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Isolation and Characterization of Cellulose Degrading Bacteria from Saliva of 'Jaffrabadi' Breed of Buffalo

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Abstract

Microorganisms with cellulolytic activity are considered as potential candidates in the processing of cellulosic biomass for biotechnological applications. This study was conducted to isolate and characterize cellulose-degrading bacteria collected from the saliva of 'Jaffrabadi' breed of buffalo by morphological and biochemical analysis. The cellulolytic bacteria were isolated and purified on CMC (Carboxy methyl cellulose) agar. Bacterial isolate produced a clear transparent zone when stained with Congo red, confirming cellulase activity. Morphological examination revealed Gram-positive, rod or bacilli- shaped, non-motile, and spore forming nature of bacterial isolate. Different biochemical tests suggested that bacterial isolate was Bacillus subtilis. The cellulase enzyme was partially purified by $(NH4)_2SO4$ *and studied using CMCase assay. The activity of partially purified cellulose was higher (8µg/ml/min) as compared to that of crude supernatant (5.5µg/ml/min). The optimum pH and optimum temperature range were found to be 6.5 - 7.5 and 40 – 50 ℃. Protein profiling using 10% SDS-PAGE further confirmed the purity of the partially purified sample.*

Keywords: *Bacillus subtilis*, Biotechnology, Cellulase, CMC agar, Ruminant, SDS-PAGE,

Introduction

The homopolysaccharide (homoglycans), cellulose is the most abundant organic compound on earth and the main constituent of the primary cell wall of green plants, many algae, oomycetes, and some species of bacteria (Tony, 2008). In green plants, it is often associated with compounds like lignin (Verma *et al.,* 2012). Chemically it is a homo polymer made up of several hundred to thousand D-glucose molecules connected by β (1→4) glycosidic linkage. Cellulose is a preferred source for energy production and has industrial and biotechnological applications (Kasana *et al.,* 2008). Being the most abundant biomass, considerable economic interest is involved to develop suitable technology to utilize it as a carbon source for various applications (Shanmugapriya *et al.*, 2012). Structural complexity makes cellulose highly resistant to enzymatic hydrolysis. Several species of bacteria and fungi produce cellulase enzyme that hydrolyzes β (1→4) glycosidic linkage of cellulose (Tomme *et al.*, 1995; Sharma *et al.*, 2016). Cellulase is considered as a multi enzyme complex with the action of three enzymes *viz.* endoglucanase, exoglucanase, and β-glucosidase. Among those, former one convert cellulose into small cello oligosaccharides and later one responsible for the formation of glucose from cello oligosaccharides (Sathya and Khan, 2014; Sindhu *et al.,* 2016).

In an ecosystem, ruminants use lignocellulosic biomass of green plants as a source of nutrients. Lignocellulosic biomass contains a vast quantity of cellulose with other carbohydrates and proteins (Leschine, 1995). The specialized foregut organ, rumen present in cows, sheep, deer, and goats is considered as a site of cellulose breakdown (Afrc, 1989). Unlike humans, ruminants can degrade cellulose because of having a cellulase enzyme in the digestive systems. For the degradation of cellulose and subsequent conversion of it into glucose, ruminants harbor a diverse group of microbes in the digestive system. One predominantly found bacterial genus that degrades cellulose is gram +ve *Ruminococcus.* Others comprise *Fibrobacter, Megasphaera, Streptococcus, Alicyclobacillus cellulosilyticus, Anaerobacterium chartisolvens, Escherichia, Chytridiomycetes fungi,* and methanogens (Weimer, 1996). However, scientific literatures suggest that about 70% of bacterial population is unexplored (Cho *et al.*, 2006). The secretions of mouth, abomasum, pancreas, and small intestine are sites of digestive enzymes in ruminants (Warner, 1956). These secretions could be explored for isolation, identification, and characterization of microbial communities. Ruminant's saliva aids in chewing and swallowing ingested biomass using fat and starch hydrolyzing enzymes (Burns, 2008). A rumen particularly of buffalo approximately produces 40-150 liters saliva per day helping in lignocellulosic biomass digestion which could be investigated for microbial diversity.

In this regard, the present investigation was aimed to explore bacterial communities with cellulolytic activity in the saliva of ruminant ('Jaffrabadi' breed of buffalo). Bacterial isolate with cellulase activity was purified and characterized by morphological and biochemical studies. Cellulases producing bacterial colonies were allowed to multiply in a suitable liquid medium and extracellular enzyme was extracted, partially purified and characterized.

