Degradation of Cellulose by Rumen Bacteria from Migratory Goats of North West Himalayan Region

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Abstract: **Total 54 bacterial isolates were obtained from the rumen of migratory goats of Himachal Pradesh. However, two isolates (CDB 2 and CDB 4) showed maximum enzymatic activity. The isolate CDB 2 showed maximum enzymatic activity at pH 7.0, temperature 40°C, incubation time 96h and yeast extract as the nitrogen source, whereas, isolate CDB 4 showed maximum activity at pH 7.0, temperature 50°C, incubation time 96h and peptone as the nitrogen source. The isolate CDB 2 was identified as *Megasphaera* species and CDB 4 as *Prevotella* species on the basis of Bergey's manual of systematic classification.**

Keywords: **Rumen, Goats, Migratory, Cellulose, Degradation**

I. INTRODUCTION

Cellulose is the most abundant biopolymer in nature and constitutes a large pool of carbon source for the microorganisms which are responsible for the decomposition of organic matter in soil (Shanker *et al.,* 2011). Cellulose degradation and its subsequent utilizations are important aspect for global carbon sources. The value of cellulose as a renewable energy source has made hydrolysis of this compound as a subject for intense research and industrial interest (Bhat *et al.,* 2000).

The rumen is a unique natural habitat for different microorganisms (bacteria, fungi, protozoans) that has evolved into a complex and efficient system. To degrade cellulose ruminants mostly depend on microorganism but very few species can degrade cellulose (Bryant, 1973). To isolate fibrolytic bacteria and identify their lignocelluloses degrading enzyme system from the rumen of variety of herbivores, considerable efforts have been made over past few decades (Flint *et al.,* 2008; Sharma *et al.,* 2017b).

Goats have an extremely varied diet including the tips of woody shrubs, trees, and lignocellulosic agricultural by-products. The rumen microbiota is highly diverse, including both prokaryotic and eukaryotic anaerobes that maintain a mutualistic relationship with its host (Doughal*et al.,* 2012; Sharma *et al.,* 2017a). Sufficient amount of hemicellulase, esterase and cellulase has been purified from these organisms (Lu *et al.,* 2006; Schwarz, 2001). On the other hand, the rumen flora is dynamic and known to adapt to changes in the host diet and age (Tajima *et al.,* 2001; Belanche*et al.*, 2012).

A complex system of cellulolytic enzyme, constituites endoglucanase (endo-1, 4-β-D-glucanase, EC 3.2.1.4), exo-glucanase(1, 4-β-D-glucan-cellobiohydrolase, EC 3.2.1.91) and β-glucosidase (β-D- glucoside glucanohydrolase, cellobiase, EC 3.2.1.21) which act synergistically to degrade cellulosic substrate (Singhania*et al.,* 2010; Chandra *et al.,* 2009)and convert into simple sugars, which are metabolized to volatile fatty acids by rumen microbes. These produced volatile fatty acids serve as energy sources for ruminants (Hegarty 1999; Guan *et al.,*2008; Hess *et al.,*2011).

In particular in North West Himalayan Region (NWHR), the goats and sheep are migrated from one region to another during winter and summer in order to overcome their forage need. During migration, they are exposed to diverse dietary resources many of which are already known to contain anti-nutritional plant metabolites. Herbivores, especially goats and sheep utilize anti-nutritional phytometabolites and polysaccharides by action of their gut microbes, which may be the major source of fibrolytic enzymes (Singh*et al.*, 2008; Sharma *et al.,* 2017a, 2017b). The attempt here is to isolate and characterize the cellulose degrading bacteria from the rumen of migratory goats of NWHR.

II. MATERIALS AND METHODS

Reagents and culture media

All the Biochemicals and reagents used were of high quality analytical grade such as microcrystalline cellulose, carboxymethylcellulose sodium salt Cobalt chloride hexahydrate ($CoCl_2.6H_2O$), Sodium Carbonate (Na_2CO_3) were purchased from Merck, Mumbai. All the necessary vitamins, Dinitrosalicyclic acid were buyed from central drug house, New Delhi (CDH) and volatile fatty acid used from Sigma (St. Louis, USA).

