62 and 5.32 µmoles/mg protein respectively), the Indian elephant (5.49 and 5.23 µmoles/mg protein respectively) and the camel (5.14 and 5.06 µmoles/mg protein respectively). Among the *Piromyces* strains isolated from cattle, buffalo, sheep and goats, the activity of the micro crystalline cellulase of the cattle strain BTP1 was found to be significantly lower ($P<0.05$) as compared to those obtained from buffalo, sheep or goat. In 14 strains of *Piromyces* the activity of this enzyme fell in the range of 4.1-4.9 µmoles/mg protein The endo β 1,4 glucanase activity was found to be highest in BTrP1(nilghai; 45.60 µmoles/mg protein and the lowest in HAP1(hippopotamus; 24.96 µmoles/mg protein). The activity of xylanase was founf to be comparable in both the strains from nilghai (BTrP1 and BTrP2), bison (BGP1), African elephant (LCP1), Indian elephant (EMP1 and EMP2) and camel (CDP1). It can be observed that these strains are efficient cellulose and xylan degraders. This study demonstrated that

nilgai, camel, elephant, rhinoceros and goats harbor highly efficient strains of anaerobic fungi with comparable ability to degrade paddy straw. It is also evident from the present study that isolates belonging to the same genus differ in their ability to degrade paddy straw and produce fibrolytic enzymes when grown on paddy straw as the substrate. Hence there is greater scope for isolating superior strains of fungi by screening more isolates.

From the results of the present study, it can be inferred that fungal strains from various host animals showed significant *in vitro* fibrolytic activities clearly suggesting that introduction of superior fibrolytic strains into the rumen could possibly improve the nutrient utilization in ruminants. Fibre degrading strains of anaerobic fungi are present in wild and domestic herbivores, but there are considerable differences in the fibre degrading potential among the fungal isolates from domestic ruminants and wild herbivores. The fibrolytic potential of these strains could also be exploited through their administration to domestic ruminants for improved nutrient utilization. However, more *in vitro* as well as *in vivo* studies are needed to verify the possibility of the successful interspecies transfer of these fungal strains and their establishment in the rumen before these fungi can be exploited as a successful additive for domesticated ruminants.

5. CONCLUSION

In India, cattle and buffaloes are used for milk production, which is an important protein source for Indians. The common factor limiting ruminant production is the dependence on quality of the feed available. Ruminants depend mainly on crop residues, straws, stovers and native pastures which are high in lignocelluloses. Feeds with a high proportion of lignocellulose are poorly digestible making it difficult for the animals to derive the nutrients they require. The dairy industry in India would benefit from new technologies that would improve the utilization of low quality roughages by treating the low quality roughages with these fungi or by selectively increasing these fungi in the rumen by using specific nutrients.

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Table 1: Fibrolytic enzyme activity of pure cultures of anaerobic fungi from zoo ruminants in Medium 10 with paddy straw as fermentable carbon