Materials and Methods

Collection of Ruminant Saliva Sample

The saliva (Approx. 20 ml) was obtained from distinct 'Jaffrabadi' breed of buffalo on the farm of village (Fulmbri), outskirts of Aurangabad city, Maharashtra, India. The collected saliva was immediately refrigerated in bottle for future use.

Isolation of Cellulose Degrading Bacteria

The collected saliva sample (5 ml) was filtered and was serially diluted in 0.85% NaCl (Apun *et al.*, 2000). The appropriately diluted sample was transferred to the CMC agar medium by spread plating technique having composition as follows: peptone 10.0 peptone gram/lit, carboxymethyl cellulose (CMC) 10.0 gram/lit, K_2HPO_4 2.0 gram/lit, $MgSO_4.7H_2O$ 0.3 gram/lit, $(NH4)_2SO_4$ 2.5 gram/lit, gelatin 2.0 gram/lit and agar 15 gram/lit. The pH was adjusted to 6.8-7.2 and the plates were incubated at 37°C for 72 hours and preserved at 4°C (Yin. et al., 2010). The colonies were isolated and purified.

Screening of Cellulase Activity

Bacterial isolates individually streaked on the plates of CMC agar and incubated at 37°C for 72 hours. After incubation, CMC agar plates were flooded with 0.1% (w/v) Congo red for 15 min at room temperature and washed with 1 M NaCl for 15 min (Apun *et al.,* 2000). The formation of cellulose hydrolysis zone around bacterial isolates indicates their cellulase activity (Lisdiyanti *et al.,* 2012). The ratio of the clear zone diameter to colony diameter was calculated in order to select the highest cellulase activity. Hydrolysis capacity (HC) value was determined according to procedure described by Sreeja *et al.,* (2013). Secondary screening of cellulose degrading bacterial colonies was done by using iodine solution (Kasana *et al.,* 2008).

Cellulase Production from Bacterial Isolates

For the quantitative estimation of cellulase enzymes, the pure culture of cellulose degrading bacteria was cultured in CMC broth containing, CMC 10.0 g/lit, peptone 10.0 g/lit, $MgSO_4.7H_2O$ 0.3 g/lit, K_2HPO_4 2.0 g/lit, $(NH_4)_2SO_4$ 2.5 g/lit and gelatin 2.0 g/lit. The pH of broth was adjusted to 6.8 - 7.2. The culture was incubated in shaking incubator at 37ºC for 72 hours with agitation speed of 160 rpm (Yin *et al.,* 2010). The cells were harvested by centrifugation at 5000 rpm for 20 min at 4°C. The supernatant was used as crude enzyme source for further studies.

Identification of Bacterial Isolates by Morphological and Biochemical Characterization

Bacterial isolates with cellulolytic activity were identified morphologically by Gram staining and microscopic viewing (Apun *et al.,* 2000). Gram staining technique differentiates bacteria into Gram +ve and Gram –ve. Motility Test (Flagella staining) and endosperm staining was performed as per standard protocol. Bacterial isolates were further identified biochemically using carbohydrate fermentation test, Catalase test, Citrate utilization test, indole production, urease test, gelatin hydrolysis, starch hydrolysis, H_2S production, Methyl-red test and Voges–Proskauer (VP) test as reported in Bergey's manual (Sneath *et al.,* 1986).

CMCase Activity Assay by DNS Method

CMCase activity of bacterial isolate was assessed by measuring the amount of reducing sugar released during enzymatic reaction by dinitrosalicylic acid (DNS) (Miller, 1959). The reaction mixture was composed of 1.0 ml phosphate buffer (0.1 M pH 6.8), 0.5 ml of 1% CMC (w/v, prepared in 0.1 M phosphate buffer (pH 6.8)) and 0.5 ml crude supernatant enzyme. The reaction mixture was incubated at 37°C for 60 minutes. The reaction was halted by adding 3.0 ml of DNSA reagent. After boiling the mixture for 10 min, the color of reducing sugar developed was measured colorimetrically at 540 nm wavelengths against a blank comprising all the reagents except the crude supernatant enzyme. Cellulase activity was expressed as micromole of glucose released per ml per min. The experiments were performed in triplicate (n=3). The results were presented as mean ± standard error of mean (S.E.M.).

