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Evaluation of Cellulases and Xylanases Production from *Bacillus* spp. Isolated from Buffalo Digestive System

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Abstract

Cellulases and xylanases have high industrial demand due to their paramount importance in biological processes. The present study was aimed to evaluate the fiber degrading potential of *Bacillus* spp. isolated from buffalo digestive system. A total of fourteen isolates from rumen and eight isolates from dung were screened on carboxymethyl cellulose (CMC; 1%) agar plates, showing clear zone of CMC hydrolysis. All screened isolates were confirmed by targeting the 16S rRNA gene, sequencing and phylogenetic analysis. The enzyme activity index (EAI) of all screened isolates was calculated and it was observed that BR28 *(Bacillus subtilis),* BR96 *(Bacillus amyloliquefaciens),* BD69 (Bacillus tequilensis) and BD92 *(Bacillus sonorensis)* exhibit 2.11, 2.05, 2.56 and 2.45 EAI, respectively. The isolates with high EAI (>2) were selected for further enzyme production studies. The results of enzyme activities showed that BD92 had higher endoglucanse (carboxymethyl-cellulase, CMCase, 240.76±4.12 U/L) and avicelase (153.56±7.28 U/L) activities after 72 h of incubation. However, BR96 showed highest xylanase (3379.27±10.58 U/L) activity than others isolates and previously reported studies. This study provides a helpful insight into the identification of *Bacillus* spp. from the buffalo digestive system with higher production of cellulases and xylanases for their application in the animal feed industry.

Keywords: *Cellulase, Xylanase, Bacillus spp., Phylogenetic analysis, Buffalo digestive system*

Bizon Sindirim Sisteminden İzole Edilen *Bacillus* spp.'den Selülaz ve Ksilanaz Üretiminin Değerlendirilmesi

Öz

Selüloz ve ksilanaza biyolojik süreçlerdeki önemlerinden dolayı yüksek endüstriyel talep vardır. Bu çalışmanın amacı, bizon sindirim sisteminden izole edilen *Bacillus* spp.'nin lifleri parçalama potansiyelinin değerlendirilmesidir. Rumenden on dört ve dışkıdan sekiz izolat karboksimetil selüloz (CMC; %1) agar plakasında CMC hidrolizin açık renkli bölge gösterme durumu bakımından incelendi. İncelenen tüm izolatlar 16S rRNA gen hedeflemesi, sekans ve filogenetik analizlerle onaylandı. İncelenen izolatların tümünün enzim aktivite endeksi (EAE) hesaplandı ve BR28 (*Bacillus subtilis),* BR96 *(Bacillus amyloliquefaciens),* BD69 *(Bacillus tequilensis)* ve BD92 *(Bacillus sonorensis)*'un sırasıyla 2.11, 2.05, 2.56 ve 2.45 EAE gösterdiği belirlendi. Yüksek (>2) EAE değerine sahip olan izolatlar daha sonra enzim üretim çalışmalarında değerlendirildi. Enzim aktiviteleri, 72 saat inkübasyon sonrasında BD92'nin daha yüksek endoglukanaz (Karboksimetil selülaz, 240.76±4.12 U/L) ve aviselaz (153.56±7.28 U/L) aktivitelerine sahip olduğunu gösterdi. Ancak, BR96 diğer izolat ve daha önceden rapor edilenlerden daha yüksek ksilanaz (3379.27±10.58 U/L) aktivitesine sahipti. Bu çalışma, hayvancılık endüstrisinde uygulama bulabilecek bizon sindirim sisteminden. yüksek düzeyde selülaz ve ksilanaz üretebilecek *Bacillus* spp. üretimine faydalı bir bakış sunmaktadır.