Sampling and isolation

The rumen contents of migratory goats were collected during upward migration in summers and downward migration in

winters when they were exposed to different anti-nutritional plants and fibrous forages present in the NWHR. The sample was collected in the thermos flask filled with CO_2 and were further processed in the laboratory. Rumen fluid glucose-cellobiose agar (RGCA) (Dehority and Scott 1965) with minor modifications (addition of cellulose in place of glucose) was used as isolation medium. The pH of the medium was adjusted to 6.7-7.0 withHCl and the medium was processed anaerobically in anaerobic workstation (IMSET, India).

Screening for cellulolytic activity

The microorganism isolated on RGCA medium were checked for their ability to degrade cellulose. The two qualitatively methods carried for the screening of isolates were Congo Red medium(Gupta *et al.*,2011) and Aesculin plus iron agar medium (Pointing, 1999). These methods are basic for screening isolates for their cellulolytic activity and will provide the primary set of putative cellulose degrading bacteria.

Enzyme assay

Dinitrosalicyclic acid (DNS) method was used for the determination of cellulose activity in the selected isolates (Ghosh, 1987). Cellulase activity was determined by estimating reducing sugars produced during enzymatic reaction by DNSwith slight modification by incubating 500 µl of CMC solution, 500 µl of crude enzyme and 500 µl of 0.05M citrate buffer pH 4.8 for 30 min at 50˚C before adding 3 ml of DNS solution. Finally, the absorbance was read at 540 nm in spectrophotometer (Miller, 1959). One unit of enzyme activity (IU) is defined as the amount of enzyme which liberates 1 micro mole of glucose/minute/ml under standard assay condition.

Optimization of enzyme activity

The isolates have been optimized for various growth factorssuch as pH, temperature, incubation period and nitrogen source by one factor at one-time analysis.

The standard production medium with pH varying from 4 to 10 using 1N NaOH and 1N HCl.The broth was inoculated with 1%inoculum and incubated at 38.5±0.5°C, for 48h. After, incubation sample was centrifuged at 15000 rpm for 10 min and supernatant was used as crude enzyme.Effect of temperature was studied on high cellulose production from the selected isolates by inoculation of standard growth medium and incubated at different temperature ranging from 30°C to 60°C for 48h to check the effect of varying temperature on growth. After, incubation the sample were collected and centrifuged at 15000 rpm for 10 min. The cell free supernatant was used as crude enzyme.

Incubation period plays an important role in microbial growth and reproduction so effect of incubating time was studied. The standard production medium was inoculated with the 1% inoculum and incubated for 24 h to 168 h to check the effect of incubation time on growth. After, incubation sample was centrifuged at 15000 rpm for 10 min and supernatant was used as crude enzyme.Effect ofdifferent nitrogen sources was observed by adding different nitrogen sources (organic and inorganic), in standard production medium *viz*. gelatin, peptone, urea yeast extract, ammonium nitrate, ammonium chloride and glycine. The growth medium with different nitrogen sources was inoculated with the selected isolates and incubated at 37°C for 48 h. After, incubation the growth measured by centrifugation of samples at 15000 rpm for 10 min and supernatant was used as crude enzyme.

Characterization of cellulose-degrading bacteria

The cellulose degrading bacteria were characterized onmorphological, physiological and biochemical profile with standard procedures according to theBergey'sManual of Systematic Bacteriology (Vos*et al.,* 2009).

