ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Isolation and Characterization of Cellulase Producing Bacteria from Rumen Fluid

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Manuscript Info

Manuscript History:

Received: 14 March 2015
Final Accepted: 18 April 2015
Published Online: May 2015

Key words:

Cellulase, Cellulose, Rumen Fluid, Growth Kinetics.

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Abstract

The rumen is known as a paunch, forms the larger part of the reticulorumen. The rumen is the first chamber in the alimentary canal of ruminant animals. The rumen is huge amount of different microbes, including bacteria, fungi, protozoa, and others. These microbes assist the bovine to digest ingested food. Bovines can eat a ample range of feeds as they have many different kinds of microbes to help them in digestion. In this study efforts were made to isolate the bacteria occur in the rumen of buffalo. Twelve cellulase producing bacteria (CPB) were isolated from eight different buffalo rumen fluid by enriching rumen specific medium which contains CMC(Carboxy Methyl Cellulose) as a substrate for cellulose degradation. To indicate the cellulase activity of the organisms, diameter of clear zone around the colony on Congo-red agar media were measured. Isolate CPB 1 &CPB4 exhibited the maximum zone of clearance around the colony. Whereas for all isolates zone of clearance and Hydrolysis Capacity (HC) value were found between 5 to 33mm and 1.3 to 3.7 respectively. Celluase enzyme assay was carried out using Miller's. The extracellular cellulase activities ranged from 0.6 to 1.3µmol/min/ml. All the cultures were further tested for their capacity to degrade CMC at various pH, temperature and NaCl concentration. The maximum CPB was estimated to be CPB 2. For the isolates CPB 1-4Growth kinetics, partial protein purification and that protein estimation were done. Cellulase activity from that partially purified enzyme is ranged from 1.2 to 1.4µmol/min/ml.

INTRODUCTION

The rumen, also known as a paunch, it is larger part of the reticulorumen, which is the first chamber in the alimentary canal of ruminants animals. Digestion in the reticulorumen is a difficult process. Digestion occurs during fermentation by microbes in the reticulorumen. The reticulorumen is one of the few of organs present in the animals in which digestion of cellulose and other recalcitrant carbohydrates .It works as the primary site for microbial fermentation of ingested food. The rumen microbial ecosystem is one of the most complex, diverse, and well-studied microbiological environments. One particularly important bacterial genus that takes part in the degradation of cellulose is gram positive *Ruminococcus. Ruminococcus* bacteria break down the plant fibre into the monosaccharide

glucose, which can then be further broken down through glycolysis. It is predicted that 70% of microbes in the rumen have yet to be identified .The proportions of microbes present vary greatly depending upon the diet of the ruminant. Robert E. Hungate, the father of rumen microbiology, began investigating this system in the 1940 (Hungate R.E. *et al.,* 1966; Krause C.E. *et al.,* 1996).

Cellulose is a linear polysaccharide of glucose residues with β-1, 4-glycosidic linkages. Plentiful availability of cellulose makes it an attractive raw material for producing many industrially important commodity products. Sadly, much of the cellulosic waste is often disposed by biomass burning, which is not only restricted to the developing countries alone, but is considered a global phenomenon. With the help of cellulolytic system, cellulose can be converted to glucose which is a multiutility product, in a much cheaper and biologically favourable process. Source for Cellulase system extraction is best suitable from microbial system found in the gut of organisms thriving on cellulosic biomasses as their major food. Insects like termites (Isopteran), bookworm (Lepidoptera), Mammals like cow, goat, sheep and buffalo and so forth, are found to have syntrophic symbiotic microflora in their guts responsible for cellulosic food digestion (Dillon R. J. *et al.,* 2004; Saxena S. *et al.,* 1993). Many microorganisms have been reported with cellulosic activities including many bacterial and fungal strains both aerobic and anaerobic. *Chaetomium, Fusarium Myrothecium, Trichoderma. Penicillium, Aspergillus*, and so forth, is some of the reported fungal species responsible for cellulosic biomass hydrolysation. Cellulolytic bacterial species include *Trichonympha, Clostridium, Actinomycetes, Bacteroides succinogenes, Butyrivibrio fibrisolvens, Ruminococcus albus,* and *Methanobrevibacter* ruminantium (Milala M. A.*et al.,* 2005; Schwarz W. H. *et al.,* 2001).