Anahtar sözcükler: *Selülaz, Ksilenaz, Bacillus spp., Filogenetik analiz, Bizon sindirim sistemi*

INTRODUCTION

Plants produce 10-50 billion tons of cellulose annually [1] which is earth's most plentiful and attractive raw material for producing many industrially important commodity products. Cellulases and xylanases have an important role in biological degradation of plant cell wall polysaccharides. These plant cell wall polysaccharides comprised of cellulose (35-50%), hemicellulose (20-35%) and lignin (5-30%). Cellulases consist of a group of enzymes such

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as endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.74) and β-glucosidase (EC 3.2.1.21) [2]. The endoglucanase also known as carboxymethyl-cellulase (CMCase) acts on reducing or non-reducing ends of the amorphous cellulose and releases glucose directly or the cellobiose dimer. The exoglucanase also known as avicelase randomly attacks at arbitrary internal amorphous sites (O-glycosidic bonds) of crystalline cellulose and cleaves the polysaccharide chain by inserting a water molecule in the 1,4-β-bond resulting in glucan chains of different lengths. The β-glucosidase hydrolyzes the cellobiose dimers and the cellodextrins of various lengths to glucose [3]. Similarly, xylanase (EC 3.2.1.8) is responsible for degradation of linear polysaccharide β-1,4-xylan into xylose. These fiber degrading enzymes (cellulases and xylanases) have attracted much interest due to their vast applications in food, animal feed, textiles, fuel, chemical and pharmaceutical industry [4,5]. The global market for these enzymes was valued at 899.19 million dollars in 2014 and is projected to reach 1371.03 million dollars by 2020, at an annual growth rate of 7.3% from 2015 to 2020. Cellulases contribute 8% of total worldwide industrial enzyme demand with an expectation of 100% increase in this demand in coming years [6] that can be fulfilled by screening and identifying novel microbes with the highest enzyme production.

Numerous microorganisms that are able to degrade cellulose include bacteria and fungi. Most of the commercially available fiber degrading enzymes have been isolated from fungi, but isolation, screening and characterization of novel fiber degrading enzymes from bacteria is still a highly active research area. As, bacteria have a higher growth rate and more complex glycoside hydrolases providing synergy with higher potency and organismal diversity of extreme niches than fungi, leading to greater production of enzymes. The bacteria belong to the genera *Clostridium*, *Cellulomonas*, *Cellulosimicrobium*, *Thermomonospora*, *Bacillus*, and *Ruminococcus* have been isolated from the variety of sources such as composting heaps, decaying agricultural wastes, elephant feces, gastrointestinal tract of buffalo and horse, soil, and extreme environments like hot-springs [7,8]. Among these, the most attractive medium in which these enzymes are present in the ruminant's digestive system because ruminants have an efficient digestive system with a unique microbial symbiosis owing to the diet of the ruminants which consists of high amounts of fibrous matter. There are at least 30 predominant bacterial species at a total concentration of 10^{10} to 10^{11} bacteria/ml of rumen liquor [9] that plays a critical role in fiber degradation [10,11]. Most attempts have been made to isolate anaerobic microbes from rumen samples that require strict anaerobic conditions and are difficult to maintain. Consequently, isolation, screening and characterization of superior aerobic/facultative thermophilic microbes is of high interest because of easy handling, maintenance and enzymatic production.

Keeping in view above mentioned significance of cellulases and xylanases, the current study was aimed to evaluate superior aerobic/facultative bacterial isolates from the buffalo digestive system (rumen and dung samples).

MATERIAL and METHODS

Sample Collection and Bacterial Isolation

The rumen fistulated adult buffalos were fed a high fiber diet (fresh fodder and wheat straw) with free access to water. The experiments were performed after approval from the institutional animal ethics committee. Representative buffalo rumen content (solid and liquid, 200 g) and dung (150 g) samples were collected using presterilized containers and spatula/plastic bags, respectively. The samples were immediately transferred to the laboratory in an ice box at 4°C. One gram of each sample was then serially diluted up to 10^{-7} in 10 mL of sterile normal saline. Each 100 µL of dilution was inoculated on enrichment medium agar plates containing (g/L) carboxymethyl cellulose (CMC; 10.0), K_2HPO_4 (1.0), K_2HPO_4 (1.0), $MgSO_4.7H_2O$ (0.2), NH_4NO_3 (1.0), $CaCl_2$ (0.02), and agar (20.0) [12]. The plates were incubated at 37°C for 48 h under aerobic conditions. The bacterial colonies were further purified by streaking onto new CMC agar plates.