III. RESULT AND DISCUSSION

Isolation and selection of cellulase producing isolates

The rumen of goats harbors a wealth of microorganisms and microbial enzymes for livestock and industrial applications due to their migratory nature (Singh *et al.,* 2008).FIbrolytic microorganisms are reported and studied from different herbivores (Sharma *et al.,* 2017a; 2017b; Haitjema*et al.,* 2017). A total of 54 isolates were obtained on Rumen Fluid Glucose-Cellobiose Agar (RGCA). Development of a zone of clearance around bacterial colonies was used as a parameter for screening and selecting the cellulolytic bacteria. The zones of clearance were observed around cellulase-producing bacterial colonies on culture media containing Carboxymethyl Cellulose (CMC) and Congo red. The appearance of clearance zone is due to the interaction of the dye with undegraded cellulose, whereas degraded cellulosic products do not interact with the dye. Of the 54 isolates, 20 bacterial isolates were found to be cellulose-degraders out of which 2 showed (CBD 2 and CBD 4) maximum zone of clearance (Fig. 1) The best possible source of cellulase system extraction is the rumen microbes as these organisms thrive on cellulosic biomass as their primary feed. (Dillon and Dillon 2004; Koeck*et al*.,2014, 2015; Mahanta*et al*.,2014).

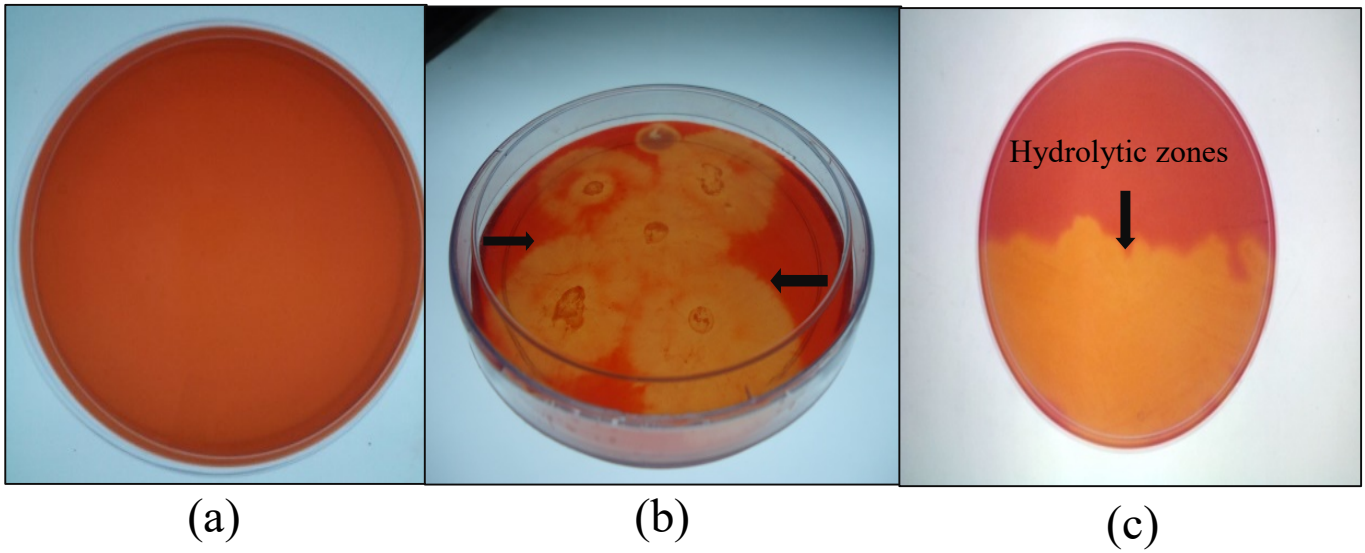


Fig. 1Bacterial isolates showing hydrolytic zone on cellulose Congo-red media

The enzyme production was also confirmed by growth of the isolates on agar medium containing aesculin as the sole carbon source. It has been reported that enzymatic degradation of aesculin, a coumarin glycoside yields glucose and coumarin that react with iron sulphate to produce a black color in the growth medium (Pointing 1999). Aesculin is used in a microbiology laboratory to aid in the identification of bacterial species. Further, 2 potential isolates were also checked on this medium. Both the isolates showed positive results as evident from blackening of the medium (Fig. 2).