Cellulase due to its massive applicability has been used in various industrial processes such as biofuels like bioethanol (Ekperigin M. M. *et al.,* 2007; Vaithanomsat P. *et al.,* 2009), triphasic biomethanation (Chakraborty N. *et al.,* 2000) agricultural and plant waste management (,Mswaka A. Y *et al.,* 1998) chiral separation and ligand binding studies (Patterson G. *et al.,* 1998). The present work concentrates on the isolation of Cellulase producing bacteria from mammals such as buffalo, and assessment of their cellulolytic activity and then co-culturing of cellulase producing bacteria and optimization of their physiological conditions. The objectives of this study are to isolate and characterize microorganisms from the rumen and to test the ability of these organisms to hydrolyze cellulose.

Materials and Methods

Sample Collection

The buffalo rumen fluid samples were collected by liquid endoscopic biopsy of buffalo rumen (Hendricks C. W. *et al.,* 1995).

Isolation and Screening of Cellulase Producing Bacteria

Medium was prepared according to composition described by Bassey (2002), for a rapid and sensitive screening test of CPB Congo-red was added.Spread plate technique was used for the isolation by spreading various dilutions (10^{-1} to 10^{-7}). Colonies showing discoloration of Congo-Red were taken as positive cellulose-degrading bacterial colonies (Lu W. J. *et al*. 2004). Cellulose-degrading potential of the positive isolates was also qualitatively estimated by calculating Hydrolysis Capacity (HC), that is, the ratio of diameter of clearing zone and colony (Hendricks C. W. *et al.,* 1995).

Bacterial Identification

The bacterial isolates were presumptively identified by means of morphological and biochemical characterizations. The results were compared with Bergey's Manual of Determinative Bacteria (Buchanan R. E. *et al.,* 1974 and Bhatt *et al.,* 2012).

Antibiotic sensitivity Testing

Antibiotic sensitivity tests were performed using disc diffusion method described by Kirby-Bauer (Bauer. K.*et al*., 1966).

Growth kinetic of isolates

Nutrient broth was inoculated with a loopful of test culture. Optical Density was measures for nine days at 620nm every 2,4,8,16,18,24,36,48 and 72 hourof incubation. Uninoculated medium used as a blank. The graph of O.D v/s time was plotted (Bhatt *et al.,* 2012)

Enzyme production

The selected CPB isolates were cultured and enzyme production was measured according to Miller's protocol.Supernatant was collected and stored as crude enzymepreparation at 4°C for further enzyme assays (Tailliez P. *et al*,. 1989).

Enzyme assay

Total cellulose activity was determined by measuring the amount of reducingsugar formed from CMC. Endoglucanase (β1-4 Endoglucanase-EC 3.2.1.4) activitywas assayed by measuring the amount of reducing sugar from amorphous cellulose (Miller G. L. *et al.,* 1959).

Process optimization for maximum Cellulase production

pH optimization

Different pH i.e. 2 4 5 7 and 9 containing Rumen specific CMCbroth was inoculated with the isolates and incubated at 37ºC temperature. At the end of incubation period, the cell-free culturefiltrate is used as enzyme source (Miller G. L *et al.,* 1959; Munro *et al.*1970).

Temperature optimization

Autoclaved Rumen Specific CMC broth was inoculated with the isolates and incubated at different temperatures i.e. -20ºC, 4ºC, 28ºC, 37ºCand 50ºC for 24 hours. At the end of incubation period, the cell-free culture filtrate obtained is used as enzyme source (Miller G. L.*et al.,* 1959; Munro *et al*.,1970).