Qualitative Screening of Fiber Degrading Bacterial Isolates

Each bacterial isolate was inoculated on CMC agar plate for qualitative screening using the Congo-red overlay method [13]. In this method, plates were flooded with 0.3% Congo-red for 20-25 min followed by de-staining with 1 M NaCl solution for 15-20 min or until the clear zones around the colonies were visualized. Colonies showing discoloration of Congo-red were taken as positive fiber degrading microbial colonies. Enzyme activity index (EAI) was calculated using the formula as follows:

$$\text{Enzyme activity index (EAI)} = \frac{\text{(Diameter of zone - Diameter of bacterial colony)}}{\text{Diameter of bacterial colony}}$$

Physiological and Biochemical Characterization of Screened Bacterial Isolates

The bacterial isolates which showed clear zones, were further identified based on morphological and biochemical characteristics using Bergey's manual of systemic bacteriology [14]. Gram's staining was done for morphological identification of bacteria, whereas biochemical identification was done using catalase, citrate utilization, indole, methyl red (MR), oxidase, starch hydrolysis, Voges-Proskauer (VP) and sugars (D-xylose, fructose, lactose, maltose, sorbitol and sucrose) fermentation tests by standard methods [14]. Further, thermo-philicity of the bacterial isolates was also checked at different temperatures (37-60°C).

DNA Extraction and Amplification of 16S rRNA Gene

The DNA was extracted from bacterial cells with conventional phenol-chloroform-isoamyl alcohol method [15] and 16S rRNA gene was amplified using universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGA GGTGATCCAGCC-3') [16]. Each reaction contained 25 µL DreamTaq Green PCR master mix, 2 µL of each primer, 2 µL isolated DNA (50-100 ng), and nuclease free water until the final volume reaches 50 µL. The PCR conditions were as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles with denaturation at 94°C for 1 min each, annealing at 55°C for 30 s, extension at 72°C for 1 min, and a final elongation at 72°C for 10 min. Amplified product was checked for size and purity on 1% (w/v) agarose gel.

Bioinformatics Analysis and Phylogenetic Tree Construction

PCR products were sequenced through commercial services provided by Macrogen, Korea. The data of 16S rRNA gene sequences was analyzed using Seq Scanner 2 software and compared with National Center Biotechnology Information (NCBI) database using BLASTN with GenBank (http://www.ncbi.nlm.nih.gov) and the closest matches to bacterial isolates were obtained. The 16S rRNA gene sequences were aligned and a phylogenetic tree was constructed using MEGA 7.0.9 software with neighbor-joining method at 1000X bootstraps [17].

Quantitative Enzyme Assays for Fiber Degrading Bacterial Isolates

Potential fiber degrading isolates (BR28, BR96, BD69, and BD92) showing maximum EAI (>2) on CMC agar plates were further cultured in broth medium containing (g/L) CMC (10.0), yeast extract (5.0), tryptone (10.0), K_2HPO_4 (1.0), KH_2PO_4 (1.0), $MgSO_4.7H_2O$ (0.2), NH_4NO_3 (1.0) and $CaCl_2$ (0.02), incubated at 37°C and 120 rpm for 5 days. Enzyme production during cultivation was assayed in triplicates at 12 h intervals. The supernatants were collected, centrifuged at 10000 × g for 10 min at 4°C and used as a crude enzyme for the enzyme assays. CMCase, avicelase, and xylanase activities were determined using the 3,5-dinitrosalicylic acid (DNS) method [18]. The reducing sugars were estimated spectrophotometrically using glucose and xylose as standards [19,20]. The reaction mixtures were prepared as follows: 500 µL of crude enzyme mixed with 500 µL of 1% (w/v) CMC for determining the CMCase activity; 500 µL of enzyme mixed with 1 mL of 1% (w/v) avicel for determining the avicelase activity; and 500 µL of enzyme mixed with 500 µL of 1% (w/v) birch wood xylan for determining the xylanase activity. The buffer used for dissolving or resuspending the substrates was 100 mM sodium citrate buffer (pH 5.5). The mixtures were incubated at 50°C for 30 min, 60 min and 15 min for CMCase, avicelase and xylanase assay, respectively. Then, the reactions were stopped by adding 1 mL of DNS reagent for CMCase and xylanase assay, while 2 mL of DNS reagent for avicelase assay. All the mixtures were heated in boiling water for 5-10 min for color development. The absorbance was measured at 540 nm [20].

