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Purification of a cellulase from cellulolytic gut bacterium, *Bacillus tequilensis* G9 and its evaluation for valorization of agro-wastes into added value byproducts

Mudasir A. Dar[a], Kiran D. Pawar[b], Bharati P. Rajput[c], Praveen Rahi[d], Radhakrishna S. Pandit[a,*]

[a] Department of Zoology, Savitribai Phule Pune University, Ganeshkhind, Pune, Maharashtra, 411007, India
[b] School of Nanoscience and Biotechnology, Shivaji University, Vidyanagar, Kolhapur, Maharashtra, 416004, India
[c] Department of Biotechnology, Shivaji University, Vidyanagar, Kolhapur, Maharashtra, 416004, India
[d] National Centre for Microbial Research, Trinity Complex, Pashan Pune, Maharashtra, 411021

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ABSTRACT

Bioconversion of lignocellulosic (LC) biomass is the most promising alternative to rapidly dwindling fossil fuels and greenhouse gas emissions. In view of this objective, *Bacillus tequilensis* strain G9 was characterized and explored for purification of its cellulase. The *B. tequilensis* G9 was found to convert LC substrates into reducing sugars and showed significant activity of 130.75 IU/ml extract on grass straw (GS). The X-ray diffraction (XRD) pattern and fourier transform infra-red (FTIR) spectroscopic analyses of the hydrolyzed GS revealed increased crystallinity index and exclusion of bands at wave numbers 898, 980 and 1543 cm^{-1} which represent digestion of cellulose content by bacterium. Liquid chromatography mass spectrometric (LC-MS) analysis revealed the identity of purified enzyme as Endoglucanase. The observed molecular mass of the purified cellulase was 43 kDa, optimally functional at acidic pH of 5.0 and temperature 40 °C. The cellulase activity was remarkably enhanced by DTT, CO^{2+}, Ca^{2+} and Zn^{2+} ions while inhibited by SDS, EDTA and Pb. Additionally, the cellulase hydrolyzed CMC into fermentable sugars like glucose and galactose which can be readily used in metabolic as well as industrial processes. Further, the co-culturing of *B. tequilensis* G9 with yeast resulted in the production of ethanol from LC waste. The above mentioned cellulolytic repertoire of *B. tequilensis* G9 signpost its potential for biotechnological applications.

1. Introduction

Rapid depletion of dwindling fossil fuels and increasing levels of greenhouse gas emission are the main challenges to present day society. These key issues are primarily related to transportation sector which largely relies on petroleum fuels for nearly 93% of its energy requirements and releases copious amounts of carbon dioxide (Zuroff et al., 2013) thereby ameliorates global warming. To address these challenges, enzyme mediated production of bio-energy from lignocellulosic (LC) wastes is a promising and pivotal alternative (Escobar et al., 2009). The LC biomass is the principal source of many polysaccharides majorly, cellulose, xylan, etc. and serves as the most abundant, chiefly unexploited, and a renewable resource of bioenergy. Unfortunately, this rich source of energy is burnt (Maki et al., 2009) in the crop fields that adds more to the environmental pollution. The biological degradation of LC biomass involves the hydrolysis by a multicomponent protein system called cellulosome. The cellulosome contains three main enzymes viz., endo-β-1, 4-glucanases (EC 3.2.1.4) that randomly breaks the cellulose chains into short stretches of residues, which are then exposed to exo-β-1, 4-glucanases (EC 3.2.1.91) releasing disaccharide units. Subsequently β-1, 4-glucosidase (EC 3.2.1.21), third enzyme of the cellulosome complex (Wilson and Irwin, 1999) produces residual sugars from these disaccharides. The key industrial applications of cellulases are biofuel generation (Lynd et al., 1991), laundry (Bhat, 2000), production of enzymes, organic acids, antibiotics (Sun and Cheng, 2003) and feedstock (Dienes et al., 2004), etc. Since the nature of the substrates used in the bioconversion processes largely effects the cellulosomes (Rastogi et al., 2010). Therefore, agricultural wastes like GS, wheat husk (WH) and sugar cane bagasse (SCB), etc. are mostly favored substrates for cellulases.

The cellulolytic microbes such as bacteria, fungi and protozoans are very important from biological and environmental view point (Pérez

* Corresponding author.
E-mail addresses: mudasir.dar@unipune.ac.in (M.A. Dar), kdp.snst@unishivaji.ac.in (K.D. Pawar), muddarym7@yahoo.com (B.P. Rajput), praveen@nccs.res.in (P. Rahi), panditrao499@gmail.com, rspandit@unipune.ac.in (R.S. Pandit).

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et al., 2002). However, bacterial cellulases have been largely ignored during the last century due to initial successes with fungal strains particularly from brown rot fungi. Further, cellulases of fungal origin such as *Trichoderma* spp. show several drawbacks like low specific activities, yield and end-product inhibition (Zaldivar et al., 2001). Therefore, bacterial cellulases that work under extreme conditions are considered as potential candidates for industrial processes (Gabani et al., 2012). Moreover, bacterial cellulases are also preferred due to their high recombinant production, stability at diverse conditions and fast growth of bacteria (Maki et al., 2009).

Despite these advantages of microbial cellulases, the high costs associated with cellulase production, difficulties in scaling up of the processes and low hydrolytic capacities are the main bottlenecks in bioconversion technology. Thus, it is immensely important to discover and develop more efficient cellulase systems that would be cost-efficient and of diverse industrial significance. In view of this, efforts were taken to investigate the cellulolytic repertoire of the *B. tequilensis* G9, previously isolated from gut fluid of giant African land snail, *Achatina fulica* while studying its potential to deconstruct/degrade various agro-wastes such as GS, filter paper (FP), WH and SCB under shaking culture conditions. Due to its putative cellulolytic potential better than many previously isolated cellulolytic bacteria, the *B. tequilensis* G9 is further characterized in this study wherein, we attempted to induce the cellulase production by using GS as substrate and subsequently purified it by ion exchange chromatography. The purified cellulase was further biochemically characterized and evaluated for maximum possible activity which could brand its importance in many industrial processes.

2. Materials and methods

2.1. Biotyping of bacterium by MALDI-TOF

The *B. tequilensis* strain G9 that was previously isolated from the gut fluid of giant African land snail, *A. fulica* (Dar et al., 2018) was further characterized in the present study. This isolate was identified by using 16S rRNA gene amplification and sequencing whose 16S rRNA gene sequence is available at NCBI under accession number KR866144. The bacterium was maintained in the laboratory and selected for the present study to further characterize its cellulolytic repertoire and its cellulolytic enzyme.

The identification of *B. tequilensis* G9 was further confirmed by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) analysis. For biotyping, a single colony of the *B. tequilensis* G9 was seeded into freshly prepared Luria Bertani (LB) broth in a 250 ml conical flask. The inoculated medium was incubated overnight at 37 °C and 150 rpm in rotary shaker to allow the bacterium to flourish. After proper growth, the biomass of the bacterium was retrieved by centrifugation (Rcf ~ 3000) for 5 min at RT. For MALDI-TOF analysis, samples were prepared by following the method of Schulthess and his colleagues (Schulthess et al., 2014) with slight modifications. Precisely, the biomass obtained was treated with 300 µl distilled water and 900 µl ethanol. The pellet containing cell biomass was recovered by repeatedly centrifuging the cell suspension at 10000 rpm (Rcf, 12857) for 2 min to remove the residual ethanol. The pellet was then air dried and thoroughly resuspended in 20 µl formic acid-water (70:30 v/v) solution followed by addition of equal volume of acetonitrile. Finally, 2 µl of the supernatant acquired after centrifuging the samples at 10,000 rpm (Rcf, 12857) for 2 min, were transferred to the target plate and allowed to dry at RT followed by overlaying with 1 µl of matrix solution. Variations in cutoff score values were measured by reducing the species cutoff values to 1.9 and the genus cutoff values to 1.6 and 1.5 followed by reinterpreting the top 2 matching database records in Bruker Biotyper systems (Bruker, USA).

2.2. Primary screening for cellulase activity

The primary screening for the secretion of cellulase enzyme was carried out on Berg minimal salt (BMS) media composed of $CaCl_2.2H_2O$, 5 g; K_2HPO_4, 0.5 g; $NaNO_3$, 2 g and 0.02 g each of $MgSO_4.7H_2O$, $MnSO_4.7H_2O$, $FeSO_4.7H_2O$ along with 5 g (W/V) of Carboxy Methyl Cellulose (CMC) sodium salt and 1.8% (w/v) bacteriological Agar per 1000 ml of solution. Upon confirmation of the cellulase production, the selected bacterium was further characterized followed by purification of its cellulase enzyme and characterization for industrial processing.

2.3. Hydrolysis of biomass and characterization

The BMS media containing GS as substrate was seeded with 1 ml of overnight grown *B. tequilensis* G9. The inoculated medium was then agitated at 37 °C and 150 rpm to visually monitor the degradation of substrate. After the proper growth on GS as evident from increased turbidity of the medium, the treated culture broth was centrifuged at 5000 rpm, to obtain the pellet of degraded biomass. The pellet acquired was washed with distilled water (DW), dried overnight in an oven at 60 °C and subjected to XRD and FTIR analyses. The XRD spectra of treated and untreated GS substrates were recorded on X-ray diffractometer (Bruker AXS D8 Advance X-ray diffractometer, Germany). Diffractions were recorded by using Cu-1.54 Å radiation at 30 kV and 10 mA and grade range between 10° to 40° in steps of 0.02°. Cellulose crystallinity index (CrI) was calculated based on intensities of crystalline portions using the following pragmatic equation (Segal et al., 1959) where I_{002} is the maximum intensity corresponding to crystalline cellulose whereas I_{am} is the amorphous portion and I_{002} is intensity diffraction at 2θ.

$$CrI\ (\%) = [(I_{002} - I_{am})/I_{002}]\ x\ 100 \quad \text{Eq. (1)}$$

Where, I_{002} represents the major peak at 2θ = 22.5 representing the crystalline region of cellulose, and I_{am} is the intensity at 2θ = 18.6 corresponding to amorphous cellulose.