Effect of pH and Temperature on Activity of Cellulase

The effect of pH on the activity of crude cellulase enzyme was examined by carrying out CMCase assay in different pH solutions with 1% (w/v) CMC as described previously (Miller, 1959). The effect of temperature ranging from 10 to 100 ºC with an interval of 10 ºC was examined by performing experiment at these temperatures by CMCase assay (Miller, 1959). The optimum values of pH and temperature were reported.

Partial Purification of Cellulase Enzyme

The clear supernatant obtained after centrifugation was subjected to partial purification by ammonium sulphate ($(NH_4)_2SO_4$) (Islam and Roy, 2018). The supernatant was 80 % saturated with ammonium sulphate at 4 ºC overnight. The protein precipitate obtained by centrifugation at 10000 rpm for 15 minutes at 4 °C was dissolved in 5 ml, 0.1 M sodium-phosphate buffer, pH 7 and dialyzed extensively against the same buffer at 4°C overnight. The dialyzed sample was considered as partially purified and used for further analysis.

Protein Profiling on 10% SDS PAGE

Qualitative analysis of proteins of partially purified sample was done by 10% Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) as described by Laemmli (1970).

Protein Estimation

The quantity of protein was estimated by Folin–phenol reagent (Lowry *et al.*, 1951) using bovine serum albumin (BSA) as standard.

Statistical Analysis

The results were presented as mean ± S.E.M. The statistical analysis and figures were generated using Graph Pad Prism 8.4.3.

Results and Discussion

The enzymes are biocatalyst which catalyzes biochemical reactions. Enzymes of bacterial origin are considered as effective tools in degrading the complex compounds. The exploration of sources of cellulase producing bacterial communities is area of interest. These bacterial colonies rapidly grows, expresses multi-enzyme complexes, works at extreme temperature and pH, has less significant feedback inhibition and ability to endure varieties of environmental stress (Lynd *et al.,* 2002; Kasana *et al.*, 2008). In present investigation, salivary secretion (saliva) of ruminant 'Jaffrabadi buffalo' was investigated for cellulase producing bacteria and reasonable bacterial colonies were observed on Bushnell-Haas media containing CMC by spread plate technique (Fig. 1A). Zone of cellulose clearance was observed around purified bacterial colonies after Congo-Red staining (Fig. 1B), confirming cellulase activity. Congo-Red is considered as an indicator of cellulose hydrolysis in agar medium and frequently used for identification of cellulolytic bacteria. The colonies which appeared colorless on red background were taken as positive cellulose-degrading bacterial colonies (Lu *et al.*, 2004). These results were in agreement with earlier findings pertaining to cellulase producing bacteria from different sources (Soares *et al.*, 2012; Ji *et al.*, 2012; Eida *et al.*, 2012; Sethi *et al.*, 2013).

A **B** **C** **D**

Figure 1: Isolation and screening of bacterial isolates: **A**) Bacterial colonies on CMC agar, **B**) Bacterial isolates with cellulase activity (shown in circle) on CMC agar stained with Congo red dye, **C**) Plate showing starch hydrolysis by bacterial isolates, **D**) Cellulase activity from crude extracellular supernatant.

In addition, bacterial isolate was found to have amylolytic activity on nutrient agar containing starch (Fig. 1C). When stained with Lugol's reagent, clear zone of hydrolysis was observed confirming the presence of amylolytic enzymes. The pure bacterial isolate was cultured in CMC broth for cellulase production at 37 °C for 72 hours. After cell harvestment, supernatant was found to have cellulase activity as depicted in Fig. 1D. Total protein content in crude supernatant was found to be 0.35 mg/ml. Bacterial isolate was subjected to various morphological and biochemical tests to identify them. Microscopic observation of the bacterial isolate revealed that bacteria is Gram positive, rod or bacilli- shaped, non-motile and spore forming (Fig. 2).

Recently, several bacterial isolates with these morphological characters were identified and reported to have cellulase activity (Hussain *et al.,* 2017). Bacterial isolates were biochemically identified using various tests as shown in Fig. 3, Fig. 4 and Table 1. The obtained results indicate that bacterial isolate belongs to *Bacillus sp.* i.e. *Bacillus subtilis*, in agreement to earlier findings (Siu-Rodas *et al.*, 2018).