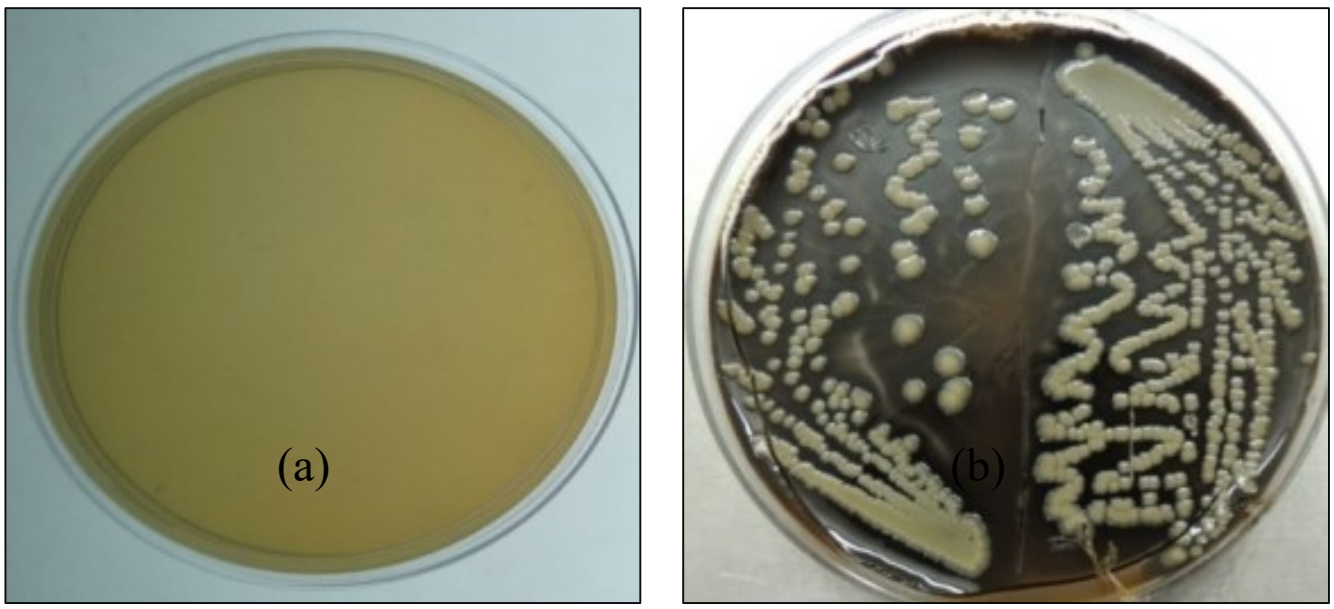


Fig. 2Bacterial isolates showing hydrolytic zone on Aesculin(plus iron) agar media.

Screening for cellulase activity

Cellulolysis is basically the biological process controlled and processed by the enzymes of cellulase system. The enzyme activity was studied with the help of Dinitrosalicyclic acid (DNS) method in which the crystal structure of the cellulose is broken down to its monomer unit i.e. glucose by action of the enzymes *viz*., endocellulase, exocellulase and β-glucosidase (Fig. 3).