One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of glucose/xylose equivalent per min under the assay conditions. The supernatant was also used for the estimation of proteins by Lowry method [21].

RESULTS

Isolation and Screening of Bacterial Isolates

Isolation and screening of fiber degrading microbes is of immense importance due to their huge demand in industrial applications. In present study initially, a total of 101 isolates were purified from enriched cultures based on shape, size and color. Two third (69) of these isolates were from rumen samples while one third (32) were from dung samples. Out of these, fourteen and eight isolates from the rumen and dung, respectively, were screened for the production of fiber degrading enzymes on CMC agar plates as showed in *Fig. 1*. EAI based on the diameter of the zone of hydrolysis and colony diameter is very useful for predicting the enzyme yield, as an aid to select isolates with a high level of fiber degrading activities. Based on EAI (mm), fiber degrading potential isolates was classified as low (0.1-0.9), medium (1-1.9) and high (>2). Two isolates from rumen samples (BR28, BR96) and two from dung samples (BD69, BD92) have high EAI (>2) as showed in *Table 1*.

Physiological and Biochemical Characterization

The physiological and biochemical characterization of selected isolates (BR28, BD69 BD92 and BR96) have been shown in *Table 2*.

Phylogenetic Analysis

Phylogenetic analysis of all the isolates and their closest related isolates by comparing the contig regions with NCBI GenBank database using BLAST algorithm (http://www.ncbi.nlm.nih.gov) showed that the isolates were the members of two major phyla namely Firmicutes and Actinobacteria with three families Enterococcaceae, Bacillaceae and Streptomycetaceae as presented in *Table 1*. Phylogenetic tree was constructed from selected bacterial isolates (BR28, BR96, BD69 and BD92) and their closest related strains from the GenBank using the neighbor-joining method in MEGA 7.0.9 software at 1000X bootstraps as presented in *Fig. 2*. BD69 and BD92 were found novel being reported for the first time as having fiber degrading activity isolated from buffalo dung samples.

Estimation of Enzyme Activities

The fiber degrading potential of promising isolates was tested by estimating enzyme activities for CMCase, avicelase,

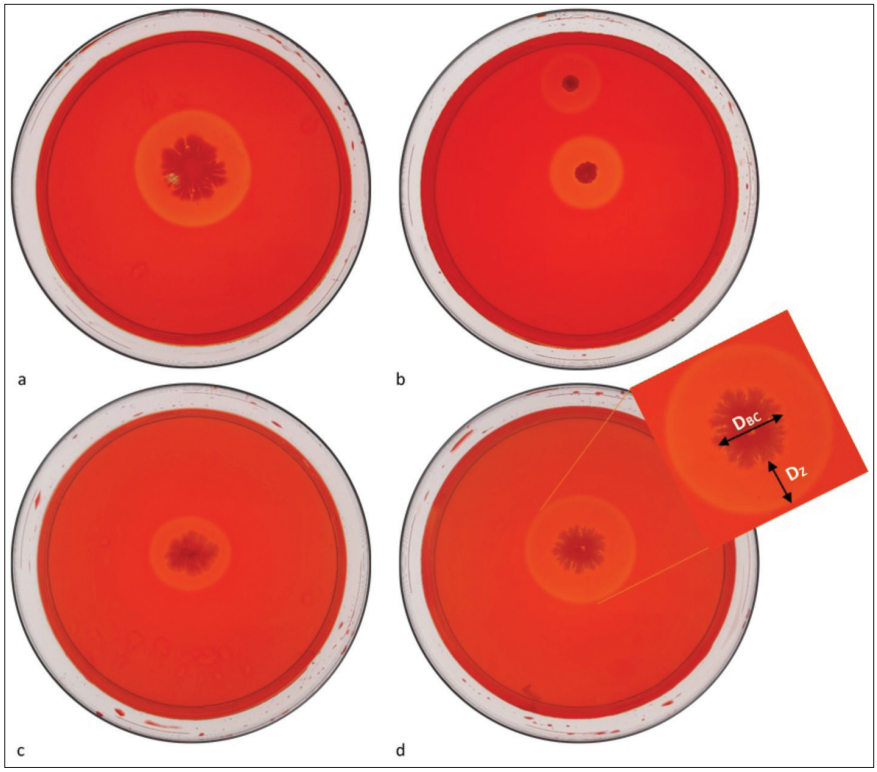

Fig 1. Plate (CMC; 1%) based screening of isolates incubated at 37°C for 48 h. (a) BR28, (b) BD69, (c) BD92 and (d) BR96 (D_{BC} = diameter of bacterial colony; D_Z = diameter of zone) after Congo-red staining (0.3%) showing zones of hydrolysis around the colonies