The XRD patterns of untreated and bacterial treated GS samples were analysed and compared using XRD peak height method. For analysis and comparison of surface functional groups, the treated and untreated samples (20 mg) were individually mixed with 2000 mg of spectroscopic grade potassium bromide (KBr) powder and then pressed (10,000 psi) into discs. The FTIR spectra were recorded from 4000 to 600 cm^{-1} at a resolution of 4 cm^{-1} on FTIR spectrophotometer (Jasco 6100, Germany). The IR crystallinity index of GS cellulose was calculated from the intensity ratios of IR absorptions between 2900 and 898 cm^{-1} (Satyamurthy et al., 2011).

2.4. Enzyme assays

The cellulase activity was determined on various agro-wastes such as GS, WH, SCB and some commercial substrates like filter paper (FP), CMC, Avicel and xylan. To study the effect of substrate and period of incubation required for the maximum enzyme production, the *B. tequilensis* G9 was grown in liquid BMS media supplemented with 1% (w/v) of above mentioned substrates at 150 rpm, 37 °C for 14 days. The sample aliquots (~2 ml) collected on alternate days, were centrifuged at 10, 000 rpm for 15 min at 4 °C to obtain the supernatant treated as extracellular enzyme extracts.

The enzyme activity was estimated by DNSA (3, 5-dinitrosalicylic acid) method (Miller, 1959) through the determination of amount of reducing sugars as described previously (Dar et al., 2015). Briefly, the reaction mixture composed of 100 µl of 1% CMC in SC buffer (pH 5.4) and 100 µl of enzyme extract was incubated for 15 min at 50 °C. The reaction was terminated by adding 300 µl of DNSA reagent followed by boiling in a water bath for 5 min. After recording the absorbances at

540 nm, reducing sugars were estimated by using glucose standards. The proteins estimation was carried out by Lowry method (Lowry et al., 1951). Finally, the enzyme activities were calculated in international units (IU) where 1 IU is described as the amount of enzyme that can liberate 1 mmol of glucose equivalents per min under the standard assay conditions.

2.5. Production and purification of cellulase

The bacterium, *B. tequilensis* G9 was induced to produce cellulase by using GS as substrate. Prior to use, GS was pretreated with alkaline solution (NaOH, 0.1 M) followed by thorough washes of DW till neutral pH and then air dried. The dried substrate was then ground finely to a particle size of ≤3 mm and passed through a nylon mesh to get the uniform sized particles. For enzyme production, 1 L of BMS media containing 1% GS (w/v) as sole source of carbon was seeded with 1 ml ($1.2x10^3$ CFU/ml) of the freshly cultured *B. tequilensis* G9 inoculum. The inoculated media was then incubated at 150 rpm and 37 °C for a period of 14 days as reported earlier (Dar et al., 2018). The small aliquots (2 ml) of the broth were periodically sampled from the cultures to discern the cell growth and production of cellulase. After the proper growth of *B. tequilensis* G9 as evident by increased turbidity and highest endoglucanase activity, the broth was centrifuged at 10, 000 rpm (Rcf, 12857) for 15 min at 4 °C to obtain crude enzyme solution. The crude enzyme was filtered through a 0.45 µm membrane filter, subjected to ammonium sulfate [$(NH_4)_2SO_4$] precipitation (70%) overnight at 4 °C. Subsequently, the precipitate was collected by centrifugation at 17000 rpm (Rcf, 15000) for 25 min at 4 °C, resuspended in phosphate buffered saline (PBS) (pH 7.4), and dialyzed overnight against the same buffer. The dialyzed enzyme was used to check the enzyme activity and processed further.

The cellulase from the dialyzed (concentrated) sample was purified by ion-exchange chromatography technique using a diethylaminoethyl (DEAE)-cellulose column (25 × 2 cm) equilibrated with 20 mM PBS (pH 7.4). The fractions were eluted with a linear NaCl gradient of 0.1–0.5 M concentration after washing the column with same buffer at a flow rate of 4 ml/5min. The eluted fractions were assayed for cellulase activity and protein concentrations were determined by measuring the absorbance at 280 nm using spectrophotometer (Carry UV 60, Agilent technologies, USA). The activity showing fractions were pooled and concentrated by lyophilization at full vacuum for 5–6 h.

2.5.1. LC-MS analysis

The purified proteins were reduced with dithiotritol (DTT) as per the method of Chidi et al. (2008) with slight modifications. Precisely, after reduction in 100 mM DTT, 50 mM ammonium bicarbonate (NH_4HCO_3) for 15 min at 60 °C, the proteins were alkylated in a freshly prepared 30 µl of Iodoacetamide (IAA, 50 mM) for 30 min under dark conditions. Finally the proteins were digested with trypsin solution for 18 h at 800 rpm and 37 °C followed by reaction termination with formic acid. The digested peptides were then subjected to mass spectrometric analysis (AB Sciex 5600, USA). The peptides were identified by using paragon software, Protein Pilot™ V 5.0 (Sciex, USA).

2.5.2. Molecular weight determination of the purified cellulase

For molecular weight determination, lyophilized enzyme was dissolved in phosphate buffer (pH 7.4). The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) using 10% resolving and 5% stacking gels. After electrophoresis, the gels containing resolved proteins were stained by a solution of 0.1% (w/v) Commassie brilliant blue, 30% (v/v) methanol and 10% (v/v) acetic acid. The broad range molecular mass markers used were from Bio-rad, (Bio-Rad, Hercules, CA, USA) and Genei, (Bangalore India) containing Myosin from rabbit muscle (205.0 kDa), phosphorylase b (97.4 kDa), serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (21.5 kDa) lysozyme (14.4 kDa), aprotinin (6.5 kDa) and insulin chains (3.5 kDa). The samples were preheated at 60 °C for 10 min with loading dye before load on the gel.

2.5.3. Zymogram analysis

To confirm the activity of the purified cellulase, zymogram analyses were performed by using PAGE, also called native gel electrophoresis. Electrophoresis was carried out with a standard procedure, using discontinuous buffer system devoid of SDS. Sixty microliters of (45–55 µg) of purified enzyme (lyophilized) were mixed with appropriate volumes of gel loading buffer and loaded on 8% (resolving gel) PAGE gel. The electrophoresis was run at a voltage of 100 V, 23 mA for an average time of 8–10 h at 4 °C. Initially the samples were loaded in duplicates in order to trace the path of the activity showing bands. After the run, the gel containing the protein of interest was blotted against a buffer system on CMC agar slabs (containing 1% CMC (w/v) with 1.8% agar (w/v) as solidifying agent) and incubated at 50 °C for 30 min. The buffer was then replaced with Congo red solution (0.1%) for staining at room temperature. After staining for 20 min, the gels were destained with 1M NaCl (w/v) solution for 10 min, which removed excess dye. The yellowish halos of activity around the protein bands were observed as measure of hydrolysis of the substrate. Sometimes acetic acid glacial (1% v/v) was also used to increase the sensitivity of the staining process.

2.5.4. Substrate specificity

The substrate specificity of purified cellulase was determined on various carbon sources such as avicel, CMC, glycogen, *p*-nitrophenyl β-D glucoside (pNPG), starch and xylan as described above. All substrates were prepared in 50 mM SC buffer (pH, 5.4) and the enzyme activities were measured as described in the enzyme assays section except pNPG. In case of pNPG, 100 µl of the substrate (10 mM) was mixed with 100 µl of the enzyme extract and incubated at 50 °C followed by a similar procedure of other substrate.

2.5.5. Kinetic parameters

To study the kinetic parameters of the purified cellulase, viz., K_m, V_{max}, K_{cat} and catalytic efficiency, the enzyme extracts were incubated with varying concentrations of CMC substrate ranging from 0.1 to 1.0%. The products in the form of reducing sugars were determined by the DNSA method. The K_m values of the enzyme were obtained graphically using the software "Sigma Plot (version 10)" of Systat Inc., USA.

2.5.6. Effect of pH on enzyme activity and stability

To find out optimal pH required for maximum activity, the purified protein was incubated with 1% (W/V) CMC in 50 mM buffers of different pHs such as SC Buffer, 2.0–5.0; Phosphate Buffer, 5.0–9.0; Glycine-NaOH, 10.6–12.6) as per assay conditions. To test for pH tolerance, the enzyme aliquots were pre-incubated in 50 mM buffers of above pHs for 1 h followed by measurement of residual activities with 1% CMC under standard assay conditions.

2.5.7. Effect of temperature on enzyme activity and stability

The optimum temperature for cellulase activity was tested at varying temperatures from 10 to 90 °C in SC Buffer (50 mM, pH 5.4) under standard conditions. For determination of thermal stability, enzyme aliquots were initially pre-incubated at various temperatures (10–90 °C) for 1 h at pH 5.0 (optimum pH) without the substrate and residual cellulase activities were estimated under standard assay conditions as stated above in the enzyme assays section.