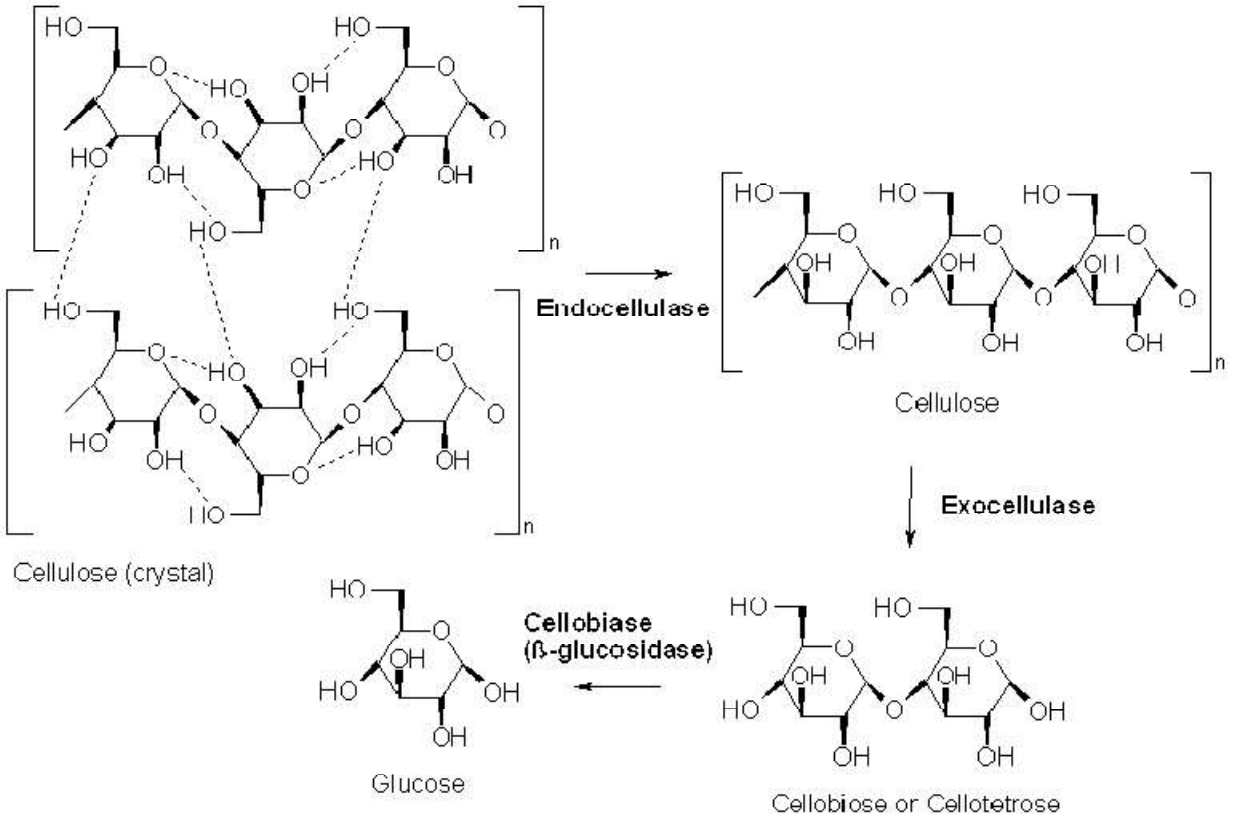


Fig. 3. Action sites of enzymes on cellulose structure

Hence, glucose was detected and measured spectrophotometrically with respect to standard curve plot (Fig. 4). The Endoglucanase activites of the isolates CDB 2 and CDB 4 were 0.846U/ml and 0.725U/ml, respectively which were found to be more than the Glucosidase activity of both these isolates as shown in Table 1.

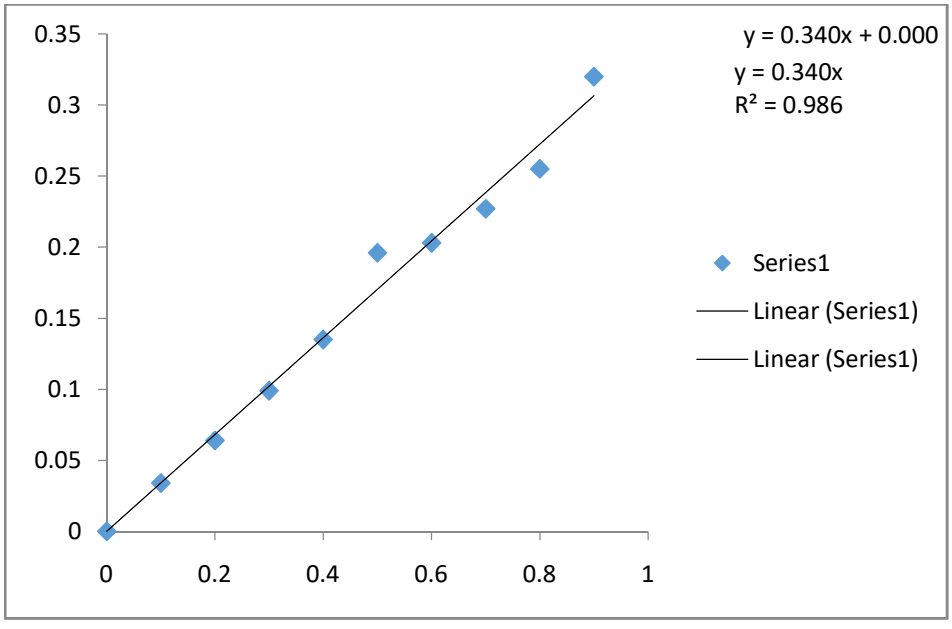


Fig. 4. The glucose concentration was taken from 0.2 mg to 1.0 mg.