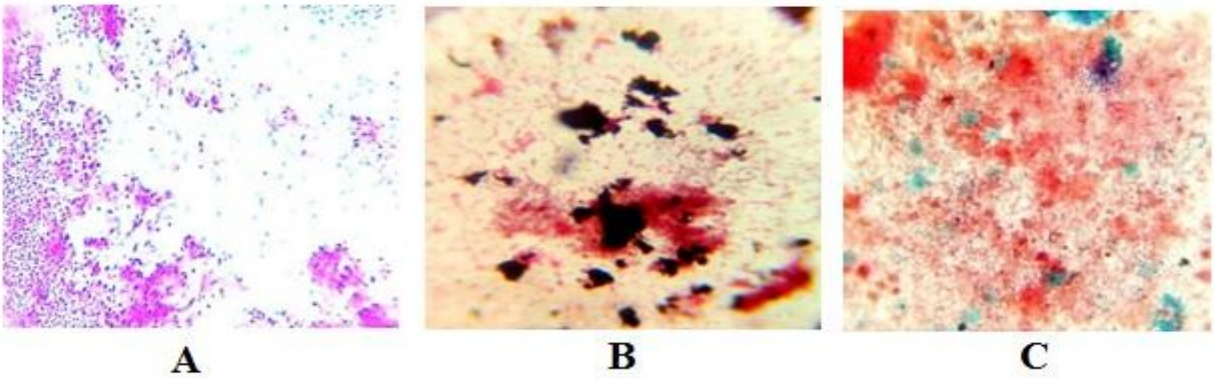


Figure 2: Morphological identification of bacterial isolates: **A**) Gram staining, **B**) Flagella staining, **C**) Endospore staining

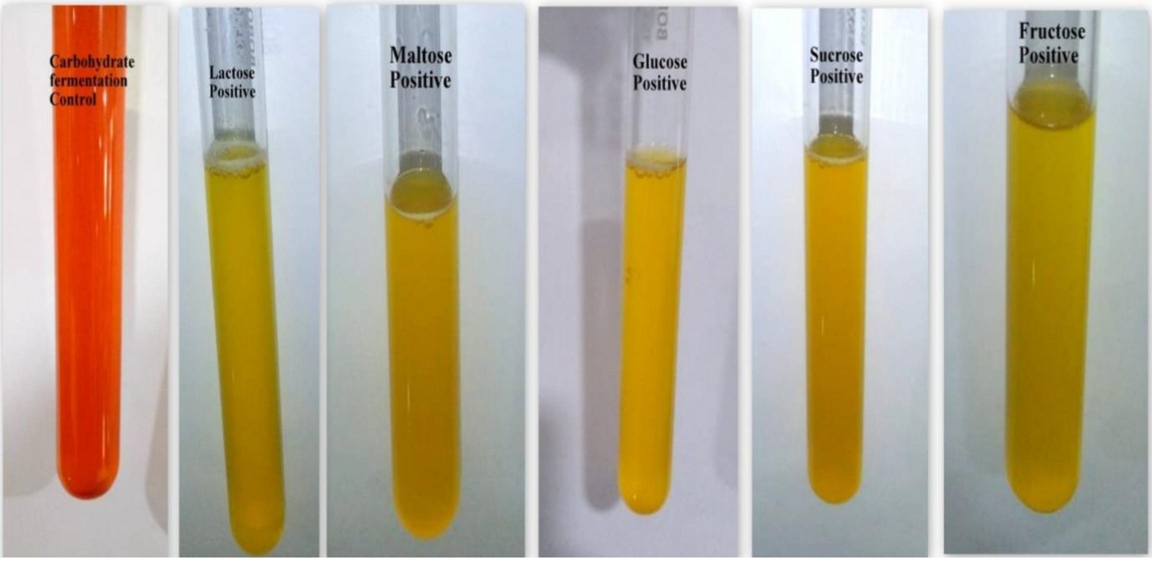
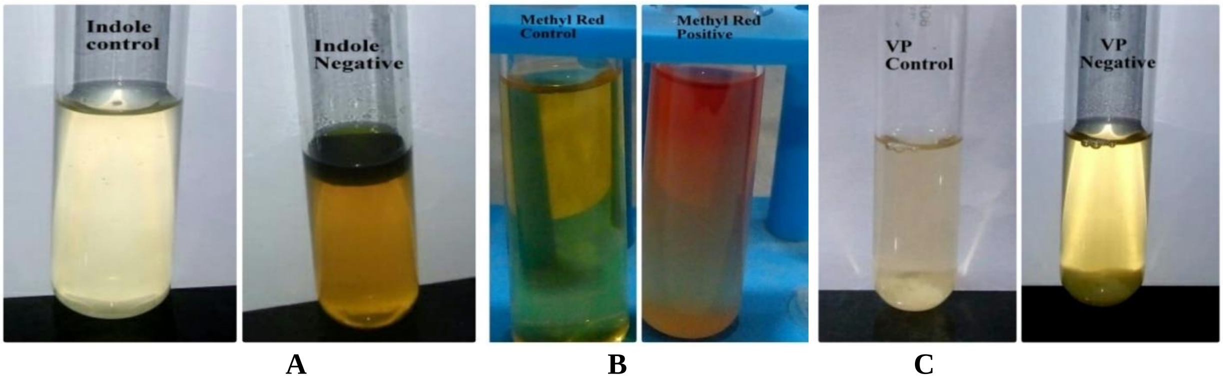