2.5.8. Effect of additives on enzyme activity

For the study of effect of various additives on enzyme activity, the purified protein was pre-incubated in 50 mM SC Buffer (pH 7.0) along with the following metal ions NH_4^+ (as NH_4Cl_2), Ca^{2+} (as $CaCl_2$), Co^{2+}

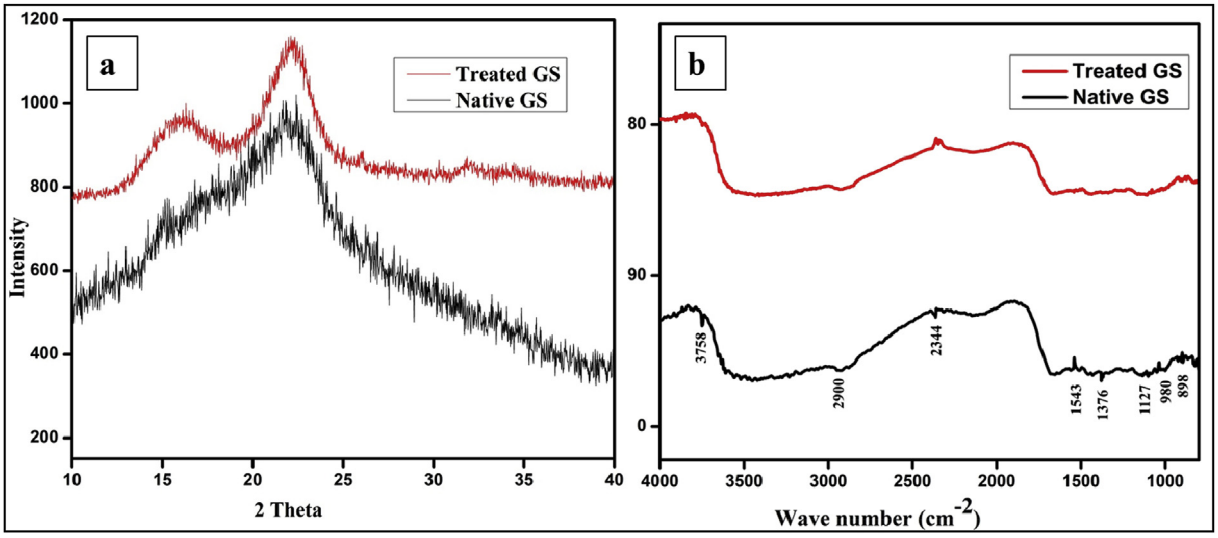

Fig. 1. The XRD (a), and FTIR (b) patterns of untreated and treated grass straw after hydrolysis by *B. tequilensis* G9.

(as $CoCl_2$), Mg^{2+} (as $MgSO_4$), Mn^{2+} (as $MnSO_4$), Hg^{2+} (as $HgCl_2$), K^+ (as KCl_2), and Zn^{2+} (as $ZnCl_2$) at 1 mM and 5 mM concentrations. In case of surfactants, the effects of 0.5% and 1% β-mercaptoethanol, EDTA, phenylmethylsulfonyl fluoride (PMSF), SDS, Tween-20 and Triton-X were tested to determine the relative activity of the enzyme. The enzyme assays were carried out as described above in enzyme assays section.

2.5.9. Analysis of hydrolyzed product

The thin layer chromatography (TLC) technique was applied for the qualitative analysis of sugars released during the enzymatic hydrolysis of CMC by purified cellulase. To accomplish this, 10 µl of hydrolysate sample and sugar standards of glucose, xylose, galactose and starch were spotted on silica plates 60 F254 (Merck, USA) by micro capillaries. The sugar standards of glucose, xylose and galactose were prepared in hydro-methanolic solutions with final concentration of 0.2 µg/ml sugars. A TLC run was performed in a closed glass chamber 10 x 5 x 10 containing mixture of acetonitrile: water (85:15 v/v) as mobile phase. For detection of separated sugars, TLC plate was derivatized and developed using solution that contained 5% H_2SO_4 and 0.3% N (1-Napthyl) ethylenediamine dihydrochloride in methanol (Bounias, 1980).

2.6. Ethanol determination and gas chromatography-mass spectrometry (GC-MS)

For ethanol production the freshly prepared BMS media containing GS as substrate was inoculated with overnight grown *B. tequilensis* G9 and incubated at 150 rpm for 12 days thereafter the culture broth containing reducing sugars was co-cultured with sugar fermenting, *Saccharomyces cerevisiae* strain. The media was then agitated at 120 rpm and 30 ^{O} C for 48 h followed by centrifugation of 10000 rpm (12835 rcf) for 10 min. The supernatant obtained was used to determine the ethanol content by GC-MS analysis. For GC-MS analysis, the supernatant was initially filtered through a 0.2 µm syringe filter and subsequently ethanol detection was carried out with method previously described by Bagewadi et al. (2016).

2.7. Statistical analysis

Results obtained are reported as mean ± standard deviation (SD) of three or more independent replicates. Analyses of data were carried out by using Microsoft office suite (version 2013) and SPSS software version 22 (IBM SPSS, NY) wherever applicable. Graphs were prepared in Origin software version 8.1 (Origin Lab. Corporation, USA).

3. Results and discussion

3.1. Biotyping by MALDI-TOF

In the light of importance and advantages offered by bacteria and their cellulases for bioconversion of LC biowastes, we previously explored gut of *A. fulica* as a source of isolation of cellulolytic bacteria (Dar et al., 2018). As a part of our endeavors, we had isolated, identified and maintained several potential bacterial isolates with cellulolytic activities in our laboratory. Though, isolation and initial screening were done, none of the isolates was used further for purification and evaluation of cellulase enzyme. Therefore, in the present study, previously isolated cellulose degrading *B. tequilensis* G9 was used and evaluated for cellulase production. To begin with, *B. tequilensis* G9 was biotyped by using MALDI-TOF analysis and evaluated for LCB degradation. The Biotyper software of the MALDI-TOF compared the sample mass spectrum with the reference mass spectra in the database, and calculated an arbitrary unit score of 2.23 reflecting the similarity between the *B. tequilensis* G9 with reference spectra and indicated *Bacillus* sp. as best match from database records (Fig. S1). The biotyping clearly confirmed that *B. tequilensis* G9 was the member of genus *Bacillus*. Scores below 1.7 are generally considered unreliable. Recently a variety of bacteria have been conveniently identified by employing the MALDI-TOF analysis (Wahl et al., 2002; Demirev et al., 1999).

3.2. Characterization of the hydrolyzed biomass

Since LCB contains enormous amount of crystalline as well as amorphous cellulose, the crystallinity of cellulose molecule greatly influences the rate of enzymatic hydrolysis. To understand the enzymatic efficiency of *B. tequilensis* G9 we employed XRD and FTIR techniques to have a detailed account of the degradation caused by bacterial treatment to GS during incubation period. The treatment of *B. tequilensis* G9 caused subtle modifications to composition of lignocellulosic waste, i.e., GS. The XRD analysis (Fig. 1a) revealed that crystallinity index of the grass straw was increased after treatment with the *B. tequilensis* G9. The crystallinity of the native cellulose was 20.8% which increased to 22.95% (Table 1) after treatment of bacteria. The possible reasons for this increased crystallinity could be the hydrolysis of the amorphous regions of cellulose leaving behind the crystalline portions together with lignin which result in the enhancement of the overall crystallinity of the molecule. These observations were similar to the inferences of

Table 1
Crystallinity index (*CrI%)* of untreated and hydrolyzed grass straw by *B. tequilensis* G9 calculated from XRD pattern.

Sample	Peak Height		Crystallinity Index (*CrI %)*
	18°	22.5°	
Untreated grass straw	775.09	978.86	20.8
Treated grass straw	889.24	1154.22	22.95

Oke and his colleagues (Oke et al., 2017) who reported a notable increase in the crystallinity of *Miscanthus sinensis* due to degradation by bacteria. The crystallinity of the cellulose is the primary factor that impedes the hydrolysis process (Kafle et al., 2015). As LCB consists of amorphous as well as crystalline portions, the amorphous regions are comparatively easier to degrade than the crystalline ones. The possible reason for increased crystallinity index could be bacterial cellulase mediated breakage of glycosidic linkages in cellulose chains leading to reduced amorphous content in the LCB (Xu et al., 2015). Recently, Zhao and co-workers (K. Zhao et al., 2012) suggested a similar reduction in peak height due to residual semi-crystalline portions in cellulose molecule after hydrolysis of the amorphous ones. The XRD pattern of the treated GS showed positive correlation with FTIR results where alterations in the functional groups, cellulose and hemicellulose contents were observed due to bacterial hydrolysis.

The FTIR spectroscopic analysis of the grass straw was carried out to measure the alteration of functional groups after treatment with *B. tequilensis* G9. The IR results of the treated GS showed absorption bands at 898, 1376, and 1543 cm^{-1} that correspond to COC, CCO, OCH deformations, C-5, C-6 stretching, bending of OH, and CH-stretching. While the broad absorption at 2900 cm^{-1} confers the stretching of C–H bonds (Binod et al., 2012). The bands corresponding to 1127, 1376 and 2900 cm^{-1} (Fig. 1b) represent distortions of C–OH, CH and OH-bond stretching respectively. The elimination of bands in treated samples at 980, 898, 1376 and 1543 cm^{-1} related to C = C stretching, CCH wagging in lignin, C–C stretching depict the functional group changes, indicating degradation by *B. tequilensis* G9.

The IR crystallinity ratios of untreated and treated GS were also determined from the peak heights such as A_{1376}/A_{1543}, A_{1127}/A_{898}, and A_{2900}/A_{898} cm^{-1} which represent cellulose content (Kacurakova et al., 2002). The IR crystallinity ratio for treated GS (0.89) (Table 2) was higher than the untreated (0.52) substrate, indicating digestion of amorphous regions of cellulose by *B. tequilensis* G9. The IR bands at wave number, 898 and 1371 correspond to β-anomer and C–H deformation of cellulose content in the LCB respectively (Haque et al., 2013). A shift of IR bands from 1371 to 1376 CM^{-1} in the treated sample signposts the deformation/deconstruction caused by bacteria. This deconstruction and hydrolysis potential demonstrates the possible industrial applications and significance of *B. tequilensis* G9 to valorize the agro-wastes for the production of added value byproducts.