Table 1. *Identification of bacterial isolates based on 16S rRNA gene homology analysis and EAI*

Type	Isolate's ID	Accession Numbers	Phylum/Family	Closest Related Strains from the GenBank	D_{BC} (mm)	D_Z (mm)	EAI
Buffalo rumen samples	BR4	MF767882	Firmicutes/ Bacillaceae	*Bacillus subtilis*	5.0	11.0	1.2
	BR9	MF767883	Firmicutes/ Bacillaceae	*Bacillus subtilis*	15.5	23.5	0.52
	BR14	MF767884	Firmicutes/ Bacillaceae	*Bacillus subtilis*	19.0	23.0	0.21
	BR17	MF767885	Firmicutes/ Bacillaceae	*Bacillus subtilis*	17.5	26.5	0.51
	BR20	MF767886	Firmicutes/ Bacillaceae	*Bacillus subtilis*	12.5	24.5	0.96
	BR21	MF767887	Firmicutes/ Bacillaceae	*Bacillus subtilis*	6.5	17.0	1.62
	BR28	MF767888	Firmicutes/ Bacillaceae	*Bacillus subtilis* subsp. *spizizenii*	9.0	28.0	2.11
	BR38	MF767889	Firmicutes/ Bacillaceae	*Bacillus tequilensis*	14.0	29.5	1.11
	BR80	MF767896	Firmicutes/ Bacillaceae	*Bacillus cereus*	21.0	31.5	0.5
	BR81	MF767897	Firmicutes/ Bacillaceae	*Bacillus subtilis*	9.5	26.5	1.79
	BR88	MF767898	Firmicutes/ Bacillaceae	*Bacillus sp.*	5.0	14.5	1.90
	BR90	MF767899	Firmicutes/ Bacillaceae	*Bacillus sp.*	18.0	36.5	1.03
	BR96	MF767901	Firmicutes/ Bacillaceae	*Bacillus amyloliquefaciens* subsp. *plantarum*	10.0	30.5	2.05
	BR98	MF767902	Firmicutes/ Bacillaceae	*Bacillus subtilis*	9.5	26.5	1.79
Buffalo dung samples	BD49	MF767890	Firmicutes/ Bacillaceae	*Bacillus subtilis* subsp. *spizizenii*	8.5	21.5	1.53
	BD55	MF767891	Firmicutes/Enterococcaceae	*Enterococcus casseliflavus*	14.0	33.0	1.36
	BD63	MF767892	Firmicutes/ Bacillaceae	*Geobacillus sp.*	6.5	8.5	0.31
	BD69	MF767893	Firmicutes/ Bacillaceae	*Bacillus tequilensis*	4.5	16.0	2.56
	BD73	MF767894	Firmicutes/ Bacillaceae	*Bacillus subtilis*	9.5	25.5	1.68
	BD77	MF767895	Firmicutes/ Bacillaceae	*Bacillus subtilis*	13.5	31.5	1.33
	BD92	MF767900	Firmicutes/ Bacillaceae	*Bacillus sonorensis*	11.0	38.0	2.45
	BD99	MF767903	Actinobacteria/ Streptomycetaceae	*Streptomyces sp.*	16.5	28.5	0.73

