A New Salt-Tolerant Thermostable Cellulase from a Marine *Bacillus* sp. Strain

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A salt-tolerant cellulase secreted by a marine *Bacillus* sp. SR22 strain with wide resistance to temperature and pH was purified and characterized. Its approximate mass was 37 kDa. The endoglucanase, named as Bc22Cel, was purified by ammonium sulfate precipitation, gel filtration chromatography, and extraction from the gel after non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The optimal pH value and temperature of Bc22Cel were 6.5 and 60°C, respectively. The purified Bc22Cel showed a considerable halophilic property, being able to maintain more than 70% of residual activity even when pre-incubated with 1.5 M NaCl for 1 h. Kinetic analysis of the purified enzyme showed the K_m and V_{max} to be 0.704 mg/ml and 29.85 $\mu\text{mol}\cdot\text{ml}^{-1}\cdot\text{min}^{-1}$, respectively. Taken together, the present data indicate Bc22Cel as a potential and useful candidate for industrial applications, such as the bioconversion of sugarcane bagasse to its derivatives.

Keywords: Sugarcane bagasse-degrading bacteria, thermostable enzymes, bioprocessing

Introduction

In the early 1970s, an energy crisis renewed the interest worldwide for bioresources aiming at a sustainable alternative for the synthesis of fuels [1]. Some countries, such as Brazil, have managed to provide new manufacturing concepts for converting sugarcane to valuable fuels and products. The decade that followed was featured by a notable increase of the bioethanol car industries in Brazil, emerging a different perspective on the energy challenges and inspiring new research on biomass conversion.

It is undeniable that the actual demand for finite petroleum resources cannot be satisfactory for a long-term projection. Meanwhile, focusing in biotechnology to improve the biological conversion of biomass may help the shifting of petroleum dependence to renewable resources and contribute to the development of a sustainable society.

Lately, one of the promising innovations of environment-friendly renewable sources is the second-generation bioethanol

produced from lignocellulosic material ordinarily discarded in agro-industrial processes [2, 3], and specialists have foreseen that the technology may positively impact the world energy scenario in the medium term.

Cellulose (β -1,4-linked glucose residues) is the most abundant polysaccharide on the biosphere and represents a promising bioresource, with the average annual production estimated at 4×10^9 tons [4]. Nevertheless, only a small portion of organisms are able to carry out its complete decomposition owing to the recalcitrance of the structure (and isomerism) of its glycosidic linkages [5, 6], requiring a pool of at least three different classes of enzymes acting synergistically to degrade the polysaccharide from the macrostructure until the release of isolated units of glucose [7].

Initially, endo- β -glucanases (E.C. 3.2.1.4) are able to cleave the cellulosic structure in internal sites, generating a large number of ends where exo- β -glucanases (E.C. 3.2.1.91) are able to act. These last enzymes are responsible for degrading

the reducing and non-reducing ends of cellulose to small glucose oligomers (cellobiose and cellodextrins), which in turn are hydrolyzed to single glucose units by enzymes denominated by β -glycosidases (E.C. 3.2.1.21) [8].

The enhanced cost of second generation bioethanol has lately moved research attention to new sources of cellulase as an important tool in depolymerization pretreatment of lignocellulosic biomass. Cellulases represent approximately 10% of the world market for enzymes with industrial application in a setting that is still booming [9]. One of the most important cellulase producers, bacteria, constitute a promising group for bioprospection due to their large ecological diversity, adaptation to extreme conditions of temperature, salinity, and pH, and the possibility to genetically engineer and optimize their secretome. Several genera of bacteria had already been characterized in the literature as extracellular cellulase producers, including *Acetivibrio*, *Alteromonas*, *Clostridium*, *Cellulomonas*, *Ruminococcus*, and *Bacillus* [10].

The microorganisms belonging to genus *Bacillus* include a wide spectra and ubiquitous group of bacteria that can be found from the forest soil, to marine ecosystems occurring in association with a variety of aquatic organisms such as scleractinian corals present in the intertidal boulders. Therefore, these microorganisms are exposed to various abiotic stresses that cause ecological selection for a physiologically adapted microbiota to such extremes of temperature and salinity. In this work, we isolated and characterized a cellulase from a marine bacterium associated with scleractinian corals and evaluated its potential for industrial purposes.