3.3. Cellulase activity

Since optimum growth of bacteria is pivotal for proper metabolism, we tested the effect of different carbon sources on cellulase production by *B. tequilensis* G9. Among the tested substrates such as FP, WH, GS, SCB, CMC, Avicel, and xylan, the maximum cellulase activity (130.75 IU/ml) was achieved on GS (Fig. S2). The cellulase activity on avicel, CMC, FP and SCB was comparatively reduced when compared with other tested substrates. Though, our results are well in agreement with the report of Sangbriba and colleagues (Sangbriba et al., 2006) for *B. megaterium* and *B. circulans*. In contrast, the cellulase activity of *B. tequilensis* G9 obtained in our study was less than that of *B. amyloliquefaciens* DL-3 (153.0 unit/ml) which could be attributed to the higher substrate concentrations (2% rice hull) used in that study (Lee et al., 2008). The cellulase activity of *B. tequilensis* G9 was much higher than the hydrolytic activity of *B. cereus* which showed a maximum activity of 28.0 IU/ml extract (Lu et al., 2005). The cellulolytic activity observed was also many fold higher than the activities shown by *C. thermocellum* and *B. subtilis* (Otajevwo and Aluyi, 2011). Hydrolysis of lignocellulosic wastes like GS, WH and SCB by *B. tequilensis* G9 demonstrated in this study indicated that B. *tequilensis* G9 can be utilized for the production of several valued products such as single cell protein, ethanol, or other industrially important chemicals.

Since the higher cellulase activities were observed with GS on 12th day of incubation as compared to other tested substrates, it was, therefore, subsequently used as substrate to induce the cellulase production by the bacterium. The increased turbidity and highest endoglucanase activity demonstrated the direct correlation between the growth of *B. tequilensis* G9 and enzyme activity. This observation might have resulted owing to the fact that, maximum enzyme secretion occurs from early to late exponential growth phases of the bacteria (Adhyaru et al., 2014) through fast utilization of the available nutrients under optimal culture conditions. Another probable reason for this positive association between cell growth and cellulase production could be absence of some metabolites that cause catabolite repression, due to easy availability and abundance of cellulose polymer in the culture media.

Table 2
The IR crystallinity ratio of untreated and hydrolyzed grass straw after treatment with *B. tequilensis* G9.

Sample	IR Crystallinity ratio		
	A_{1376}/A_{1512}	A_{1137}/A_{898}	A_{2900}/A_{898}
Untreated grass straw	0.42	0.32	0.52
Treated grass straw	0.89	0.72	0.82

3.4. Purification and molecular weight determination

It was observed that *B. tequilensis* G9 could grow on a mineral based medium containing GS as the only source of carbon. Since cellulase activity was found highest on 12th day of incubation on GS, it was considered as optimum incubation period for higher yields along with the previously tested parameters. The *B. tequilensis* G9 produced cellulase extracellularly, which was precipitated by 70% ammonium sulfate salt and further purified by positively charged DEAE-cellulose through ion exchange chromatography, an effective step for enzyme purification. The acetone and ammonium sulfate precipitations resulted to a yield of 21.7% which was then further reduced after employing the DEAE-cellulose column chromatography. The elution profile of DEAE-cellulose chromatography provided multiple fractions among which fraction number 19 showed highest protein content (Fig. 2), therefore fractions 17–21 were pooled together and concentrated by applying the technique of lyophilization. Moreover, two groups of fraction, such as 17–21 (group I) and 28–31 (group II) showed cellulase activities among which the activity of group I was much higher than the other group. The use of only group I fractions in subsequent purification experiments led to purified cellulase with a yield of 10% and 9.89 purification fold (Table 3). This yield of cellulase purified in our study is comparatively higher than many earlier reported studies (Kim et al., 2009; Asha et al., 2012). The SDS-PAGE analysis of the purified enzyme showed a single band of apparent homogeneity, corresponding to the molecular mass of 43 kDa (Fig. 3a). The bacterial cellulase of similar molecular mass was also reported recently by Hakamada and coworkers, (2002) in case of *B. circulans*. However, the molecular mass of our purified cellulase was larger than the previously reported cellulases e.g. 37–40 kDa from *B. licheniformis* (Bischoff et al., 2006), and *Bacillus* strains CH43 and HR68 (Mawadza et al., 2000), but comparatively lesser than the cellulases (53 and 67 kDa) secreted by other members of *Bacillus* such as *Bacillus amyloliquefaciens* DL-3 (Lee et al., 2008) and *Bacillus* sp. AC-1 (Li et al., 2006) respectively. These observations together with ours clearly

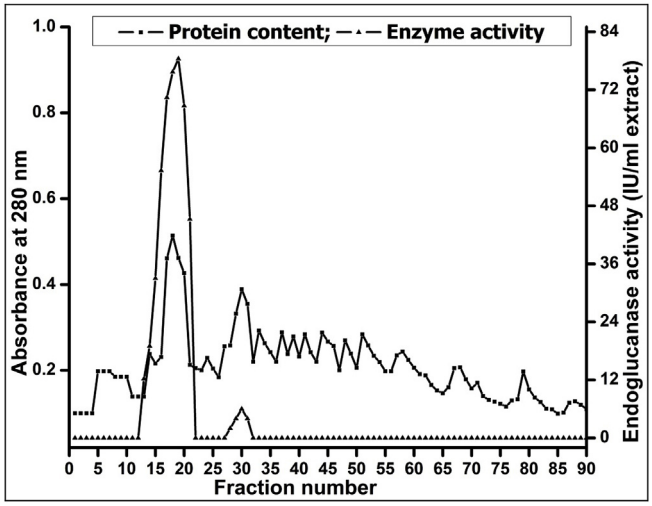

Fig. 2. Elution profile of cellulase using ion exchange chromatography stage (-■-) refers to the λ_{max} = 280 nm and enzyme activity (-▲-).

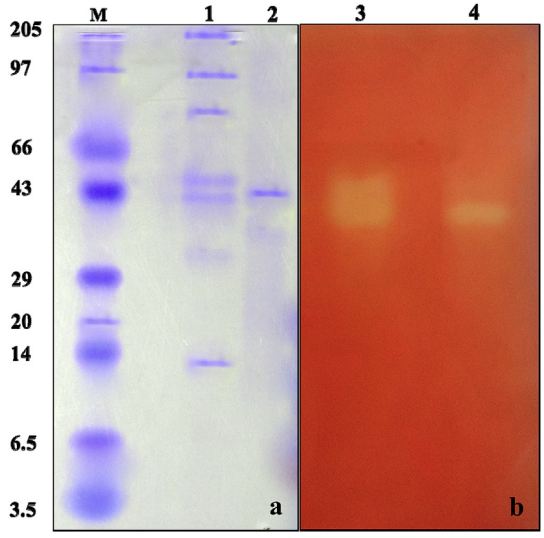

Fig. 3. Zymogram analysis of the purified cellulase; (a) SDS-PAGE analysis of *B. tequilensis* G9 proteins. Lane M: standard molecular weight marker proteins in kDa and, Lane 1: partially purified protein extract, Lane 2: DEAE-cellulose chromatography fraction of endoglucanase; (b) Zymogram of partially purified (Lane 3) and purified endoglucanase (Lane 4) of *B. tequilensis* G9.

support the findings based on molecular analysis that cellulase of *Bacillus* spp. are transcribed as precursor proteins with over 55 kDa of molecular masses, which involve the removal of a peptide sequence from the C-terminus thereby yielding an extracellular enzyme of over 35 kDa (Mawadza et al., 2000).

3.4.1. Mass spectroscopy of the purified cellulase

The appropriate identification of the purified protein fragments were carried out by mass spectrometric analysis. The protein pilot database produced matches against several cellulases of bacterial origin (Fig. S3). The analyzed peptides matched cover 33.8% of the *B. subtilis* endoglucanase. Further, proteomic analysis applied by LC-MS to the purified enzyme extract of *B. tequilensis* G9 allowed us to recognize the peptides present in cellulase that are responsible for endocellulase activity in the bacterium. Some of the oligopeptides showed similarity with β-glucanase as well as endo-1, 4- β-mannosidase of the *B. subtilis* 168 (Kunst et al., 1997) while few peptides resembled with the peptides from β-galactosidase of *B. megaterium* DSM 319 (Eppinger et al., 2011).

3.4.2. Zymogram analysis

The zymogram activity determined by blotting the enzyme containing native PAGE gel against the CMC-agar slab at 50 °C indicated the presence of several protein bands in partially precipitated extracts. These different sized proteins ranged from 14 to 205 kDa in molecular weight (Fig. 3b). On zymography analysis, two adjacent bands of 43 and ~50 kDa showed cellulase activity which merged with each other even after 15 min of incubation only. However, this bias was eliminated after DEAE column purification step where a single and distinct band was observed with fractions 17–21. Based on the zymogram analysis, it was observed that the purified cellulase showed a clear band (a yellow region) of CMC digestion after staining with Congo red solution. A similar type of activity has been stated recently by several researchers for *Bacillus* species (Sriariyanun et al., 2016).

3.4.3. Substrate specificity

The substrate specificity of the purified cellulase was determined towards different polysaccharides viz., Avicel, CMC, glycogen, pNPG, starch, xylan, laminarin, chitin, chitosan and disaccharide i.e., cellobiose. Among the commercial substrates, the purified cellulase showed higher preference towards CMC followed by Avicel depicting the activities of 242.5 ± 23.6 and 233.3 ± 3.6 IU/ml of extract respectively. Further it was observed that purified enzyme showed least preference towards the starch substrate (Fig. 4). The activity on cellobiose, starch, pNPG and Glycogen was very low showing a positive correlation with the cellulolytic nature of the enzyme. The preference of purified cellulase for tested carbon sources followed the order as CMC > Avicel > Chitosan > Laminarin > Xylan > Chitin > pNPG > Cellobiose > Glycogen and lastly starch. Our observations were in line with the results of Zhao et al. (2012) and Asha et al. (2012) who observed more preference of purified cellulase towards CMC substrate and low activity towards non-cellulosic substrates. In addition, our results are also similar to the inferences of Lee et al. (2008) with *B. amyloliquefaciens* DL-3 except the activity on pNPG. The microcrystalline cellulose (Avicel) and cellobiose having β-1, 4-linkages are produced from cellulose upon partial hydrolysis by enzymes. The pentose sugar, xylan is main component of the hemicelluloses predominant in hard woods and other plants like grasses, cereals, and herbs (Petzold et al., 2006) etc. The enzyme also showed notable sensitivity towards Laminarin containing β-1, 3 linkages thereby showing the capacity to hydrolyze both the β-1, 4 and β-1, 3 glycosidic linkages.