D_{BC} = diameter of bacterial colony; D_Z = diameter of zone; EAI = enzyme activity index

and xylanase using CMC, avicel and birch wood xylan as substrate, respectively. The mean CMCase activities ranges from 22.24-55.82, 17.56-66.19, 48.60-240.76 and 38.46-118.62 U/L for BR28, BD9, BD92 and BR96, respectively as presented in *Fig .3a*. Whereas, BD92 showed highest mean CMCase activity which increases to its maximum (240.76±4.12 U/L) at 72 h of incubation while BR28, BD69 and BR96 showed maximum mean activities 55.82±2.22, 66.19±3.62 and 118.62±5.65 U/L at 24, 36 and 60 h of incubation, respectively. The mean avicelase activities ranges from 31.54-90.32, 37.16-116.03, 54.70-153.56 and 31.58-65.88 U/L for BR28, BD69, BD92 and BR96, respectively as presented in *Fig .3b*. The isolate BD92 showed highest mean activity (153.56±7.28 U/L) at 72 h of incubation while BR28, BD69 and BR96 showed maximum mean activities 90.32±4.34, 116.03±4.01 and 65.88±3.88 U/L at 60, 72 and 48 h, respectively, of incubation. Similarly, the mean xylanase activities ranges from 7.65-955.74, 23.32-1009.57, 771.54-2921.54, and 167.97-3379.27 U/L for BR28, BD69, BD92 and BR96 respectively as presented in *Fig. 3c*. BR96 showed highest mean activity (3379.27±10.58 U/L) at 24 h of incubation when compared with BR28, BD69 and BD92 which showed highest mean activity 955.74±7.52, 1009.57±11.57 and 2921.54±59.12 U/L at 24, 24 and 36 h of incubation, respectively.

Table 2. *Identification of physiological and biochemical characteristics of promising bacterial isolates*

Characteristic Features	Isolate's ID			
	BR28	BD69	BD92	BR96
Gram's staining	+	+	+	+
Growth on MacConkey	-	-	-	-
Morphology	B	B	B	B
Spores	+	+	+	+
Methyl red	-	-	-	-
Voges-Proskauer	+	+	+	+
Aerobic	+	+	+	+
Catalase	+	+	+	+
Oxidase	-	-	-	-
Starch hydrolysis	+	+	+	+
Indole	-	-	-	-
Citrate utilization	+	+	+	+
Growth at 50°C	+	-	+	-
Growth at 60°C	-	-	+	-
Lactose	+	+	+	+
D-xylose	+	+	+	+
Fructose	+	+	+	+
Maltose	+	+	+	+
Sucrose	+	+	+	+
Sorbitol	+	+	+	+

B = bacilli; - negative; + positive

DISCUSSION

Approximately 70% of plant biomass is locked up in 5 and 6 carbon sugars, which are found in lignocellulosic biomass comprised of mainly cellulose (35-50%), lesser hemi-celluloses (20-35%) and least of all lignin (5-30%) [22]. The degradation of plant cellulosic material is not an easy task. There are a lot of chemical, physical and biological pretreatment methods available for this purpose, but these methods are not environmentally safe, they generate toxic substances inhibitory to fermentation that makes process environmentally unfavorable and uneconomical [23]. Degradation of plant cellulosic materials using microbial enzyme systems is an economical and environment friendly process. There has been increasing interest for the degradation of plant cellulosic material using bacteria because of their faster growth than fungi. Habitats that contain plant-based substrates are the best sources to find these microorganisms. The buffalo digestive system has been selected as a source for obtaining novel desirable fiber degrading microbes because there is a rich assemblage of fiber degrading microbes owing to the diet of the ruminants that primarily consists of huge amounts of cellulosic matter.