NaCl concentration optimization

Autoclaved and different NaCl concentration i.e. 2% 4% 6% 8% containingrumen Specific CMC broth with pH 7 was inoculated with the isolates and was incubated at 37ºC and for 24 hours. At the end of incubationperiod, the cell-free culture filtrate is used as source of enzyme (Miller G. L. *et al*., 1959; Munro *et al.*, 1970).

Partial purification of protein

Partial purification of protein were done by using acetone precipitationit was further used to checking the enzyme activity byDNS method (Miller G. L.*et al*., 1959; Munro *et al*.,1970)**.**

Molecular Identification

Genomic DNA was isolated using Phenol Chloroform Iso-amyl (PCI) method. Genomic DNA isolation was performed in two main steps (Bhatt *et al.*2012**).** Genomic DNA was visualized by agarose gel electrophoresis (Vilber-Lormat). 16S rDNA region was amplified by Polymerase Chain Reaction using universal forward and reverse primers (Tajima *et al.* 2001). 2 µl of genomic DNA was mixed with 48 µl of PCR mixture, the final reaction mixture was taken 50 µl. For the amplification of 16S rDNA region of isolates, forward primer is complementary to the 5'end of 16S rDNA, and the reverse primer is complementary to the 3' end of 16S rDNA region (Bhatt *et al.* 2011). PCR reaction mixture was prepared in total of 25 µl which consisting of 1.0 µl of both forward and reverse primers (10 mM), 2.5 µl PCR Taq Buffer with MgCl2, 2.5 µl dNTP mix (25mM), 0.5 µl Taq DNA polymerase (3 U/µl) and 1 µl Template DNA (50ng). Thermo- cycler settings included a 3 min denaturation step at 94 oC followed by 32 cycles of 94 oC for 40 sec, 56 oC for 45 sec, and 72 oC for 75 sec. A final extension step was done at 72 oC for 10 min (Bhatt, V. D. *et al*., 2012).

Sequencing of the amplified products was done by Sanger sequencing at Eurofins, (Bangalore). Further the sequence generated was BLAST against the NCBI nr database and searched for the utmost homology for the sequence identification. The phylogenetic tree was constructed using MEGA 6.0. All the sequences are submitted into Genbank NCBI.

Result and Discussion

Isolation and screening of Cellulase Producing Bacteria

A total twelve bacterial isolates were found to be positive while screening using specific media i.e. rumen specific CMC congo-red agar, producing clear zone. Clear zone and hydrolytic capacity endorses cellulase producing capacity of isolates.

The result showed that clear zone and Hydrolysis Capacity (HC) value ranges between 5 to 33 mm and 1.3 to 3.7 respectively for all isolates (Table 1). It was observed that, CPB 1 has produced higher amount of cellulase followed by CPB 4, CPB 2, and CPB 3. The range of HC value obtained is similar to range reported by Lu *et al*. whereas Hatami *et al*. found the hydrolytic value between 1.38 to 2.33 and 0.15to 1.37 of cellulolytic aerobic bacterial isolates from farming and forest soil, respectively.

Growth kinetic study

Growth kinetics of isolates was studied using pattern of growth curve. It represents that isolates enter the growth phase after 2 hours and remains there up to 48 hours after which they stayed in stationary phase from 48 to 168 hours and finally move into decline phase (figure 1).

Gram staining of cellulase producing bacteria

The result of gram's staining was show morphology like size, shape, and arrangement of bacteria. CPB-1 to CPB-4 isolates were observed pink coloured rod shape so it can be concluded that they are Gram negative bacillus.

Biochemical characterization

Biochemical characterization of all the Cellulase producing isolates was performed and results obtained were used to identify cellulase producing bacterial referring Bergey's manual (Table 2)

Antibiotic sensitivity testing

The diameter of inhibition zones (Table 3) showed that all the isolates have sensitivity against various

antibiotics. The main aim behind checking the antimicrobial activity is for the treatment of the disease caused by those particular organisms. The average of diameters of the zone was considered. CPB-1, CPB-2, CPB-3, and CPB-4 were found to be more sensitive to Gentamycin, Streptomycin, Kanamycin and Tetracycline respectively and resistant to Lincomycin, Chloramphenicol, Rifampcin, and Penicillin G respectively.