Materials and Methods

Isolation, Identification, and Screening of Cellulase-Producing Bacteria

The bacterial strains were obtained from aseptically collected tissues of different *Siderastrea stellata* colonies at Cabo Branco coral reefs, Paraiba State, Brazil (7°08'50"S; 34°47'51"W). For bacterial isolation, ecto- and mesoderms from the anthozoan were suspended in sterile saline solution, agitated until homogenization, and then spread over marine agar plates (pH 7.0) containing 0.5% peptone, 0.1% yeast extract, and 1.5% agar diluted in sterile marine water and incubated at 40°C until adequate growth. Each colony was individually isolated and phenotypically identified before being screened for its cellulolytic activity. The strains were grown for 48 h at 40°C on pH 7.0 carboxymethylcellulose (CMC) agar plates (containing 0.1% CMC; 0.05% NaNO₃; 0.1% K₂HPO₄; 0.05% MgSO₄·7H₂O; 0.001% FeSO₄·7H₂O; 0.1% Yeast extract; 1.5% agar) and then overlaid with 0.1% Congo-Red solution for 30 min and

washed with 1 M NaCl for equal time as well as stained with 0.2% potassium iodine for 5 min. Bacterial colonies showing clear zones were considered to be cellulase producers and selected for agro-waste degradation experiments [11].

Bacterial Molecular Identification

In order to identify the cellulase producer isolates, the 16S rRNA gene sequence was amplified from the previously extracted DNA. Bacterial universal primers 26F (5'-GAGTTTGATCMTGGCTCAG-3') and 1492R (5'-ACGGCTACCTTGTTACGACTT-3') [12] were used to amplify the 16S rDNA gene by polymerase chain reaction (PCR) performed in a MWG-Biotech Primus 96 Plus Thermal Cycler (Primus, USA). Each reaction mixture (50 μ l) contained reaction buffer, 2 mM MgCl₂, 0.2 μ M of each primer, 0.2 mM of dNTPs, 2U of Taq DNA polymerase (Invitrogen, USA), and 40 ng of genomic DNA. The 1.5 Kb amplification products were directly purified from the PCR using the AxyPrep DNA Gel Extraction kit (AxiGen Biosciences, USA) according to the manufacturer's instructions. Purified reactions were sequenced by ACTGene (UFRGS, Porto Alegre, RS, Brazil) using the automated sequencer ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems, USA). The obtained 16S rRNA gene sequence for the SR22 isolate was compared with deposited sequences in the GenBank database (NCBI). For the local alignment, which was based on a BLASTn search and bootstrapping of 1.000 replicates of the 16S rRNA gene sequence, MEGA 6.0 software was used for multiple sequence monitoring and for the construction of a dendrogram by the neighbor-joining method [13].

Effect of Agro-Waste Material and Salt Tolerance on Cellulase Production

To evaluate the effect of sugarcane bagasse as the sole carbon source on cellulase production, one CMC-degrading isolate (afterward denominated *Bacillus* sp. SR22) was selected to grow on basal medium containing 0.05% ammonium sulfate and 2.0% (w/v) sugarcane bagasse supplemented with crescent molarity of NaCl (0–2 M) for 48 h at 40°C, and then stained with 0.2% potassium iodine as previously described.

Crude Enzyme Production and Enzymatic Assay

In order to obtain the crude enzyme, cultures of *Bacillus* sp. SR22 were first grown in 100 ml of CMC broth. After the exponential phase of growth, the cells from seed cultures were re-inoculated (aliquot of 500 μ l at 0.5 McFarland standard suspension) in 500 ml of medium, and the inoculated flasks were incubated at 40°C under shaking conditions (2.5 G-force) for a period of 1–4 days and then centrifuged at 1.118 G-force for 10 min at 4°C in order to collect supernatant. The sample was freezed at –20°C. Cellulase was assayed by measuring the reducing sugar released by 3,5-dinitrosalicylic acid (DNS) [14]. Briefly, 500 μ l of substrate (1% soluble CMC in 0.05 M phosphate buffer, pH 7.0) and 500 μ l of crude enzyme were incubated for 60 min at 40°C, and the reaction was stopped by adding 1 ml of DNS followed by boiling for

5 min. The optical density was taken at 520 nm in a microtiter reader (BioTek, USA). One unit of enzyme activity was defined as the amount of enzyme that liberates 1.0 μmol of glucose per minute.