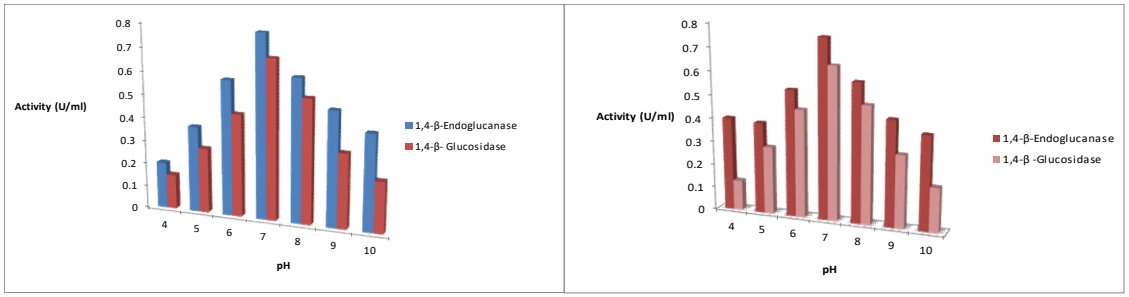
Table 1 Enzyme activities for both isolates

ENZYME ↓ / ISOLATES →	CDB 2	CDB 4
Endoglucanse activity	0.846 U/ml	0.725 U/ml
Glucosidase activity	0.652 U/ml	0.532 U/ml

Optimization for enzyme activity

Effect of different pH on cellulase activity

The pH of the medium is an important parameter for the enzyme activity of microorganisms as low and very high pH might kill rumen microbes and that may affect their cellulose degrading efficiency. pH plays an important role in inducing morphological changes in the microbes and also their enzyme production potential (Sethi and Gupta 2014). Both the isolates CDB 2 and CDB 4 were found to be very effective at pH 7.0. This is probably due to the fact that cellulose degradation is primarily carried out in rumen at neutral pH and activity starts decreasing below this pH as reported by Sung *et al.*, (2007). Hussain *et al.*, 2017 reveals that the minimum cellulase production was detected at pH 3 for all selected bacterial isolates while the optimum cellulase production was at pH 7(Fig. 5).



Effect of temperature on cellulase activity

Temperature plays a very vital role in the metabolism of microorganisms. A very high or low temperature might cease the activity of enzyme involved in degradation of cellulose. The isolate CDB 2 and CDB 4 were very active at 40°C and50°C, respectively. Irfan et al., (2012) studied Cellulomonassp.ASN2 cellulases showed optimum activity at 60˚C. As the temperature increased from 30˚C enzyme activity increased but activity started to decline as temperature increased above 60˚C and became completely denatured at 100˚C. Cellulases from some species of *Bacillus subtilis*subsp subtilis A-53, B. subtilis YJ1 and Bacillus strains RH68 and CH43 have optimum temperature of 50˚C (Yin et al .2010), 60˚C (Yin *et al.*,2010), 70˚C (RH68) and 65˚C (Mawadza*et al.*,2004), respectively. The optimum temperature of crude cellulase of the present isolate was higher than that of *M. circinelloidesi.e.*55˚C (Saha,2010). Ibrahim *et al.*, (2012), also reported high cellulase activity in thermophilic bacteria at 40°C and 55°C, respectively. Seo*et al.*, (2013) suggested that three enzymes were stable at a range of 20°C-50°C, and beyond 50°C, the enzyme activity had declined(Fig. 6).

