aJava

Asian Journal of Animal and Veterinary Advances

Academic
Journals Inc.

www.academicjournals.com

Asian Journal of Animal and Veterinary Advances 9 (9): 587-592, 2014
ISSN 1683-9919 / DOI: 10.3923/ajava.2014.587.592

Isolation and Characterization of Superior Fibre Degrading Fungus from Rumen of Mithun (*Bos frontalis*)

[1]K.C. Das, [1]R. Sharma, [2]S.S. Paul, [3]K. Dhama, [1]N. Haque, [1]K.K. Baruah and [1]C. Rajkhowa

[1]National Research Centre on Mithun, Jharnapani, Medziphema, Nagaland, 797106, India
[2]Central Institute for Research on Buffaloes, Hissar, Haryana, 125001, India
[3]Division of Pathology, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, 243122, India

Corresponding Author: K.C. Das, Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar, Odisha, 751002, India

ABSTRACT

Six fibre degrading fungus were isolated from rumen of mithun (*Bos frontalis*) at National Research Centre on Mithun, Jharnapani, Medziphema, Nagaland using specific media for rumen fungus. These isolated fungal cultures showed higher fibre degrading enzymes like CM cellulase, Xylanase and Avicelase activity. Addition of fungal culture in the mixed rumen liquor of mithun increased *in vitro* gas production. The identification and characterization of these isolates was done by DNA isolation, Polymerase Chain Reaction (PCR) amplification, sequencing and plotting of phylogenic tree. The result showed that two isolates were homologus to *Orpinomyces* species and four isolates were homologus to *Neocallimastix* species. These isolates can have potential to be used as microbial feed additives in ruminants.

Key words: Fungus, rumen, mithun, characterization

INTRODUCTION

Mithun (*Bos frontalis*) is an important massive ruminant of the North-Eastern Hilly Region (NEHR) of India. This unique bovine species is believed to be domesticated more than 8000 years ago and is mainly confined in the north-eastern hilly states of India and is also found, though in lesser number, in Myanmar, China, Bangladesh and Bhutan. In India it is mainly available in the four North-Eastern hilly states of India i.e. Arunachal Pradesh, Nagaland, Mizoram and Manipur. In recent years the bacterial diversity of rumen microbes of Mithun (*Bos frontalis*) has been extensively studied by cloning and sequencing of 16S rRNA gene of bacteria (Das *et al*., 2014). Some of the bacteria/fungus have been isolated in growth media followed by DNA isolation, Polymerase Chain Reaction (PCR) amplification, sequencing and phylogenic characterization. In this experiment, six fungal isolates obtained from the rumen of mithun (*Bos frontalis*) of superior *in vitro* gas production and fibre degradation activity were identified and characterized by molecular method.

MATERIALS AND METHODS

Maintenance of animals: The experiment was carried out on three adult Mithun (Fig. 1) reared at the Mithun Farm, Medziphema, Nagaland, India. All the animals were maintained on paddy

Fig. 1: A Nagaland strain of Mithun (*Bos frontalis*) Bull

straw based diet for continuously long period. The diets consisted of paddy straw with limited quantity of concentrate mixture and tree leaves. Samples of rumen liquor were collected from three animals, filtered through muslin cloth and strained rumen liquor was used for further isolation study.