Using CMC as substrate, the enzyme showed a 21 fold increase in activity than starch. The CMCase, also called endoglucanase activity shown by the purified cellulase was also much higher than many commercial cellulases such as (14.9 ± 0. 10 U/ml) Accellerase® 1500 (Yang et al., 2017), (6.75 ± 0.34 U/ml) Accellerase™ 1000 (Fujii et al.,

Table 3
Purification steps of cellulase from *B. tequilensis* G9.

Purification step	Total activity (IU)	Total Protein (mg)	Specific activity (IU)	Yield (%)	Purification fold
Crude enzyme	2,15082 ± 181	28.95 ± 0.8	7428 ± 62	100	1
Ammonium Sulfate precipitation	46794 ± 159	1.76 ± 0	26505 ± 90	21	3.6
Acetone precipitation	44602 ± 50	1.67 ± 0	27839 ± 64	21	3.74
DEAE Cellulose Chromatography	21518 ± 25	0.292 ± 0	73210 ± 86	10	9.89

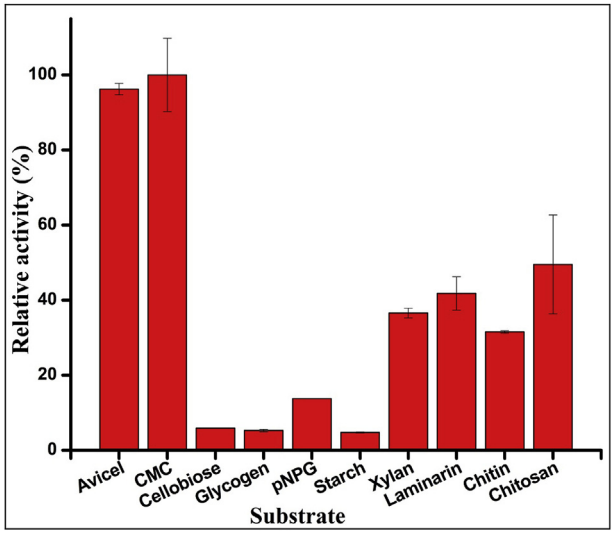

Fig. 4. Substrate specificity of the cellulase towards the tested substrates. CMC: Carboxymethyl cellulose; pNPG: ρ-Nitrophenyl N-acetyl-β-D-glucosaminide. n = 3.

2009), (18.33 ± 5.77 U/ml) Spezyme® CP (Nieves et al., 1998). The slightly higher preference for CMC as substrate indicated the endoglucanase nature of the purified enzyme for which it was easier to degrade CMC than its crystalline counterpart, i.e., Avicel. Likewise, the endoglucanases of many other *Bacillus* species are also known to depict avicelase activity (Kim et al., 2005; Han et al., 1995). The bifunctional nature could be attributed to the carbohydrate binding domain present in the tertiary structure of the protein, which enables these enzymes to degrade CMC as well as micro-crystalline cellulose (Ogawa et al., 2007). Since this enzyme degrades CMC as well as Avicel, therefore it is more appropriate to refer this enzyme as cellulase rather than endo or exo-glucanase alone.

3.4.4. Kinetic parameters

The kinetic parameters like K_m, V_{max}, K_{cat} and catalytic efficiency of the purified cellulase were studied for the hydrolysis of CMC as a substrate (Table 4). The kinetic parameters of purified enzyme were determined by Lineweaver Burk double reciprocal plot at varying concentrations of CMC as substrate and found as 0.25 mg/ml. The K_m, V_{max}, K_{cat} and catalytic efficiency of purified cellulase for carboxymethyl cellulose sodium salt were found to be 3.50 mg/ml, 1.09 mol $l^{-1}min^{-1}$, 0.34 s^{-1} and 1.98 $s^{-1}M^{-1}$, respectively. The K_m value of the purified cellulase was much higher than the K_m value of cellulase from *Bacillus* sp., (Sadhu et al., 2013), indicating less saturation of the enzyme even at high substrate concentrations. A similar K_m value of cellulase (3.6 mg/ml) was also reported by Bakare et al. (2005) in *Pseudomonas fluorescens*. In comparison, the K_m value was less than cellulases of *Actinobacteria anitratus* and *Branhamella* sp. (Ekperigin, 2007). This variation of the K_m values could be a consequence of source of the bacteria as well as the cellulosic substrate used.

3.4.5. Effect of pH on enzyme activity and stability

The effect of pH on purified enzyme was observed by incubating with buffers of different pH ranging from pH 3.0 to 10.0. The enzyme was recorded to show increased activity at pHs 4.0 and 5.0. The optimum pH for the cellulase activity was 5.0 (Fig. 5a) but there was an 18% loss of enzyme activity after 1 h of preincubation in the same buffer. However, the enzyme showed lesser activities at higher pH (above pH 5.0) which indicates the acidic nature of the enzyme. In case of enzyme stability, the cellulase activities were reduced at all tested pH and retained more than 30% activity on pHs 5.0 to 9.0 different pHs. A similar pH 5.0 for maximum cellulase activity was also found optimum in *Thermomonospora* (George et al., 2001), and *Bacillus* strain, M-9 (Bajaj et al., 2009). The stability of the purified cellulase under acidic conditions was in congruence with the reports of Bischoff et al. (2006) for *Bacillus licheniformis*. The optimum pH of the enzyme was slightly less than the optimum pH of *Bacillus amyloliquefaciens* which was more stable at neutral pH i.e., 7.0 (Lee et al., 2008).

Table 4
Kinetic parameters of the purified cellulase from *B. tequilensis* G9.

	K_m(mg ml^{-1})	V_{max} (moles $l^{-1}min^{-1}$)	K_{cat} (s^{-1})	Catalytic efficiency ($s^{-1}M^{-1}$)
Cellulase	3.509	1.093	0.3427	0.0982

3.4.6. Effect of temperature on enzyme activity and stability

The activity of purified cellulase was determined at various temperatures ranging from 10 to 90 °C keeping all other variables constant. Under tested conditions, the enzyme showed increased activity up to 40 °C while the activity at higher temperatures declined slightly. The highest 50.92 ± 0.6 IU/ml (100% relative) activity was estimated at 40 °C indicating it as optimum temperature for the enzyme. Though the enzyme activity was not affected much at temperatures in the range 10–50 °C, it showed abrupt reduction of 12–15% in its activity at temperatures > 60 °C (Fig. 5b). Possibly, this abrupt reduction in enzyme activity can be attributed to the denaturation of the enzyme caused at higher temperatures. Majority of the cellulase enzymes from *Bacillus* strains are known to have optimum activity in the range 40–60 °C (Hakamada et al., 2002; Kim et al., 2005). However, the optimum temperature of cellulase purified in this study was comparatively lower than other strains of *Bacillus* such as 60 °C in M-9 (Bajaj et al., 2009), BY-4 (Ma et al., 2015), 50 °C in C1 (Sadhu et al., 2013) and HSH-810 (Kim et al., 2005).

In case of thermal stability, the enzyme showed marked reductions in its activity after pre-incubation for 1 h at higher temperatures. However, the enzyme maintained 30–85% activity after preincubation for 1 h at 10–50 °C. Recently, a similar trend has been stated for *Bacillus* strain isolated from the cow dung (Sadhu et al., 2013). The reduced activity at higher temperatures can be attributed to the same effect as that of increased temperature leading to enzyme denaturation. The preincubation at 40 and 50 °C did not affect the enzyme activity retaining more than 80% activity, which was also reported for the cellulase of *Bacillus* sp. AC-1 (Li et al., 2006) revealing the mesophilic nature of the enzyme.

3.4.7. Effect of various additives on cellulase activity

Since additives are vital for regulation of enzyme activity, we tested the effect of different metal ions on cellulase activity at 1 and 5 mM concentrations whereas in case of surfactants the tested concentrations were 0.5 and 1% only. The lower concentration (1 mM) of metal ions such as Ca^{2+} and Hg^{2+} did not affect the enzyme activity but at 5 mM concentration, there was a notable change (decrease) in the activity (Table 5). The probable reason for this reduction could be interaction of metal ions with tryptophan residues or carboxyl groups of the amino acid chains that are pivotal for substrate binding (Lamed et al., 1994). The inhibition of cellulase activity by Hg^{2+} ions could have followed a similar mechanism (Lusterio et al., 1992) preventing the enzyme-substrate binding. The Pb showed an inhibitory effect on the purified cellulase causing 46 and 62.2% reduction in cellulase activity at tested concentrations of metal ion respectively. Further, 48% decrease in the activity was also observed due to Hg^{2+} at 5 mM concentration whereas metals like Mn^{2+} and Co^{2+} remarkably improved the enzyme activity by 1.5 and 2 folds respectively. Our observation showed close resemblance with the results achieved by Lee et al. (2008) who reported a similar inhibition of enzyme activity by Hg^{2+} (74.3%) and Pb (36.8%) at 5 mM concentration; whereas, some researchers (Kim et al., 2005;