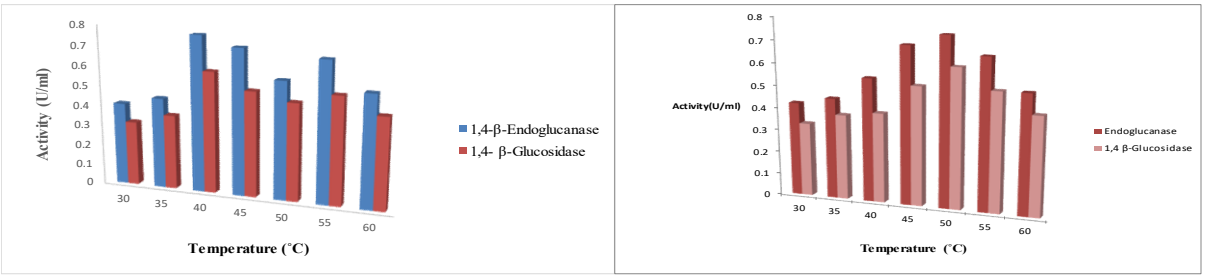


Fig. 6 (a) Activities of enzymes of CDB 2 isolate at different temperatures

(b) Activities of enzymes of CDB 4 isolate at different temperatures

Effect of incubation period on cellulase activity

Incubation time is necessary for optimal activity of enzymes. To complete the life cycle and reproduce, microorganism need to have proper incubation time. Both the isolates showed highest activity after of 96 hours i.e. 4 days(Fig. 7).The verdictsimitate to the earlier studies where maximum yield of endoglucanase was noticed on 4th day of incubation (Ibrahim *et al.*, 2012).

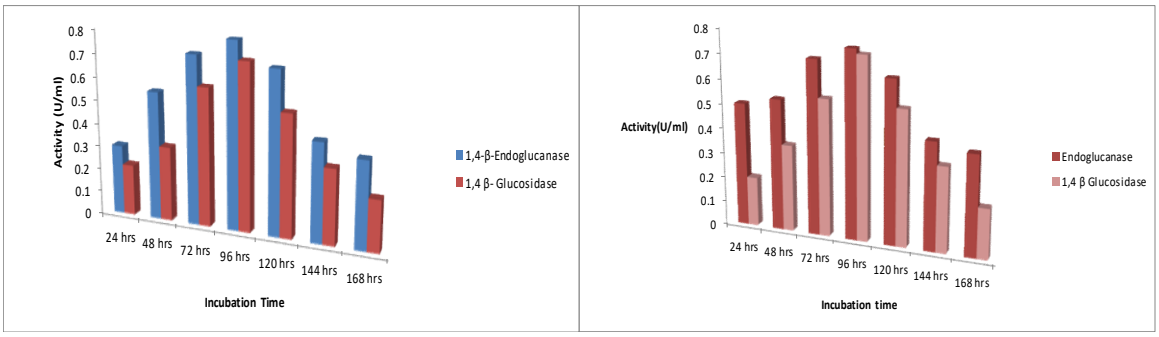


Fig. 7 (a) Activities of enzymes of CDB 2 isolates at different incubation time

(b) Activities of enzymes of CDB 4 isolates at different incubation time

Effect of different nitrogen sources on cellulase activity

Every microorganism has their ability to utilize different nitrogen sources according to their enzyme system. Isolate CDB 2 utilized yeast extract as nitrogen sources and isolate CDB 4 utilized peptone more efficiently(Fig. 8). Using inorganic nitrogen sources, the enzyme activity was found to be low. This could be due to improper metabolism of inorganic nitrogen by the microorganisms (Yang *et al.*, 2014). However, further studies should be carried out to evaluate utilization of unconventional nitrogen sources *vis-à-vis* synthesis of commercially important enzymes in rumen bacteria.