S.No	Fungal strain	Activity of the enzyme in µmoles / mg protein					
		Avicelase	Filter Paper Cellulase	β- 1,4 endoglucanase	Xylanase	β-Glucosidase	Acetyl esterase
1	BTP1	3.22^{a} ± 0.06	2.97^{a} ± 0.04	26.80^{ab} ±0.39	32.08^{de} ±0.69	30.27^{f}± 0.67	14.59^{abc}± 0.34
2	BBP1	3.58^{b} ±0.19	3.23^{c} ±0.1	26.55^{ab}±0.74	32.89^{def}±0.56	30.26^{f}±0.82	14.83^{abc}±0.13
3	CHP1	3.55^{b}± 0.08	$3.33b^{cd}$±0.07	30.19^{bc}±0.24	27.61^{bc}±0.42	33.46^{gh}±0.1	20.77^{g}±0.36
4	OAP1	3.61^{b}±0.03	3.42^{bcd}±0.07	31.77^{cde}±0.42	26.51^{b}±0.64	32.25^{f}±0.98	19.53^{fg}±0.73
5	EMP1	5.49^{h}±0.13	5.23^{k}±0.1	42.24^{h}±0.75	35.67^{fg}±1.47	33.92^{gh}±0.46	18.49^{defg}±1.40
6	EMP2	4.66^{d}±0.15	4.41^{hi}±0.13	38.52^{fg}±0.80	33.90^{efg}±0.40	20.03^{a}±0.18	15.40^{abcd}±0.55
7	HAP1	4.45^{cd}±0.08	$3.97f^{g}$±0.22	24.98^{a}±0.35	32.16^{de}±0.84	23.81^{de}±0.34	17.49^{cdef}±0.32
8	HAP2	3.69^{b}±0.15	3.31^{bc}±0.80	31.19^{bcd}±0.21	30.72^{d}±0.24	22.51^{bcd}±0.35	16.78^{cdef}±0.13
9	LCP1	4.94^{ef}±0.53	4.82^{jk}±0.49	41.38^{gh}±0.34	33.96^{efg}±0.22	23.66^{de}±0.54	17.02^{cdef}±0.57
10	RUP1	4.33^{c}±0.07	4.14^{gh}±0.07	33.99^{cdef}±0.39	30.12^{cd}±2.39	22.46^{bcd}±0.14	16.48^{bcdef}±0.14
11	CDP1	5.14^{fg}±0.05	5.06^{k}±0.02	41.40^{gh}±0.51	34.36^{efg}±0.76	22.55^{bcd}±0.14	13.10^{a}±0.445
12	ECP1	4.62^{d}±0.02	3.48^{cd}±0.07	36.30^{ef}±0.15	22.83^{a}±0.69	18.93^{a}±0.19	18.23^{defg}±0.26
13	ZEP1	3.62^{b}±0.18	3.29^{bc}±0.02	29.19^{abc}±1.60	20.58^{a}±2.14	20.52^{abc}±2.23	13.44^{ab}±3.73
14	GCP1	4.21^{c}±0.05	3.61^{de}±0.15	35.99^{def}±0.39	26.54^{b}±0.21	20.36^{ab}±0.35	15.39^{abcd}±0.23
15	BTrP1	5.62^{g}±0.06	5.32^{i}±0.11	45.60^{i}±6.76	36.31^{g}±0.17	34.98^{h}±0.48	18.73^{efg}±0.13
16	BTrP2	$4.89d^{e}$±0.04	4.65^{ij}±0.07	42.91^{f}±.31	34.29^{efg}±0.71	24.33^{de}±0.35	14.92^{abc}±0.21
17	ACP1	4.44^{cd}±0.05	4.23^{gh}±0.05	35.83^{def}±0.38	35.18^{efg}±0.86	25.77^{e}±1.24	17.49^{cdef}±0.31
18	ACP2	4.27^{c}±0.02	4.07^{fg}±0.02	37.12^{fg}±0.70	35.13^{efg}±0.56	23.52^{d}±0.69	16.75^{cdef}±0.99
19	BGP1	4.35^{c}±0.03	4.14^{gh}±0.03	35.26^{def}±0.09	35.96^{fg}±0.34	22.98^{d}±0.62	16.05^{abcd}±0.50
20	BGP2	4.36^{c}±0.05	4.14^{gh}±0.05	36.06^{def}±0.27	34.71^{efg}±0.56	22.43^{bcd}±0.42	17.07^{cdef}±0.25
21	CAP1	4.30^{c}±0.06	3.81^{ef}±0.07	29.24^{abc}±0.39	23.15^{a}±0.76	22.65^{bcd}±0.37	14.63^{abc}±0.35
22	CAP2	4.25^{c}±0.05	3.14^{ab}±0.17	35.11^{def}±1.01	20.98^{a}±1.42	23.45^{d}±0.59	15.11^{abc}±0.30
23	CAP3	4.24^{c}±0.04	3.17^{ab}±0.09	37.07^{fg}±0.71	21.58^{a}±1.19	22.71^{cd}±0.56	16.26^{bcde}±0.46