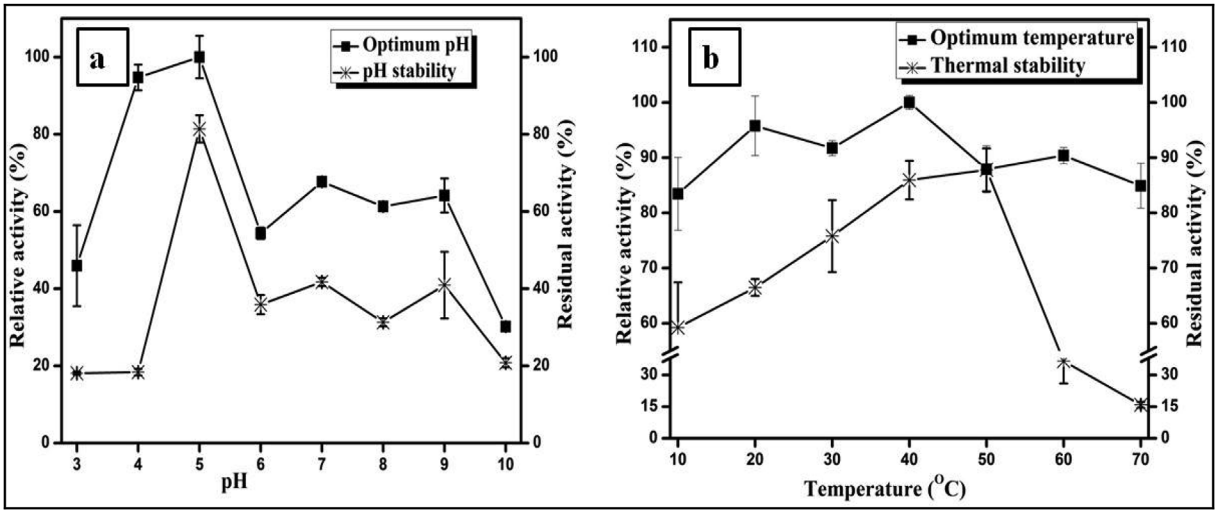

Fig. 5. Effect of pH (a) and temperature (b) on endoglucanase activity of purified cellulase from *B. tequilensis* G9. The symbol (-■-) indicates optimum pH and temperature required for enzyme activities that corresponds to left axis of the respective graphs. Whereas the symbol (-⁎-) depicts the residual activities of the purified cellulase at different pH values and temperatures corresponding to the right axes of the graphs respectively.

Mawadza et al., 2000) also stated 30 and 36% reduction in activity by Hg^{2+} and Pb at 1 mM concentration respectively. The other ions such as NH_4^+, Mg^{2+}, Zn^{2+}, and K^+ did not show any significant effect on the enzyme activity but $CaCl_2$ does show a 122% increase in activity at 1 mM concentration. Most of the enzymes like amylases and endoglucanases require divalent cations like Ca^{2+} which plays a major part in the protein stability and tertiary structure maintenance thereby causing activation of the enzyme and leading to enhancement of activity. Similarly, some divalent cations like Ca^{2+} and Mg^{2+} interact with imidazole and carboxyl groups in proteins causing enhancement of activity. Our observations were contrasting with *B. subtilis* BY-4 where the activity of cellulase was 28.6 and 20.1% inhibited by Mn^{2+} and Co^{2+} respectively (Ma et al., 2015). Depending upon the nature of metal salts used, the effect of metal ions on cellulases varies from strain to strain (Mawadza et al., 2000; Bajaj et al., 2009). Nonetheless, the purified enzyme showed considerable activity in presence of majority of the tested metals, demonstrating robust nature of cellulase which is a prerequisite for many industrial applications. We have also tested the effect of ascorbic acid on the enzyme which showed a remarkable enhancement (191%) in the activity of the purified cellulase (Table 5). Thus these elements viz., ascorbic acid, Mn^{2+} and Co^{2+} proved as important co-factors for the increased stability and enhanced activity of the purified cellulase.

Among the tested surfactants, DTT and β-mercaptoethanol proved effective by causing 191 and 133% increase in cellulase activity at 1% concentrations (Table 5). However SDS and EDTA were the only compounds that inhibited the cellulolytic potential of the enzyme to greater extents at both concentrations by 55.3 and 41.4% respectively. The effect of other compounds like DMSO, PMSF, and Triton X −100 showed no or least effect on cellulase activity. The Tween 20 and Tween 80 were found inhibitory at low concentration showing slight reduction viz., > 10% in the catalytic capacity of the enzyme. The inhibition of cellulases from *Bacillus* spp. by EDTA is already well reported by several authors (Asha et al., 2012; X. Zhao et al., 2012) which contradicts with few reports where it doesn't exert any inhibition on the enzymes (Hakamada et al., 2002; Li et al., 2006). This could be due to the low concentration of the surfactant used in the assays. Moreover, EDTA also inhibited the activity of *Pseudomonas fluorescens* cellulase (Bakare et al., 2005). The EDTA is known to inhibit the divalent-dependent enzymes, thereby causing chelation of the divalent cations like Ca^{2+} and Mg^{2+} which ultimately leads to the decrease in the enzyme activity. The anionic surfactant, SDS induces the negative charge by interaction with hydrophobic group of proteins, resulting in perturbation of the protein structure thereby decreasing cellulase activity (Bhuyan, 2009). On the other hand, marginal concentrations of some surfactants alter the cellulose surface properties, minimize irreversible binding of the enzyme with cellulose during hydrolysis process, and thereby prevent inactivation (Wu and Ju, 1998) which ultimately leads to enhancement of

Table 5
Effect of various additives on the endoglucanase activity of purified cellulase from B. tequilensis G9.

Sr.no.	Metal ion	Relative activity (%)		Sr.no.	Surfactant	Relative activity (%)	
		1 mM	5 mM			0.5%	1.0%
1	None	100	100	12	DMSO	97.3	105.0
2	NH_4^+	108.0	99.0	13	DTT	198.7	191.3
3	Ca^{2+}	122.0	92.0	14	EDTA	58.6	71.9
4	Co^{2+}	172.9	200.4	15	BME	134.3	133.6
5	Pb	53.6	37.8	16	PMSF	100.3	109.9
6	Mg^{2+}	110.4	96.1	17	SDS	44.7	50.8
7	Mn^{2+}	140.0	158.5	18	Triton X 100	94.2	91.5
8	Hg^{2+}	107.8	52.6	19	Tween 20	88.7	90.9
9	K^+	108.0	94.9	20	Tween 80	89.5	89.8
10	Na^+	104.4	97.5	21	Ascorbic acid	170.1	166.0
11	Zn^{2+}	118.8	94.9				

DMSO: dimethyl sulfoxide; DTT: dithiothreitol; EDTA: ethylene diamine tetra acetic acid; BME: β-mercaptoethanol; PMSF: phenyl methyl sulfonyl fluoride; SDS: sodium dodecyl sulfate.

the cellulolytic activity.

3.4.8. Analysis of hydrolyzed product

The products of the enzymatic hydrolysis of CMC were also qualitatively analyzed by TLC method. The TLC analysis confirmed the production of 6C hexose or 5C pentose sugars like glucose and xylose respectively after enzymatic hydrolysis of CMC by purified enzyme (Fig. S4). Upon comparison with loaded standard sugars, the hydrolyzed products showed similarity with galactose and glucose. Since the purified cellulase had a low affinity for starch, some products were formed upon hydrolysis but were poorly resolved on TLC plates. Therefore, further analysis with more sensitive techniques is needed which will help to resolve the sugar profile of the hydrolysate more clearly. Recently, a similar TLC profile has been reported for products released from hydrolysis of CMC by a recombinant cellulase C18 of *B. licheniformis* (Kumar et al., 2016). Since the enzyme showed more affinity towards CMC and the products obtained in the hydrolysis reaction of CMC confirm the endo-β-1, 4-D-glucanase nature of the enzyme (Girfoglio et al., 2012).

3.4.9. Ethanol determination and GC-MS analysis

The ethanol produced by co-culturing the *B. tequilensis* G9 with *S. cerevisiae* was qualitatively determined by GC-MS method. The mass spectrometric analysis of the sample showed 2 peaks at 43 and 44 m/z representing ethanol (Fig. S5) and CO_2 respectively. The first peak showed a retention time of 2.325 with a molecular mass of 43 which is a characteristic of ethanol content also observed by Bagewadi et al. (2016). The second peak at 44 molecular mass with retention time of 1.48 was a typical of CO_2. However the most frequent peak for ethanol is observed on a molecular mass of 31 but due to the ionization caused by the breakage of C–C bond near the oxygen atom it is also shown at m/z = 43 (Kar and Cheng, 2011). The co-culturing technique involving simultaneous saccharification and fermentation by microbes has always been the preferred choice for several industrial applications (Saratale and Oh, 2015). This process could also be helpful to reduce the overall production costs of ethanol as well as feedstock generation from lignocellulosic biomass together with the creation of more revenue.

4. Conclusion

In this report, the cellulolytic bacterium *Bacillus tequilensis* G9 was biotyped by MALDI-TOF analysis, showed close resemblance with *Bacillus* sp. which was also determined by 16S rRNA gene sequencing. The isolate showed capability to degrade down various agro-industrial wastes such as WH, GS, SCB, etc. Cellulase of *B. tequilensis* G9 was purified and characterized to find the optimum conditions and kinetic properties of enzymatic hydrolysis with cellulosic substrates. The enzyme showed tolerance to a wide range of conditions which signpost its industrial applications. The purified cellulase showed more preference towards the cellulosic substrates as compared to non-cellulosic polysaccharides indicating its possible use in biorefinery and bio-ethanol industry. Further, the catalytic efficiency of the purified cellulase was enhanced by metal ions and surfactants which might attract attention for its exploitation in industrial usage. The future endeavors will be to thoroughly characterize the *B. tequilensis* G9 for efficient production of bio-ethanol from agro-industrial wastes through fermentation technologies.

Conflicts of interest

The authors declare no conflict of interest with respect to the research, authorship, and/or publication of this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101219.