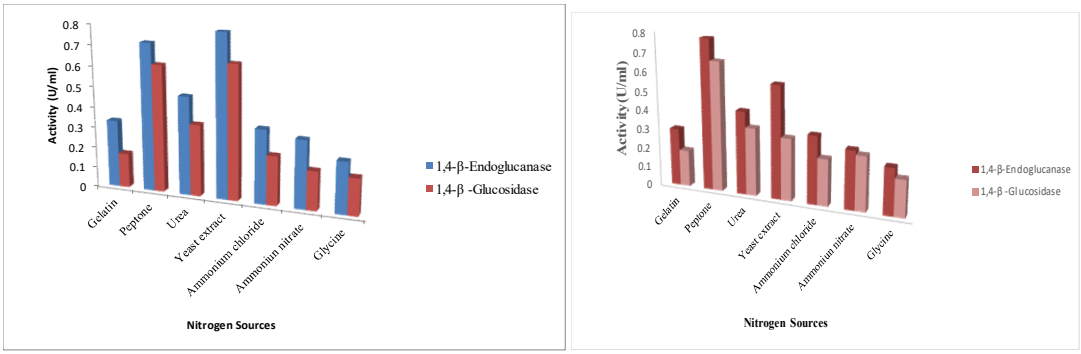


Fig. 8 (a)Activities of enzymes of CDB 2 isolates on different nitrogen sources

(b)Activities of enzymes of CDB 4 isolates on different nitrogen sources

Morphological identification

The isolates were tentatively identified on the basis of their morphological, cultural and biochemical characteristics following Bergey's Manual of Determinative Bacteriology (Holt et al. 1994). Their colonial, morphological and biochemical characteristics are tabulated in Table 2. The two bacterial isolates were found to be gram negative coccus (CDB2) and gram negative bacilli (CDB 4) when observed under compound microscope(Fig. 9). CDB1 and CDB5 were identified to be *Megasphearasp.* and *Prevotellasp.*, respectively.

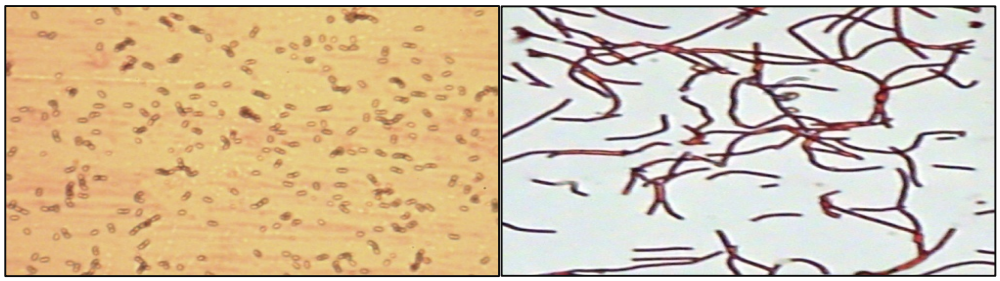


Fig. 9 Bacterial isolates observed under compound microscope

(a) CDB2 (gram negative coccus)

(b) CDB4 (gram negative bacilli)

Table 2 Colony, morphological and biochemical characteristics of most efficient isolates

Isolate no.	Colony characteristics on media	Substrate used	Hydrolytic Zone	Morphology
CDB 2	Larger white , 2mm diameter	Cellulose, hemicellulose and other carbohydrates	5.49	Gram positive, cocci
CDB 4	Small yellowish pin pointed 3mm diameter	Cellulose, pectin, other carbohydrates, proteins	5.35	Gram negative, bacilli

IV. CONCLUSION

In conclusion, the present study highlights the bacterial diversity of culturable cellulolytic bacteria in the bovine rumen and rapidly isolated a wide range of cellulolytic rumen bacteria. The study revealed that ruminants (goat) harbors various organisms that are active cellulose degraders, out of which *Megasphearaand Prevotella*species grow best on Rumen Fluid Glucose-Cellobiose Agar. Rumen should be a site for isolation of microorganisms that are capable of cellulose hydrolysis in order to reduce the coast of purchasing commercial enzymes as well as to provide direct fed microbial in the form of probiotics. These results provide a better understanding of the mechanisms of cellulose degradation in the rumen for improving feed efficiency, although further studies for and sequencing their genomes need to be performed.

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