Isolation procedure: About 2-3 samples of rumen content were collected from rumen of each mithun and then mixed together to form a composite sample which was brought to the laboratory in strict anaerobic conditions for further processing. The rumen content was properly mixed with dilution medium and isolation was carried out by using the specific media for rumen fungus. Many isolates were obtained in this experiment and then screened for selection of best isolates. Strict anaerobic conditions were maintained during the isolation procedure. The growth media included Mineral Solution I (KH_2PO_4 3 g, $CaCl_2.2H_2O$ 0.8 g, $MgCl_2.7H_2O$ 0.6 g, NH_4SO_4 0.5 g) 15 mL, Mineral Solution II (K_2HPO_4 3 g L^{-1}) 15 mL, NaCl (3 g L^{-1}) 5 mL, NH_4Cl (4.5 g L^{-1}) 5 mL, yeast extract 0.5 g, Tryptone 0.2 g, Glucose 0.5 g, Resazurine (0.1%) 0.1 mL, Hemin (0.1%) 0.1 mL, Sodium carbonate 0.8 g, clear rumen fluid 10 mL, distilled water 50 mL and cysteine hydrochloride 30 mg. The pH of the solution is maintained to 6.9-7.0 by adding orthophosphate solution. The antibiotic solution (Ampicillin, Chloramphenicol, Streptomycin and Kanamycin) was used in appropriate dose in preventing bacterial contamination.

Enzyme estimation: For enzyme estimation, pure cultures of fungus were incubated for 72 h followed by addition of lysozyme and carbon tetrachloride. The culture was then incubated at 39°C for 3 h after which the reaction was terminated by keeping the sample at 4°C. The sample was sonicated, centrifuged and supernatant was used for different enzyme estimation.

Carboxymethyl cellulase: The enzyme activity was determined by measuring the amount of reducing sugar released from carboxymethyl cellulose. The reaction mixture contained 1.0 mL phosphate buffer (0.1 M pH 6.8), 0.5 mL of 1% carboxymethyl cellulose solution prepared in 0.1 M phosphate buffer (pH 6.8), 0.5 mL enzyme and incubated for 60 min at 39°C. The reaction was halted and reducing sugars were determined by the addition of 3.0 mL of dinitrosalicylic acid reagent (Miller, 1959). Glucose was used as standard for determination of reducing sugars.

Xylanase: Xylanase activity was estimated by measuring the amount of reducing sugars released from oat spelt xylan. The reaction mixture contained 1.0 mL phosphate buffer (0.1 M pH 6.8). One milliliter of 0.5% oat spelt xylan solution prepared in 0.1 M phosphate buffer (0.1 M pH 6.8), 0.5 mL enzyme and incubated for 30 min at 39°C. The reaction was halted by the addition of 3.0 mL of dinitrosalicylic acid reagent. Samples were centrifuged to pellet residual xylan and 2 mL of supernatant was removed and analysed for reducing sugars. The absorbance values were read against Xylose as the standard.

B-glucosidase: Activity was determined by measuring the absorbance of the p-nitrophenol released from the substrate p-nitrophenylglucopyranoside. The assay mixture contained 0.1 mL enzyme, 0.9 mL substrate (0.1% p-nitrophenylglucopyranoside dissolved in phosphate buffer 0.1 M, pH 6.8) and 1.0 mL phosphate buffer (0.1 M, pH 6.8). After incubation of 10 min at 39°C, OD was measured at 410 nm.

Avicelase: Microcrystalline cellulose was estimated by measuring the amount of reducing sugars released from Avicel (micro-crystalline cellulose). The substrate (1% Avicel) suspension in the buffer (phosphate buffer 0.1 M, pH 6.8) was kept standing at 10°C for 48 h before use. The assay mixture consisted of 1.0 mL substrate suspension and 1.0 mL enzyme and incubated for 60 min at 40°C with continuous gentle shaking. The reaction was halted by addition of 3.0 mL of dinitro-salicylic acid. The samples were centrifuged to pellet residual Avicel and 2.00 mL of supernatant was removed and analysed for reducing sugars.