References

Adhyaru, D.N., Bhatt, N.S., Modi, H.A., 2014. Enhanced production of cellulase-free, thermo-alkali-solvent-stable xylanase from *Bacillus altitudinis* DHN8, its characterization and application in sorghum straw saccharification. Biocatalysis. Agri. Biotechnol. 3, 182–190. https://doi.org/10.1016/j.bcab.2013.10.003.

Asha, Malini, B., Revathi, M., Yadav, A., Sakthivel, N., 2012. Purification and characterization of a thermophilic cellulase from a novel cellulolytic strain, *Paenibacillus barcinonensis*. J. Microbiol. Biotechnol. 22, 1501–1509. https://doi.org/10.4014/jmb.1202.02013.

Bagewadi, Z.K., Mulla, S.I., Ninnekar, H.Z., 2016. Purification and characterization of endob-1,4-D-glucanase from Trichoderma harzianumstrain HZN11 and its application in production of bioethanol from sweet sorghum bagasse. 3 Biotech 6, 101. https://doi.org/10.1007/s13205-016-0421-y.

Bajaj, B.K., Pangotra, H., Wani, A.M., Sharma, P., Sharma, A., 2009. Partial purification and characterization of a highly thermostable and pH stable endoglucanase from a newly isolated *Bacillus* strain M-9. Indian J. Chem. Technol. 16, 382–387.

Bakare, M.K., Adewale, I.O., Ajayi, A., Shonukan, O.O., 2005. Purification and characterization of cellulase from the wild-type and two improved mutants of *Pseudomonas fluorescens*. Afr. J. Biotechnol. 4, 898–904.

Bhat, M.K., 2000. Cellulases and related enzymes in biotechnology. Biotechnol. Adv. 18, 355–383. https://doi.org/10.1016/S0734-9750(00)00041-0.

Bhuyan, A.K., 2009. On the mechanism of SDS-induced protein denaturation. Biopolymers 93, 186–199. https://doi.org/10.1002/bip.21318.

Binod, P., Satyanagalakshmi, K., Sindhu, R., Janu, K.U., Sukumaran, R.K., Pandey, A., 2012. Short duration microwave assisted pretreatment enhances the enzymatic saccharification and fermentable sugar yield from sugarcane bagasse. Renew. Energy 37, 109–116. https://doi.org/10.1016/j.renene.2011.06.007.

Bischoff, K.M., Rooney, A.P., Li, X.L., Liu, S., Hughes, S.R., 2006. Purification and characterization of a family 5 endoglucanase from a moderately thermophilic strain of *Bacillus licheniformis*. Biotechnol. Lett. 28, 1761–1765. https://doi.org/10.1007/s10529-006-9153-0.

Bounias, M., 1980. N-(1-Naphthyl)ethylenediamine Elihydrochloride as a new reagent for nanomole quantification of sugars on thin-layer plates by a mathematical calibration process. Anal. Biochem. 106, 291–295.

Chidi, S.B., Godana, B., Ncube, I., Van Rensburg, E.J., Cronshaw, A., Abotsi, E.K., 2008. Production, purification and characterization of celullase-free xylanase from *Aspergillus terreus* UL 4209. Afr. J. Biotechnol. 7, 3939–3948.

Dar, M.A., Pawar, K.D., Jadhav, J.P., Pandit, R.S., 2015. Isolation of cellulolytic bacteria from the gastro- intestinal tract of *Achatina fulica* (Gastropoda: pulmonata) and their evaluation for cellulose biodegradation. Int. Biodeterior. Biodegrad. 98, 73–80. https://doi.org/10.1016/j.ibiod.2014.11.016.

Dar, M.A., Pawar, K.D., Pandit, R.S., 2018. Prospecting the gut fluid of giant African land snail, *Achatina fulica* for cellulose degrading bacteria. Int. Biodeterior. Biodegrad. 126, 103–111. https://doi.org/10.1016/j.ibiod.2017.10.006.

Demirev, P.A., Ho, Y., Ryzhov, V., Fenselau, C., 1999. Microorganism identification by mass spectrometry and protein database searches. Anal. Chem. 71, 2732–2738. https://doi.org/10.1021/ac990165u.

Dienes, D., Egyhazi, A., Reczey, K., 2004. Treatment of recycled fiber with *Trichoderma* cellulases. Ind. Crops Prod. 20, 11–21. https://doi.org/10.1016/j.indcrop.2003.12.009.

Ekperigin, M.M., 2007. Preliminary studies of cellulase production by *Acinetobacter anitratus* and *Branhamella* sp. Afr. J. Biotechnol. 6, 28–33.

Eppinger, M., Bunk, B., Johns, M.A., Edirisinghe, J.N., Kutumbaka, K.K., Koenig, S.S.K., et al., 2011. Genome sequences of the biotechnologically important *Bacillus megaterium* strains QM B1551 and DSM319. J. Bacteriol. 193, 4199–4213. https://doi.org/10.1128/JB.00449-11. PMID: 21705586.

Escobar, J.C., Lora, E.S., Venturini, O.J., Yáñez, E.E., Castillo, E.F., Almazan, O., 2009. Biofuels: environment, technology and food security. Renew. Sustain. Energy Rev. 13, 1275–1287. https://doi.org/10.1016/j.rser.2008.08.014.

Fujii, T., Fang, H., Inoue, H., Murakami, K., Sawayama, S., 2009. Enzymatic hydrolyzing performance of *Acremonium cellulolyticus* and *Trichoderma reesei* against three lignocellulosic materials. Biotechnol. Biofuels 2, 24. https://doi.org/10.1186/1754-6834-2-24.

Gabani, P., Copeland, E., Chandel, A.K., Singh, O.V., 2012. Ultraviolet-radiation-resistant isolates revealed cellulose-degrading species of *Cellulosimicrobium cellulans* (UVP1) and *Bacillus pumilus* (UVP4). Biotechnol. Appl. Biochem. 59, 395–404. https://doi.org/10.1002/bab.1038.

George, P.S., Ahmad, A., Rao, M.B., 2001. Studies on carboxymethyl cellulase produced by an alkalo-thermophilic actinomycetes. Bioresour. Technol. 77, 171–175. https://doi.org/10.1016/S0960-8524(00)00150-4.

Girfoglio, M., Rossi, M., Cannio, R., 2012. Cellulose degradation by *Sulfolobus solfataricus* requires a cell anchored endo- β-1-4-glucanase. J. Bacteriol. 194, 5091–5100. https://doi.org/10.1128/JB.00672-12.

Hakamada, Y., Endo, K., Takizawa, S., Kobayashi, T., Shirai, T., Yamane, T., Ito, S., 2002. Enzymatic properties, crystallization, and deduced amino acid sequence of an alkaline endoglucanase from *Bacillus circulans*. Biochim. Biophys. Acta 1570, 174–180. PMID: 12020807. https://doi.org/10.1016/S0304-4165(02)00194-0.

Han, S.J., Yoo, Y.J., Kang, H.S., 1995. Characterization of bifunctional cellulase and its structural gene. The cell gene of *Bacillus* sp. D04 has exo and endoglucanase activity. J. Biol. Chem. 270, 26012–26019. https://doi.org/10.1074/jbc.270.43.26012.

Haque, M.A., Barman, D.N., Kang, T.H., Kim, M.K., Kim, J., Kim, H., Yun, H.D., 2013. Effect of dilute alkali pretreatment on structural features and enhanced enzymatic hydrolysis of *Miscanthus sinensis* at boiling temperature with low residence time. Biosyst. Eng. 114, 294–305. https://doi.org/10.1016/j.biosystemseng.2013.01.006.

Kacurakova, M., Smith, A.C., Gidley, M.J., Wilson, R.H., 2002. Molecular interactions in bacterial cellulose composites studied by 1D FT-IR and dynamic 2D FTIR spectroscopy. Carbohydr. Res. 337, 1145–1153. https://doi.org/10.1016/S0008-6215(02)00102-7.

Kafle, K., Shin, H., Lee, C.M., Park, S., Kim, S.H., 2015. Progressive structural changes of Avicel, bleached softwood, and bacterial cellulose during enzymatic hydrolysis. Sci. Rep. 5, 15102. https://doi.org/10.1038/srep15102.

Kar, K., Cheng, W., 2011. Using mass spectrometry to detect ethanol and acetaldehyde emissions from a direct injection spark ignition engine operating on ethanol/gasoline blends. SAE International 2011–01–11959, 1–14. https://doi.org/10.4271/2011-01-1159.

Kim, B.K., Lee, B.H., Lee, Y.J., Jin, I.H., Chung, C.H., Lee, J.W., 2009. Purification and characterization of carboxymethylcellulase isolated from a marine bacterium, *Bacillus subtilis* sub sp. *subtilis* A-53. Enzym. Microb. Technol. 44, 411–416. https://doi.org/10.1016/j.enzmictec.2009.02.005.

Kim, J.Y., Hur, S.H., Hong, J.H., 2005. Purification and characterization of an alkaline cellulase from a newly isolated alkalophilic *Bacillus* sp. HSH-810. Biotechnol. Lett. 27, 313–316. https://doi.org/10.1007/s10529-005-0685-5.

Kumar, M., Singh, P., Sukla, L.B., 2016. Addition of expansin to cellulase enhanced bioethanol production. Process Biochem. 51, 2097–2103. https://doi.org/10.1016/j.procbio.2016.09.012.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A.M., Alloni, G., Azevedo, V., et al., 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature 390, 249–256. https://doi.org/10.1038/36786. PMID: 9384377.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685. https://doi.org/10.1038/227680a0.

Lamed, R., Tormo, J., Chirino, A.J., Morag, E., Bayer, E.A., 1994. Crystallization and preliminary X-ray analysis of the major cellulose-binding domain of the cellulase from *Clostridium thermocellum*. J. Mol. Biol. 244, 236–237. https://doi.org/10.1006/jmbi.1994.1721.