Figure 3: Carbohydrate fermentation test: Individual liquid medium was prepared for glucose, fructose, maltose, lactose, and sucrose as given in Bergey's manual. Bacterial isolate~~s~~ was inoculated in the medium with control for each without microorganism.

For the biochemical characterization of extracellular cellulase (evaluation of effect of pH and temperature), bacterial isolate was grown in CMC broth media. Extracellular medium (crude supernatant) containing cellulase enzyme and partially purified sample with ammonium sulphate was assessed for CMCase activity. Partially purified sample showed slightly improved activity i.e. 8µg/ml/min (protein 250µg/ml) compared to that of crude supernatant i.e.; 5.5µg/ml/min (protein 350µg/ml). The optimum pH of crude cellulase enzyme was found to be in the range of 6.5 - 7.5 whereas optimum temperature was recorded to be between 40- 50 °C (Fig. 5).



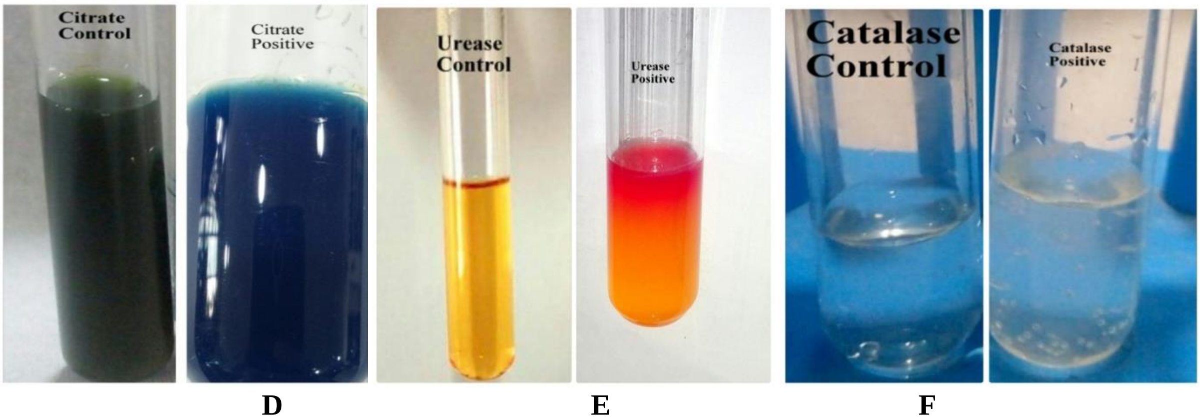


Figure 4: Biochemical tests for purified bacterial isolates: **A**) Indole test, **B**) Methyl red test, **C**) Voges–Proskauer (VP), **D**) Citrate test, E) Urease test, F) Catalase test.