Measurement of cellulase production

Experiments were carried out to determine the cellulase production ability of 4 bacterial isolates CPB 1, CPB 2, CPB 3, and CPB 4. Maximum cellulase production ability of the bacterial isolates was in the following order, CPB 2(1.363 µmol/min/ml), CPB 1(1.29 µmol/min/ml), CPB 3 (1.207µmol/min/ml), CPB 4(1.119µmol/min/ml) after 48 hours of incubation. Their cellulase production efficiency ranged between 1.2 to 1.4µmol/min/ml. Based upon this enzyme assay it can be concluded that the isolates do maximum enzyme production at 48 hours which is the stationary phage for all isolates. Glucose standard curve for cellulase enzyme activity is shown figure 2.

Optimization of cellulase enzyme activity at different pH, temperatures, NaCl concentrations

Cellulase enzyme activity at different pH

Cellulase enzyme activity was measured after 48 hours and it was observed that all had the ability to grow at different pH. Results showed that isolates can produce maximum cellulase at 4 then 7 pH. Lest enzyme production was observed at pH 2 whereas optimal pH was found to be 4. Generally, Rumen has acidic pH, so the optimum cellulase activity has been observed at pH 4 (Figure 3).

Cellulase enzyme activity at different temperatures

All the isolates have ability to grow at different temperatures. After incubating for 48 hours it was found that cellulose enzyme activity of isolates was maximum between 40˚C to 55˚C and optimum was 40˚C for all isolates, at 0˚C and 28˚C, least enzyme production was observed. Cellulase showing maximum activity in the range of 40ºC to 55ºC falls between the normal ranges of temperature in Rumen (Figure 4).

Cellulase enzyme activity at different NaCl concentrations

All the isolates have ability to grow at different NaCl concentration. After 48 hours, CPB 3 gave maximum cellulose activity with 4% NaCl concentration whereas CPB 1 gave maximum activity with 6% NaCl concentration. Least enzyme production by all isolates was observed at 2% and 8%NaCl concentration (Figure 5).

Partial protein estimation

Experiments were carried out to determine the protein production ability of 4 bacterial isolates CPB 1, CPB 2, CPB 3, and CPB 4. Maximum Cellulase protein production ability of the bacterial isolates in the production medium were in the following order, CPB 2(1.4µmol/min/ml) CPB 1(1.3 µmol/min/ml) CPB 3 (1.2µmol/min/ml) CPB 4(1.2 µmol/min/ml) after 48 hours of incubation. Their cellulase production efficiency ranged between 1.2 to 1.4µmol/min/ml (Figure 6).

Molecular Identification

Genomic DNA isolation was done by PCI method, obtain DNA was used for amplification of 16S rDNA region. After DNA isolation the 16S rDNA region was amplified by PCR, 5 µl of PCR products were visualized by agarose gel electrophoresis in UV light. The amplified product of 1500bp was observed in all samples (Figure 7).

DNA sequencing

The resultant sequence from the sangar sequencing was BLAST against the NCBI nr database (Table 4). Phylogenetic tree was generated using MEGA Version 6.5 of the resultant sequences from the rumen of buffalo and reported sequences present in NCBI database. All the 5 isolates are forming separate lines then the reported ones. Moreover all the isolates sequences are forming a group in the phylogenetic tree indicating that they are closely related (Figure 8).

Table1: Clear zone and hydrolytic capacity (HC) value of CPB on CMC Congo redagar media.