In the present study, out of 101 isolates, 14 from rumen

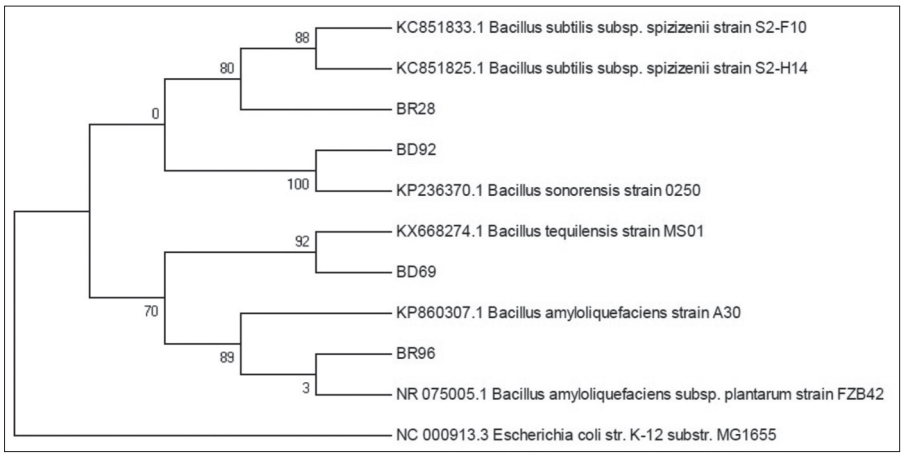

Fig 2. Phylogenetic tree of 16S rRNA gene sequences from promising fiber degrading bacterial isolates and their closest related strains from the GenBank. The tree was generated by using the neighbor-joining method in MEGA 7.0.9 software at 1000X bootstraps

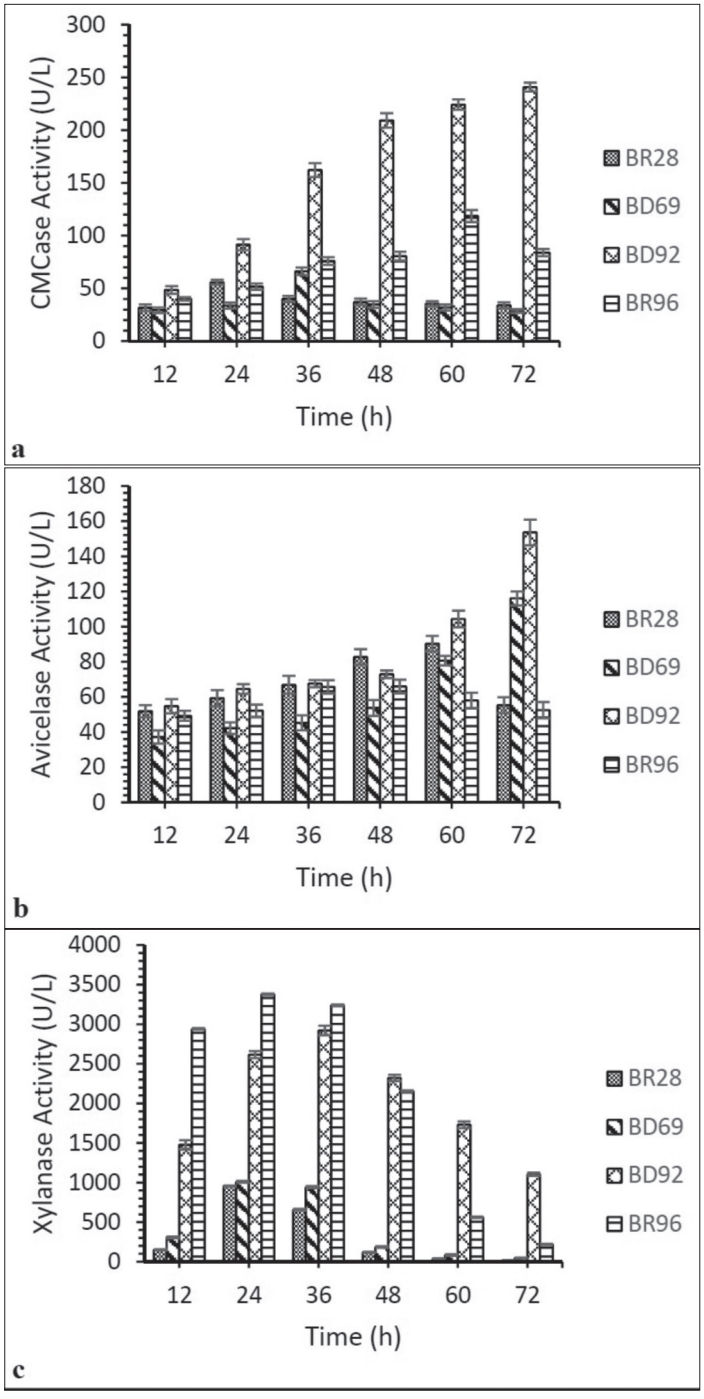

Fig 3. Mean fiber degrading enzyme production (n=3) profile of BR28, BD69, BD92 and BR96 isolates in time course of 120 h showing (a) CMCase, (b) avicelase and (c) xylanase activities