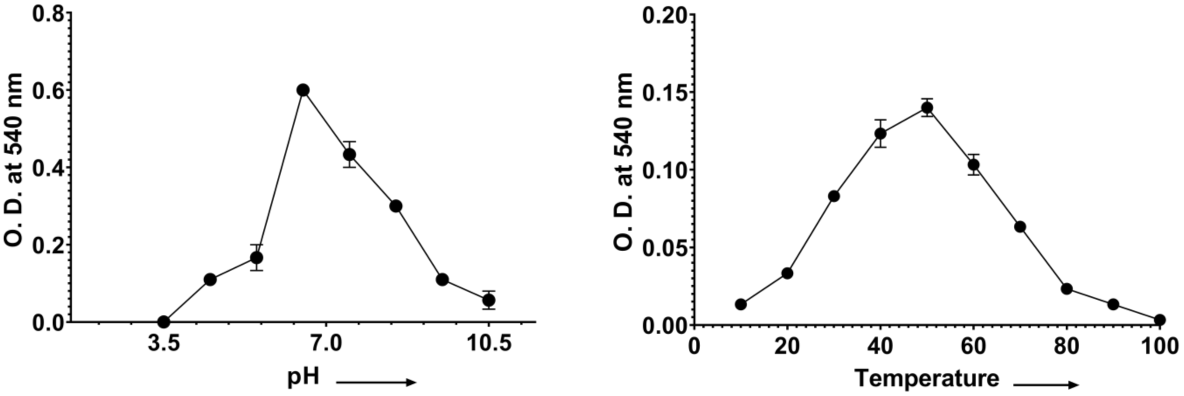


Figure 5: Effect of pH and temperature on crude cellulase activity. The data were expressed as mean ± S.E.M (n = 3).

Table 1: Morphological and biochemical properties of purified bacterial isolates

Characteristics	Reaction
Motility	-
Endosperm	+
Gram Staining	+
Catalase	+
Citrate	+
Voges–Proskauer (VP)	-
Methyl red	+
Urease	+
Indole	-
H_2S production	-
Hydrolyzing ability	
a. Starch	+
b. Gelatin	+
Carbohydrate fermentation	
a. Lactose	+
b. Maltose	+
c. Glucose	+
d. Sucrose	+
e. Fructose	+

This finding is in agreement with study of Islam and Roy (2018) who reported optimal pH 7 and temperature 40 °C for cellulase activity. For further characterization, crude and partially purified samples were resolved on 10% SDS-PAGE. The total numbers of protein bands were visually counted as four in crude supernatant of bacterial isolate whereas single band (likely cellulase) was observed in the partially purified sample (Fig. 6).

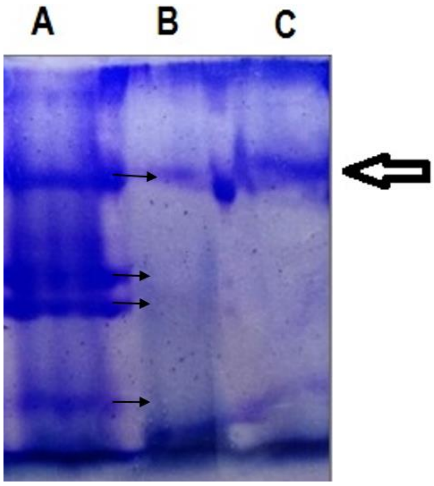


Figure 6: Protein profile of bacterial supernatant on10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE): A) Crude bacterial supernatant (30 µg protein), B) Partially purified sample (15 µg protein) and C) Partially purified sample (30 µg protein). Arrow indicates probable protein band of cellulose enzyme.

Earlier reports on cellulose-degrading micro-organisms from ruminants suggest that, these bacterial isolates could be subjected to bioremediation of organic wastes to obtain useful by-product (Vidali, 2001). Furthermore, ruminant's microorganisms (especially from saliva) with cellulase activity may find application in biotechnological approaches used for processing cellulosic biomass (Shelke *et al.*, 2015; Seki *et al.*, 2015; Nikam *et al.*, 2017). In this prospective, our findings on cellulolytic activity from buffalo saliva may assist to design biotechnological approaches lay down to cellulosic biomass processing using ruminant's microorganisms.

Conclusion

Cellulase-producing bacteria from the saliva of 'Jaffrabadi' breed of buffalo were successfully isolated, purified, and characterized. The isolated microorganism was Bacillus subtilis with optimum cellulase activity in neutral pH and physiological temperature. These microorganisms with cellulolytic activity could be further explored in the prospective of lignocellulosic biomass digestion for industrial and biotechnological applications.

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Conflict of Interests

There is no conflict of interest.

Publisher Disclaimer

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