Sr. No	Total Size(mm)	Colony Size(mm)	Zone Size(mm)	Hydrolysis Capacity
CPB1	33	7	26	3.7
CPB 2	30	8	22	2.8
CPB 3	28	9	19	2.11
CPB 4	20	5	15	3.0
CPB 5	12	5	7	1.4
CPB 6	10	4	6	1.5
CPB 7	5	2	3	1.5
CPB 8	9	4	5	1.25
CPB 9	7	2	5	1.5

CPB 10	9	5	4	1.25
CPB 11	11	5	6	1.2
CPB 12	8	3	5	1.7

Table 2: Biochemical test

Biochemical Test	CPB-1	CPB-2	CPB-3	CPB-4
Citrate Utilization Test	+ve	-ve	+ve	+ve
Triple Iron Sugar Test	Redish pink	yelllow	yellow	red
Starch Hydrolysis Test	-ve	+ve	-ve	+ve
Casein Hydrolysis Test	+ve	-ve	+ve	+ve
Phenyl Alanine Test	-ve	-ve	-ve	-ve
Methyl red test	+ve	+ve	-ve	-ve
V-P test	-ve	- ve	+ ve	+ve
Indole test	-ve	- ve	+ ve	+ve
H2S production test	+ve	- ve	- ve	-ve
Urea hydrolysis test	+ve	- ve	+ ve	-ve
Gelatin hydrolysis test	-ve	- ve	- ve	+ve
Dehydrogenase test	-ve	+ve	- ve	-ve
Catalase test	+ve	+ve	+ ve	+ve
Oxidase test	+ve	+ve	+ve	+ve

+ve indicates positive-ve indicates negative

Table 3: Antibiogram

Antibiotics	Diameter of inhibition zone (mm)			
	CPB-1	CPB-2	CPB-3	CPB-4
Amoxicillin AM^{10}	-	-	-	30
Trimethoprim TR^{5}	25	18	15	28
Amikacin AK^{10}	20	15	20	25
Streptomycin S^{10}	22	22	20	29
Tetracycline TE^{10}	15	7	5	35
Lincomycin L^{10}	-	-	-	10
Penicillin G P^{10}	-	-	-	-
Rifampcin R^{5}	18	6	-	6
Kanamycin K^{5}	12	8	25	33
Oxytetracyclin O^{30}	22	18	11	22
Oxacillin OX^{10}	-	-	-	4
Chloramphenicol C^{10}	-	-	12	13
Erythromycin E^{15}	25	30	-	16
Gentamycin Gen^{10}	28	18	18	32

Table 4: Identification of organism

Sr No.	Isolate Code	Name of Organism
1	**CPB1**	*Pseudomonas stutzeri*
2	**CPB2**	*Sphingobacteriumalimentarium*
3	**CPB3**	*Serratiamarcescens*
6	**CPB4**	*Staphylococcus saprophyticus*