Lee, Y.J., Kim, B.K., Lee, B.H., Jo, K.I., Lee, N.K., Chung, C.H., Lee, Y.C., Lee, J.W., 2008. Purification and characterization of cellulase produced by *Bacillus amyloliquefaciens* DL-3 utilizing rice hull. Bioresour. Technol. 99, 378–386. https://doi.org/10.1016/j.biortech.2006.12.013.

Li, Y.H., Ding, M., Wang, J., Xu, G.J., Zhao, F., 2006. A novel thermos-acidophilic endoglucanase, Ba-EGA, from a new cellulose-degrading bacterium, *Bacillus* sp. AC-1. Appl. Microbiol. Biotechnol. 70, 430–436. https://doi.org/10.1007/s00253-005-0075-x.

Lowry, O.H., Rosebrough, N.J., Farri, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

Lu, W.J., Wang, H.T., Yang, S.J., Wang, Z.C., Nie, Y.F., 2005. Isolation and characterization of mesophilic cellulose-degrading bacteria from flower stalks-vegetable waste co-composting system. J. Gen. Appl. Microbiol. 51, 353–360. PMID: 16474195. https://doi.org/10.2323/jgam.51.353.

Lusterio, D.D., Suizo, F.G., Labunos, N.M., Valledor, M.N., Ueda, S., Kawai, S., Koike, K., Shikata, S., Yoshimatsu, T., Ito, S., 1992. Alkali-resistant, alkaline endo-1, 4-b-glucanase produced by *Bacillus* sp. PKM-5430. Biosci. Biotechnol. Biochem. 56, 1671–1672. https://doi.org/10.1271/bbb.56.1671.

Lynd, L.R., Cushman, J.H., Nichols, R.J., Wyman, C.E., 1991. Fuel ethanol from cellulosic biomass. Science 251, 1318–1323. https://doi.org/10.1126/science.251.4999.1318.

Ma, L., Yang, W., Meng, F., Ji, S., Xin, H., Cao, B., 2015. Characterization of an acidic cellulase produced by *Bacillus subtilis* BY-4 isolated from gastrointestinal tract of Tibetan pig. J. Taiwan. Institute. Chem. Eng. 56, 67–72. https://doi.org/10.1016/j.jtice.2015.04.025.

Maki, M., Leung, K.T., Qin, W., 2009. The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. Int. J. Biol. Sci. 5500–516 PMID: PMC2726447.

Mawadza, C., Hatti-Kaul, R., Zvauya, R., Mattiasson, B., 2000. Purification and characterization of cellulases produced by two *Bacillus* strains. J. Biotechnol. 83, 177–187. https://doi.org/10.1016/S0168-1656(00)00305-9.

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

Nieves, R.A., Ehrman, C.I., Adney, W.S., Elander, R.T., Himmel, M.E., 1998. Technical communication: survey and analysis of commercial cellulase preparations suitable for biomass conversion to ethanol. World J. Microbiol. Biotechnol. 14, 301–304. https://doi.org/10.1023/A:1008871205580.

Ogawa, A., Suzumatsu, A., Takizawa, S., Kubota, H., Sawada, K., Hakamada, Y., Kawai, S., Kobayashi, T., Ito, S., 2007. Endoglucanase from *Paenibacillus* spp. from a new clan in glycoside hydrolase family 5. J. Biotechnol. 129, 406–414. https://doi.org/10.1016/j.jbiotec.2007.01.020.

Oke, M.A., Suffian, M., Annuar, M., Simarani, K., 2017. Mixed lignocellulosic biomass degradation and utilization for bacterial cellulase production. Waste biomass valor 8, 893–903. https://doi.org/10.1007/s12649-016-9595-0.

Otajevwo, F.D., Aluyi, H.A.S., 2011. Cultural conditions necessary for optimal cellulase yield by cellulolytic bacterial organisms as they relate to residual sugars released in broth medium. Mod. Appl. Sci. 5, 141–151. https://doi:10.5539/mas.v5n3p141.

Pérez, J., Muñoz-Dorado, J., de la Rubia, T., Martínez, J., 2002. Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. Int. Microbiol. 5, 53–63. https://doi.org/10.1007/s10123-002-0062-3.

Petzold, K., Schwikai, K., Heinze, T., 2006. Carboxymethyl xylan-synthesis and detailed structure characterization. Carbohydr. Polym. 64, 292–298. https://doi.org/10.1016/j.carbpol.2005.11.037.

Rastogi, G., Bhalla, A., Adhikari, A., Bischoff, K.M., Hughes, S.R., Christopher, L.P., Sani, R.K., 2010. Characterization of thermo-stable cellulases produced by *Bacillus* and *Geobacillus* strains. Bioresour. Technol. 101, 8798–8806. https://doi.org/10.1016/j.biortech.2010.06.001.

Sadhu, S., Saha, P., Sen, S.K., Mayilraj, S., Maiti, T.K., 2013. Production, Purification and Characterization of a Novel Thermotolerant Endoglucanase (CMCase) from Bacillus Strain Isolated from Cow Dung, vol. 2. Springer Plus, pp. 1–10. https://doi.org/10.1186/2193-1801-2-10.

Sangbriba, R.U., Duan, C.J., Tang, J.L., 2006. Isolation and characterization of mesophilic *Bacillus* species cellulase genes from black liquor. Bioresour. Technol. 14, 2727–2733.

Saratale, G.D., Oh, M.K., 2015. Improving alkaline pretreatment method for preparation of whole rice waste biomass feedstock and bioethanol production. RSC Adv. 5, 97171–97179. https://doi.org/10.1039/C5RA17797A.

Satyamurthy, P., Jain, P., Balasubramanya, R.H., Vigneshwaran, N., 2011. Preparation and characterization of cellulose nano whiskers from cotton fibres by controlled microbial hydrolysis. Carbohydr. Polym. 83, 122–129. https://doi.org/10.1016/j.carbpol.2010.07.029.

Schulthess, B., Bloemberg, G.V., Zbinden, R., Böttger, E.C., Hombach, M., 2014. Evaluation of the Bruker MALDI Biotyper for identification of gram positive rods: development of a diagnostic algorithm for the clinical laboratory. J. Clin. Microbiol. 52, 1089–1097. https://doi.org/10.1128/JCM.02399-13.

Segal, L., Creely, J.J., Martin Jr., A.E., Conrad, L.M., 1959. An empirical method for estimating the degree of crystallinity of native cellulose using X-ray diffractometer. Textil. Res. J. 29, 786–794. https://doi.org/10.1177/004051755902901003.

Sriariyanun, M., Tantayotai, P., Yasurin, P., Pornwongthong, P., Cheenkachorn, K., 2016. Production, purification and characterization of an ionic liquid tolerant cellulase from *Bacillus* sp. isolated from rice paddy field soil. Electron. J. Biotechnol. 19, 23–28. https://doi.org/10.1016/j.ejbt.2015.11.002.

Sun, Y., Cheng, J., 2003. Hydrolysis of lignocellulosic materials for ethanol production: a review. Chem. Inf. 34, 1–11. https://doi.org/10.1016/S0960-8524(01)00212-7.

Wahl, K.L., Wunschel, S.C., Jarman, K.H., Valentine, N.B., Petersen, C.E., Kingsley, M.T., Zartolas, K.A., Saenz, A.J., 2002. Analysis of microbial mixtures by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Anal. Chem. 74, 6191–6199. https://doi.org/10.1021/ac0203847.

Wilson, D.B., Irwin, D., 1999. Genetics and properties of cellulases. In: In: Tsao, G.T. (Ed.), Recent Progress in Bioconversion of Lignocellulosics. Advances in Biochemical Engineering/biotechnology, vol. 65. Springer, Berlin, Heidelberg, pp. 1–21. https://doi.org/10.1007/3-540-49194-5_1.

Wu, J., Ju, L.K., 1998. Enhancing enzymatic saccharification of waste newsprint by surfactant addition. Biotechnol. Prog. 14, 649–652. https://doi.org/10.1021/bp980040v.

Xu, K., Feng, J., Zhong, T., Zheng, Z., Chen, T., 2015. Effects of volatile chemical components of wood species on mould growth susceptibility and termite attack resistance of wood plastic composites. Int. Biodeterior. Biodegrad. 100, 106–115. https://doi.org/10.1016/j.ibiod.2015.02.002.

Yang, J.E., Kim, J.K., Lee, S.H., Yu, J., Kim, K.H., 2017. Evaluation of commercial cellulase preparations for the efficient hydrolysis of hydrothermally pretreated empty fruit bunches. BioRes 12, 7834–7840.

Zaldivar, M., Velasquez, J.C., Contreras-Perez, L.M., 2001. *Trichoderma aureovi* aureoviride 7–121, a mutant with enhanced production of lytic enzymes: its potential use in waste cellulose degradation. J. Biotechnol. 4, 1–6. https://doi.org/10.2225/vol4-issue3-fulltext-7.

Zhao, K., Guo, L.Z., Lu, W.D., 2012. Extracellular production of novel halotolerant, thermostable and alkali-stable carboxymethyl cellulase by marine bacterium *Marinimicrobium* sp. LS-A18. Appl. Biochem. Biotechnol. 168, 550–567. https://doi.org/10.1007/s12010-012-9796-3.

Zhao, X., Zhang, L., Liu, D., 2012. Biomass recalcitrance. Part I: the chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocellulose. Biofuels Bioprod. Bioref. 6, 465–482. https://doi.org/10.1002/bbb.1331.

Zuroff, T.R., Xiques, S.B., Curtis, W.R., 2013. Consortia-mediated bioprocessing of cellulose to ethanol with a symbiotic *Clostridium phytofermentans*/yeast co-culture. Biotechnol. Biofuels 6, 59. https://doi.org/10.1186/1754-6834-6-59.