***In vitro* gas production procedure:** The isolates were screened for *in vitro* gas production (Menke and Steingass, 1988). The amount of gas produced during the incubation of isolates with rumen liquor is closely related to degradability of isolates. Hundred milliliter calibrated glass syringes (haeberle Labortechnik, Germany) were used for this purpose and 24 h gas production was recorded in triplicate for each isolate. Rumen fluid was obtained from three adult mithun. The rumen liquor was collected into the thermo flask that had been prewarmed to a temperature of 39°C. Incubation procedure was done by using special type of calibrated transparent glass syringes with fitted silicon tube. Thirty milliliter inoculums containing strained rumen liquor, buffer and mineral solution under continuous flushing with CO_2 was dispensed using 50 mL plastic calibrated syringe after which 2 mL of pure isolates were pushed into the syringe. The syringe was tapped and pushed upward by the piston in order to completely eliminate air in the inoculums. The silicon tube in the syringe was then tightened by a metal clip so as to prevent escape of gas. Incubation was carried out at 39±1°C and the volume of gas production was measured after 24 h of incubation. The average of the volume of gas produced from the blanks was deducted from the volume of gas produce per sample against the incubation time.

DNA isolation and PCR amplification: Genomic DNA was extracted from six fungal isolates by using Himedia fungal DNA mini kit. Polymerase Chain Reaction (PCR) amplification of fungal Internal Transcribed Spacer (ITS) region was performed using universal fungal primer forward: 5'-GTTTCCGTAGGTGAACCTGC-3' and reverse: 5'-ATATGCTTAAGTTCAGCGGGT-3'. The composition of PCR was 2.5 μL 10x buffer, 0.5 μL dNTP, 0.5 μL *Taq*, 2.0 μL $MgCl_2$, 0.5 μL BSA, 14 μL NF H_2O, 1 μL F primer, 1 μL R primer, 3 μL DNA templates. The protocol of fungal samples were as follows: 5 min of initial denaturation at 94°C followed by denaturation at 94°C for 1 min,

annealing at 52°C for 40 sec, extension at 72°C for 2 min followed by a 20 min extension step at 72°C with the last cycle 29 times and finally 4°C. The PCR product was visualized on an agarose gel. The purified PCR product was sent for sequencing. The sequences were queried online in the NCBI BLAST database.

Identification and characterization of isolates: The DNA was amplified by universal and specific primers and amplification product of required band size was obtained. The bands were excised and purified PCR products were sent for sequencing. The sequences were queried online in the NCBI BLAST database. The sequences of the isolates were compared with those available in the database. The reference sequences were downloaded from the nucleotide sequence databases and were used in further analysis for comparison. The obtained sequences were aligned using clustal V method of megAlign software (DNASTAR) and then phylogenetic tree was plotted.

RESULTS AND DISCUSSION

The isolation was carried out using specific media of rumen fungus with antibiotics and strict anaerobic condition was maintained during the isolation procedure. After growth of fungus in solid media, the fungus was transferred to liquid media.

***In vitro* gas production:** On addition of these fungal isolates to the rumen fluid of Mithun (Fig. 2), there was increase in gas production. The gas production of isolates after 24 h incubation varied from 14-18.0 mL mL^{-1} of isolate, highest in MFD 5 and lowest in MFD 6. Increased gas production in this experiment indicated that, these isolates have potential to be used as microbial feed additives (Table 1). Sehgal *et al.* (2008) reported that the digestibility of dry matter, crude protein, neutral detergent fibre and acid detergent fibre were higher ($p<0.05$) in fungus (*Neocallimastix* sp.) fed buffaloes calves. Similarly, Lee *et al.* (2000) isolated *Orpinomyces* strain (KNGF-2) from a Korean native goat and it increased nutrient digestibility and nitrogen (N) retention in the rumen of sheep, resulting from an increase in numbers of bacteria and fungi.