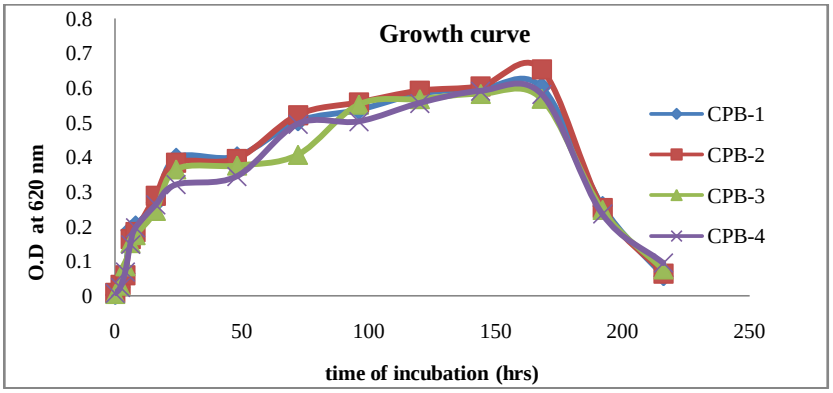

Figure 1: Study of growth kinetics

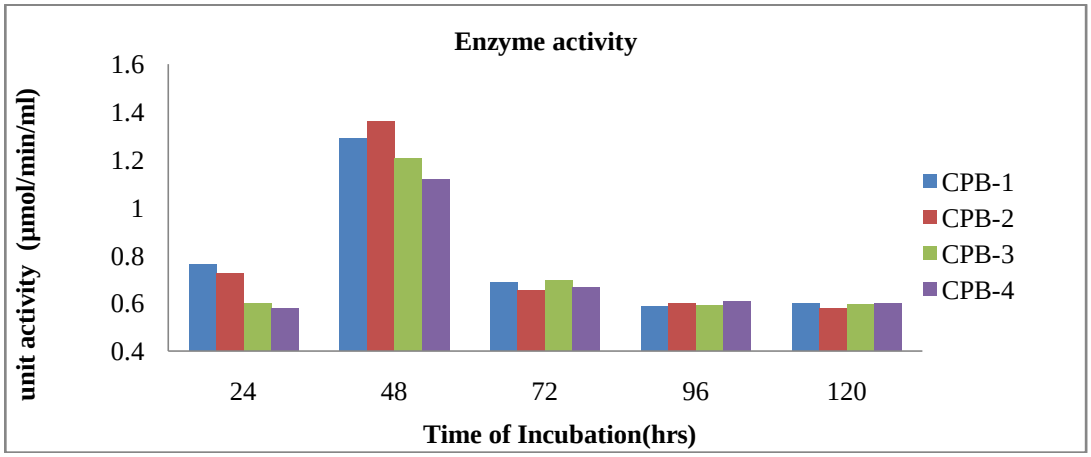

Figure 2: Cellulase production ability of the four isolates isolated from buffalo rumen

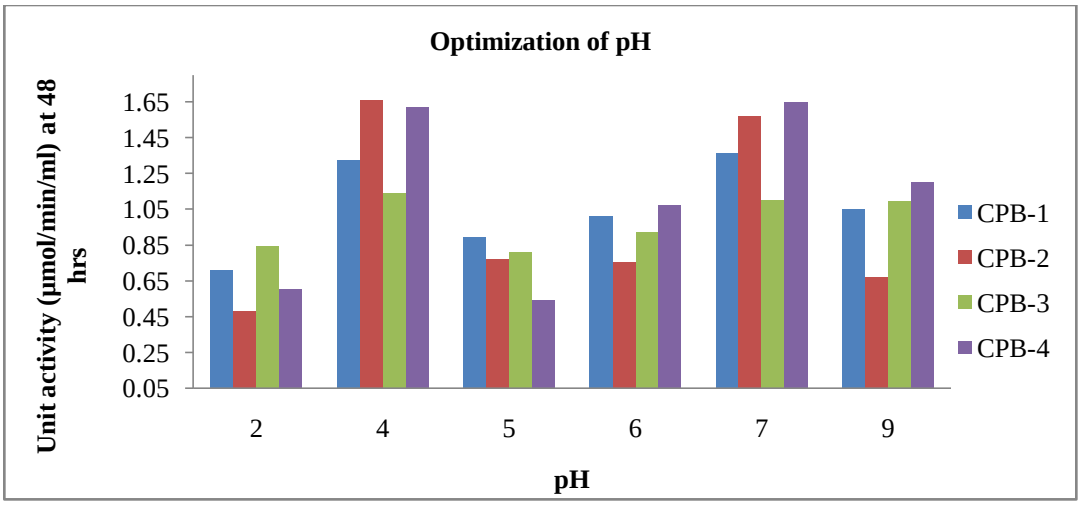

Figure 3: Cellulase enzyme activity at various pH.