Purification Procedure

The crude enzyme supernatant from *Bacillus* sp. SR22 culture was precipitated overnight with 60–90% $(\text{NH}_4)_2\text{SO}_4$ and the pellet was recovered by centrifugation at 1.500 G-force for 30 min. After ammonium sulfate precipitation, the sample was solubilized in pure water. Dialysis was performed against this same solvent in order to remove all salts before storing the lyophilized enzyme for the next experiments. The concentrated sample was applied to a G-75 Superdex column (GE Healthcare, USA) on an FPLC system equilibrated with borax buffer (200 mM, pH 7.0) and the flow rate was 15 ml/h. Tubes containing high cellulosic activity within the same chromatographic peak were jointed, lyophilized, and rechromatographed at the same conditions. In order to concentrate the purified enzyme, the rechromatographed sample was submitted to 12.5% SDS-PAGE containing 0.2% CMC, followed by silver and activity staining. Then, the gel was cut into two parts for local identification of the cellulase position, and its corresponding band was spotted in order to eluate the purified enzyme, which was denominated Bc22Cel. The protein concentration was determined by the Bradford method using bovine serum albumin as a standard [15].

Polyacrylamide Gel Electrophoresis, Protein Weight Determination, and Activity Staining

The purified protein from the culture supernatant were observed by SDS-PAGE according to Laemmli [16]. The protein bands also were visualized by silver staining [17]. The approximate molecular mass of the cellulase was estimated by SDS-PAGE using as reference the molecular mass marker Spectra Multicolor Broad Range (Thermo Scientific, USA). For activity staining, samples were separated by SDS-PAGE at 4°C in a gel containing 0.2% CMC; after running, the gel was washed with 0.05 M acetate buffer (pH 6.5) containing 0.1% Triton X-100 for 30 min in order to remove the SDS. After that, the sample was washed in distilled water for equal time, followed by soaking the gel for 3 h at 60°C in 0.05 M acetate buffer (pH 6.5). Subsequently, the gel was stained with 0.1% Congo-Red dye for 30 min and the excess dye was removed by 1 M NaCl until the appearance of clear bands demonstrating that CMC was hydrolyzed, as previously described [8].

Effect of pH, Temperature and Halophilic Properties of Bc22Cel

The relative activity and stability of purified Bc22Cel were assayed by measuring the enzymatic activity at varying pH (ranging 3–9) and temperatures (ranging 20–80°C) as well as in different NaCl molarities. In order to evaluate the effect of pH on enzymatic activity, the reaction was conducted at 40°C for 60 min in the following buffers with 1% CMC: citrate-phosphate buffer (pH 3.0–6.0), sodium acetate buffer (pH 6.5–7.0), and glycine-NaOH

buffer (pH 8.0–9.0). To evaluate the pH stability, Bc22Cel was pre-incubated in different pH buffers for 60 min at 4°C and the residual activity was measured in sodium acetate buffer (pH 7.0) for 60 min at 40°C.

The thermal activity was determined by incubating the purified enzyme in sodium acetate buffer (pH 7.0) like previously described with 1% CMC at different temperatures for 60 min and the stability was verified by pre-incubating for 30 min at the above specified temperatures following by the measurement of the residual activity. The effect of NaCl was evaluated by incubating the Bc22Cel in pH 7.0 buffer with 1% CMC in a crescent saline molarity (0–2 M) for 60 min at 40°C. All enzymatic activity was determined by Miller's method using DNS [14].

Enzymatic Kinetics

The influence of substrate concentration on the reaction velocity was determined by incubating the purified Bc22Cel in a crescent concentration of CMC ranging from 0.25 to 4.0 mg/ml. For each CMC concentration, the enzymatic activity was assayed under standard conditions (sodium acetate buffer, pH 7.0, at 40°C for 1 h). K_m and V_{max} were determined from the Lineweaver-Burk plot.

Results and Discussion

Bacterial Identification

Sixty-nine bacterial strains were isolated from *Siderastrea stellata* tissue samples collected at Cabo Branco shore, and among those, only 10 strains were positive at cellulolytic screening on CMC agar plates, with one strain (named SR22) showing the best growth and cellulolytic activity even at high salt concentrations chosen for further studies. The SR22 isolate showed to be a gram-positive spore-forming bacilli, facultative anaerobe, and catalase positive, as well as negative for indole, H_2S production, and citrate utilization (Table 1). The same morphological characteristics were found by previous studies with cellulolytic *Bacillus*

Table 1. Morphological and biochemical characteristics of isolated *Bacillus* sp. SR22.

Parameter	Result
Gram staining	Positive
Morphology	Bacillus
Arrangement	Ausent
Endospore	Positive
Catalase	Positive
Urease	Negative
Citrate utilization	Negative
H_2S production	Negative
Indole production	Negative

Fig. 1. Phylogenetic tree of isolated SR22 and other related species based on 16S rDNA sequences, constructed using the maximum-likelihood principle.