Table 1: *In vitro* gas production of isolated fungus

	Twenty four hours gas production	
Isolate No.	Corrected by blank sample	mL per 1 mL isolate
MFD 1	28	14
MFD 2	34	17
MFD 3	30	15
MFD 4	32	16
MFD 5	36	18
MFD 6	27	14

Table 2: Enzyme activity of isolated fungus

	CM cellulase	Avicelase	Xylanase	β-glucosidase
Isolate No.	mIU mL^{-1}			
MFD 1	2.8406	49.3596	105.2186	0.6952
MFD 2	10.2903	47.7766	76.8035	0.6681
MFD 3	2.6221	35.0012	124.4676	0.8191
MFD 4	1.7479	25.4219	26.5946	0.7616
MFD 5	4.5764	32.5864	78.8974	0.5683
MFD 6	2.8756	31.9854	23.9871	0.8649

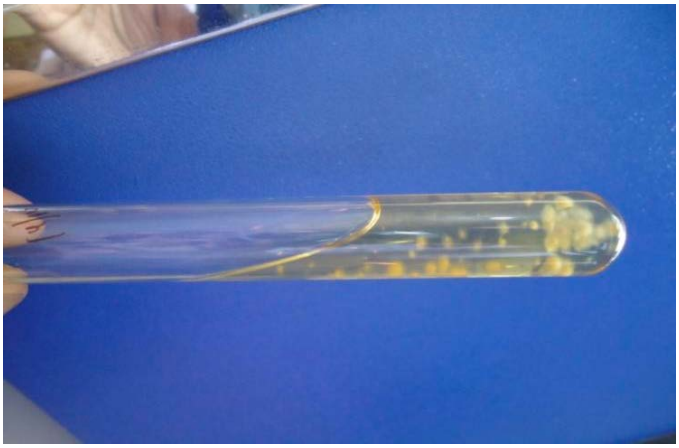

Fig. 2: A *Orpinomyces* strain of fungus isolated during the experiment

Table 3: Identification of fungal isolates

Isolate No.	Name	Accession No. of closest sequence in GenBank
MFD 1	*Neocallimastix* species	FJ501261
MFD 2	*Orpinomyces* species	AJ864475
MFD 3	*Orpinomyces* species	JN560952
MFD 4	*Neocallimastix* species	AY997064
MFD 5	*Neocallimastix* species	FJ501261
MFD 6	*Neocallimastix* species	AY429664

Enzyme production potential of isolates: The culture medium of pure culture of fungal isolates were subjected to enzymatic analysis like carboxymethyl cellulose (CMC), xylanase, β-glucosidase, avicelase and acetyl esterase. All the six isolates showed higher fibre degrading activity highest in isolate No. MFD 2 (Table 2). Similar to this experiment, it has been reported that, isolated *Neocallimastix* sp. from goat rumen showed a maximum activity of CMCase and *in vitro* digestibilities of DM and NDF (Thareja *et al*., 2006). The production of carboxymethyl cellulase, xylanase, acetyl esteraseand β-glucosidase was significantly ($p<0.05$) higher in the anaerobic fungus (FNG 5) isolated from Nilgai inoculated incubation medium (Paul *et al*., 2004).

Identification of fungal isolates: For identification of isolates, DNA was isolated from all pure isolates and DNA was found to be sufficient for PCR reaction as quantified by spectrophotometer. The DNA was amplified by universal fungus primers and amplification product of required band size was obtained. The bands were excised and purified PCR products were sent for sequencing. Two isolates were homologus to *Orpinomyces* species (GenBank accession number AJ864475, JN560952) and four isolates were homologus to *Neocallimastix* species (GenBank accession number FJ501261, AY997064, FJ501261 and AY429664) (Table 3). This indicated that these isolates may be used as microbial feed additive in mithun (*Bos frontalis*) for better growth and production. There is also ample scope in using the enzymes of these fungi for biofuel production.