and 8 from dung samples were found positive for fiber degrading potential on CMC agar plates using Congo-red staining. Congo-red interacts with cellulose in agar medium, but when bacteria secrete fiber degrading enzymes that degrade cellulose into cellobiose/glucose and organic acids which lowered the pH of medium [3]. This pH difference affected the color of the medium and form a clear zone around the colony indicating enzyme production. EAI is a very useful parameter for predicting the enzyme yield [24]. In the present study, the isolates showed EAI >2 proved to be more prolific producers of these enzymes. Our results on the morphological/ biochemical characteristics were similar to those reported by Ozkan and Ahmet [8], who reported *Bacillus* strains from rumen samples. While, it has also been reported that buffalo has higher fiber degrading bacteria than other farming animals may be due to the fact that buffalo used fibrous feed more efficiently as compared to other farming animals. The fiber degrading potential of lower tract microbial population is high as compared to rumen microbial population which would affect the type of fermentation and the end-products [25].

Molecular characterization was done using 16S rRNA gene sequencing because 16S rRNA region is highly conserved and protected from mutation in the evolutionary period. Results confirmed that the isolates were members of two major phyla namely Firmicutes (95.65%, the most dominant culturable rumen and dung microbiota) and Actinobacteria (4.35%) and three families Streptomycetaceae, Enterococcaceae and Bacillaceae. Our findings were parallel to those in earlier studies on microbiota from the gastrointestinal tract of ruminants [8,26]. Our results were also consistent with previous studies those isolated *Bacillus* species from the rumen, cow dung and Tibetan pig's intestine [27,28]. There are continuous efforts to find novel and efficient microbial isolates with fiber degrading potential. To our best knowledge we have isolated two *Bacillus* spp., BD69 *(Bacillus tequilensis)* and BD92 *(Bacillus sonorensis)* from dung samples with the highest EAI reported first time, which indicated their ability to produce fiber degrading enzymes. Moreover, the isolate BD92 *(Bacillus sonorensis)* has the ability to survive up to 60°C which highlights its industrial importance.

The enzyme production in liquid culture medium has been determined at different time intervals using the DNS method. In this method, free carboxyl groups were released by the oxidation of aldehyde groups of the glucose molecule, which is formed by the action of fiber degrading enzymes. These reduce 3,5-DNS (yellow) to 3-amino-5-nitro salicylic acid (orange) under alkaline conditions [18]. In the present study, BD92 has higher mean CMCase (240.76±4.12 U/L) and avicelase (153.56±7.28 U/L) activity at 72 h, when compared to BR28, BD69 and BR96. Liang et al.[29] reported CMCase activity of 0.01 U/mL from *Bacillus subtilis* which was lower than the CMCase activity of the isolates in present study. They also showed that enzyme activity could be improved by optimizing carbon and nitrogen components in the media as ME27-1 *(Paenibacillus terrae)*. They improved production from 0.17 to 2.08 U/mL when using wheat bran and NH_4Cl as carbon and nitrogen source,

respectively in their study. The production of fiber degrading enzymes generally depends on a variety of growth parameters which include inoculum size, pH, temperature, medium additives (carbon and nitrogen sources), aeration, growth and time [30-32] and also on the presence of various metal ions as activators and inhibitors [33].

In present study, it was proved that *Bacillus* spp. have capability to produce CMCase and avicelase which was comparable to another study conducted by Ladeira *et al*.[34] whereas, Fukumori et al.[35] reported that alkalophilic *Bacillus subtilis* strains 1139 and N-4 were capable of hydrolyzing CMC, but could not degrade avicel significantly. Xylanase is also an important fiber degrading enzyme, BR96 produces high mean xylanase activity in this study, which was comparable to previously reported studies [36,37]. This proves that novel subspecies of *Bacillus* isolated in this study showed higher fiber degrading enzyme production compared to previously reported studies [7,22,29,38,39]. Further, growth optimization and enzyme characterization of these novel microbes are necessary for large scale production and offer a promising approach for its possible use in the animal feed industry.

In conclusion, present study demonstrates that the buffalo digestive system contains a novel community of fiber degrading isolates which play an important role in the degradation of fibers. Moreover, we have reported *Bacillus* spp. which demonstrates that the rumen and dung have a great potential to be a source of fiber degrading microbes.

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