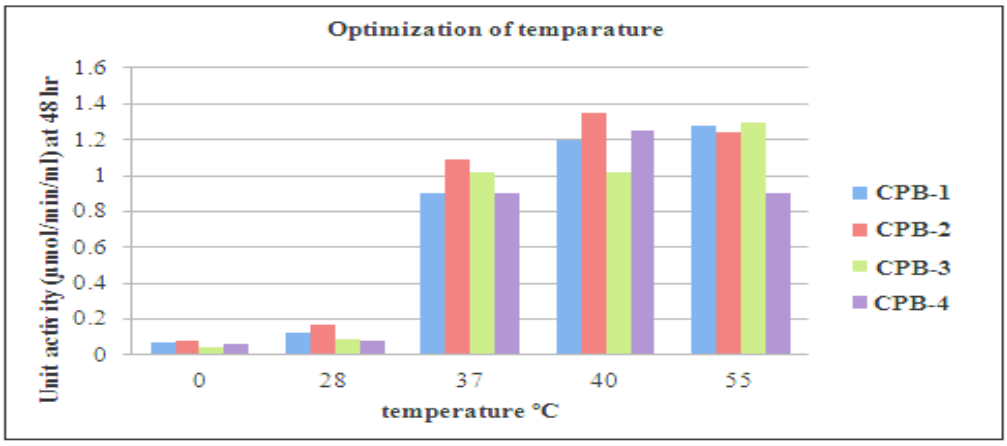

Figure 4: Cellulase enzyme activity at various Temperatures

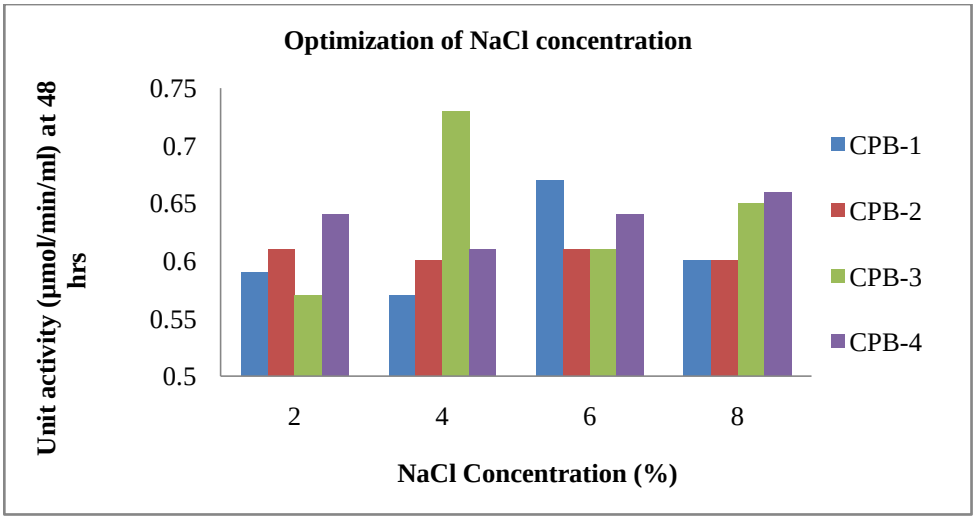

Figure 5: Cellulase enzyme activity at various NaCl concentrations

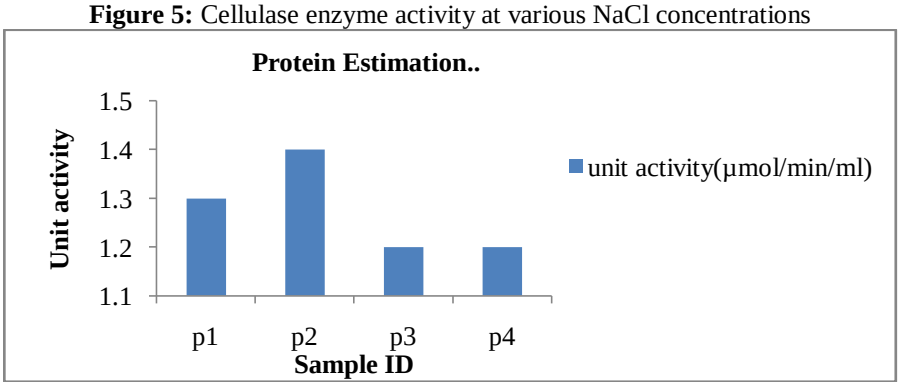

Figure 6: Partially purified Cellulase enzyme activity

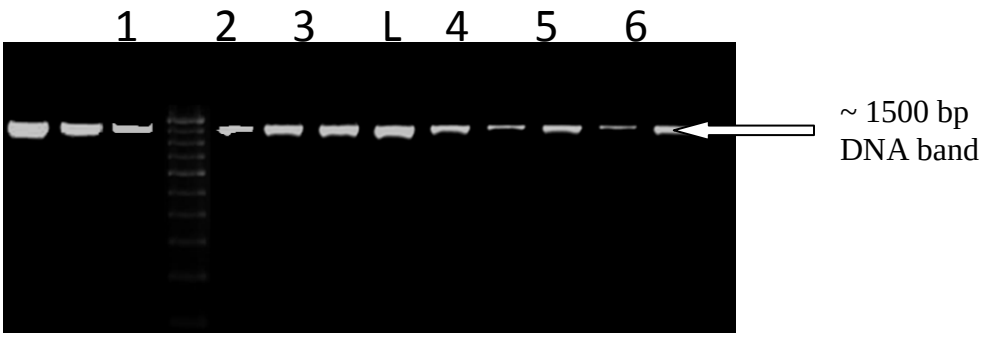

Figure 7: Visualization of PCR amplified products by agarose gel electrophoresis

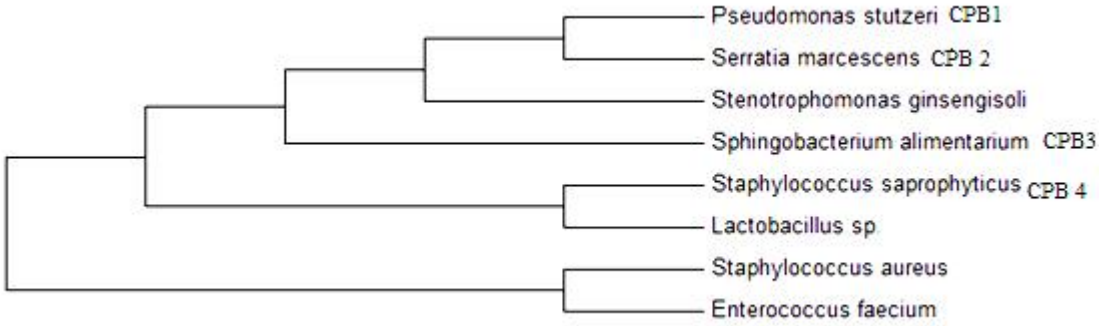

Figure 8: Phylogenetic tree

Conclusion

Bovines have four stomach compartments to digest their food. Among them, rumen is the largest compartment, and it can hold as much as 50 gallons of food and other ingested substances. The most important thing about rumen is that, it contains huge amount of different microbes, including bacteria, fungi, protozoa, and others. These microbes help the bovine to breakdown ingested food, while the bovine provides them with shelter and nutrients. Bovines digest plant fibres and starches and produce useful fatty acids. Bovines can eat a wide range of food because they have many different kinds of microbes to help them in digestion. In the present study efforts were made to isolate the bacteria present in the rumen of buffalo. Total 12 bacteria were isolated out of which four were optimums cellulolytic. The morphological and physiological characterization of the isolates was done. The endoglucanase activity of all the isolates was studied and it was found that CPB2 gives more activity followed by CPB1, CPB3 and then CPB4. It was found that after 48 hours of incubation in rumen specific CMC medium at 37°C temperature with pH 4 isolate CPB2 gave maximum activity followed by CPB1, CPB3 and then CPB4. Upon 16s RNA gene amplification the isolates were identified asCPB2- *Pseudomonas stutzeri*, CPB3- *Sphingobacteriumalimentarium*and CPB4- *Serratiamarcescens*. Also efforts were made to extract crude enzyme but it still required to standardization.

Acknowledgement

We are thankful to CVM and ARIBAS for giving wonderful opportunity to carry out the work and also AAU (Anand Agriculture University) for provide Rumen Fluid samples.

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