Phylogenetic characterisation of fungal isolates: The phylogenic tree displayed two groups of fungus, i.e., *Neocallimastix* species and *Orpinomyces* species. MFD1, MFD4 and MFD5 were grouped in one cluster whereas MFD 2, MFD 3 and MFD 6 were grouped in a separate cluster

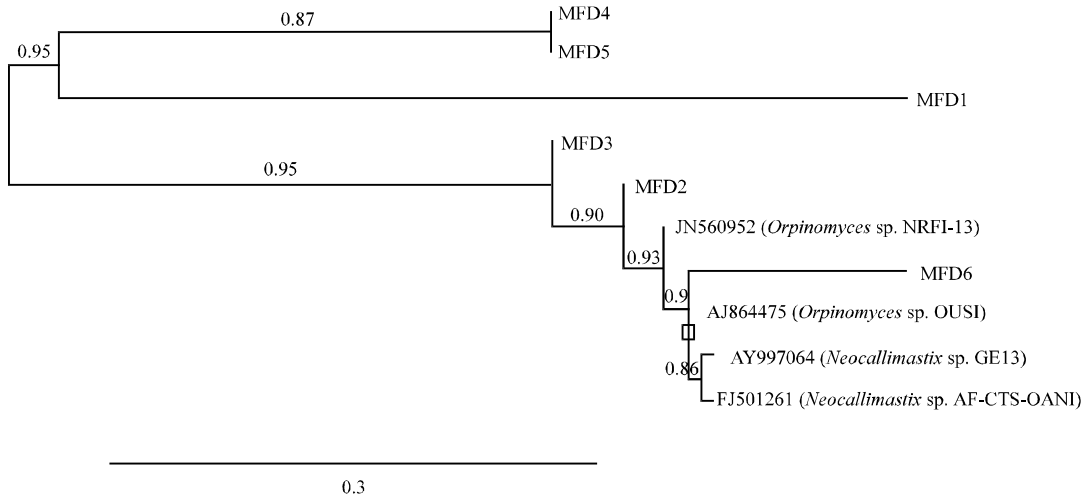

Fig. 3: Phylogenetic characterisation of fungal isolates

(Fig. 3). The MFD 6 was closely related to the fungus isolated from the rumen fluid of sheep (Accession No. FJ501261) and MFD 2 was closely related to the fungus isolated from the rumen fluid of cattle (Accession No. JN560952).

CONCLUSION

The study showed that two isolates of *Orpinomyces* species and four isolates of *Neocallimastix* species isolated from the rumen of Mithun (*Bos frontalis*) have high fibre degrading enzyme activity and these isolates have potential to be used as microbial feed additives.

ACKNOWLEDGMENT

We are thankful to DBT, New Delhi for financial help for this study.

REFERENCES

Das, K.C., S.S. Paul, L. Sahoo, K.K. Baruah, P.K. Subudhi, K. Ltu and C. Rajkhowa, 2014. Bacterial diversity in the rumen of mithun (*Bos frontalis*) fed on mixed tree leaves and rice straw based diet. Afr. J. Microbiol. Res., 8: 1426-1433.

Lee, S.S., J.K. Ha and K.J. Cheng, 2000. Influence of an anaerobic fungal culture administration on *in vivo* ruminal fermentation and nutrient digestion. Anim. Feed Sci. Technol., 88: 201-217.

Menke, K.H. and H. Steingass, 1988. Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. Anim. Res. Dev., 28: 7-55.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31: 426-428.

Paul, S.S., D.N. Kamra, V.R.B. Sastry, N.P. Sahu and N. Agarwal, 2004. Effect of anaerobic fungi on *In vitro* feed digestion by mixed rumen microflora of buffalo. Reprod. Nutr. Dev., 44: 313-319.

Sehgal, J.P., D. Jit, A.K. Puniya and K. Singh, 2008. Influence of anaerobic fungal administration on growth, rumen fermentation and nutrient digestion in female buffalo calves. J. Anim. Feed Sci., 17: 510-518.

Thareja, A., A.K. Puniya, G. Goel, R. Nagpal, P.K. Singh, J.P. Sehgal and K. Singh, 2006. *In vitro* degradation of wheat straw by anaerobic fungi from small ruminants. Arch. Anim. Nutr., 60: 412-417.