The scale bar represents 0.01 substitutions per site. The evolutionary distances were computed using the Kimura two-parameter method. Bootstrap values are indicated at the branches from 1.000 replications. GenBank accession numbers of the sequences are given in parentheses.

sp. strains [6, 18, 19]. Those findings led us to consider the isolate belonging to the genus *Bacillus*, which was confirmed by the phylogenetic analysis, which revealed that the SR22 strain formed a clade with *Bacillus subtilis* (Fig. 1). Its nucleotide sequence was deposited in GenBank as Accession No. MH119099 and the degree of sequence similarity of strain SR22 to *Bacillus* sp. was 99%.

Bacterial Growth on CMC and Agro-Waste Material with Crescent NaCl Molarity

The growth of the SR22 marine isolate on CMC and sugarcane bagasse as sole carbon source was compared on solid media at different salinity ranges (Fig. 2). The isolate was able to grow on high NaCl molarities on both media ranging from 0 to 2.0 M on CMC and 0 to 1.5 M on sugarcane

Fig. 2. *Bacillus* sp. SR22 culture containing bagasse (top row) and carboxymethylcellulose (bottom row) as sole carbon sources under crescent saline molarity.

Halos around bacterial colonies are indicative of cellulose degradation.

bagasse agar plates, showing a remarkable property to access and degrade sugarcane bagasse even at stressful saline conditions, like 4-fold greater than the ocean's NaCl molarity (where this organism is supposed to occur), thus demonstrating a considerable capacity to survive and produce its hydrolytic enzymes, like previously described for *Bacillus* species [20]. The occurrence of this polysaccharide in the marine environment as a constituent of the cell wall of many organisms could explain the predisposition of cellulolytic bacterial isolates to degrade that kind of substrate. Those microorganisms play an important role in the biogeochemical cycle of carbon as decomposers in marine ecosystems [21].

Purification of Bc22Cel from *Bacillus* sp.

The extracellular proteins from *Bacillus* sp. culture were fractionated and the cellulase activity was detected in the 60–90% ammonium-sulfate fraction. After a successive dialysis, the sample was concentrated and loaded onto a

Fig. 3. Purification and enzyme activity of Bc22Cel.

Bc22Cel chromatographic purification profile on a Superdex 75 10/300 GL column (A) equilibrated with borax buffer (200 mM, pH 7.0). Molecular exclusion chromatography (B) showing a unique peak when the protein concentration (closed circles) and endo-β-1,4-glucanase activity (opened circles) were measured.

gel filtration chromatography (Table 2) where the cellulolytic activity was detected in a single chromatographic peak (Fig. 3A). The sample was rechromatographed and a single protein signal was also detected with cellulolytic activity (Fig. 3B), and SDS-PAGE showed a single band (Fig. 4) with an approximate molecular mass of 37 kDa, similar to a previously found cellulase from soil bacterium *B. licheniformis* [22] but differing in saline resistance and kinetic parameters and possessing the ability to degrade CMC, as visualized after zymographic staining of the sample. The protein showed to be a monomer, which is consistent with the molecular pattern previously described of bacterial cellulases, ranging from monomers with 185 kDa from *B. subtilis* to 36 kDa in *Pseudomonas fluorescens* strains [19, 23].

Determination of the Optimal pH and Temperature

The optimal temperature for Bc22Cel activity was estimated to be 60°C, retaining more than 30% of activity at higher temperatures like 80°C (Fig. 5A). When pre-incubated for 1 h at different temperatures, the enzyme showed more than 40% of residual activity until 70°C, demonstrating considerable thermal stability when compared with other purified cellulases [8].

Fig. 4. Size characterization of purified Bc22Cel.

Electrophoretic profile and activity staining of purified Bc22Cel. SDS-PAGE with silver showing the purified cellulase (B) with an approximate molecular mass of 37 kDa (A corresponds to an empty lane). (C) Zymography stained with 0.1% Congo-Red showing enzymatic activity as a single degradation band even after treatment with SDS and Triton X-100. MW, molecular weight markers.

Table 2. Purification summary of the cellulase produced by *Bacillus* sp. SR22.

Purification step	Volume (ml)	Protein concentration (mg/ml)	Total activity (IU)	Specific activity (IU/mg)	Yield (%)
Culture broth	50	0.525	295.36	11.24	100
Ammonium-sulfate fraction	12	0.354	157.76	34.28	53.41
G-75 Superdex	5	0.276	59.38	43.02	20.10

The purified enzyme showed the best activity at pH 6.5 (Fig. 5B), differing considerably from standard ocean's water pH, which is around 8.1 [24, 25], although it was effective in a larger range of pH, retaining more than 80%

of activity at pH 4 as well as being stable (conserving more than 60% of residual activity) after 1 h pre-incubation at pH 4–8. Other cellulases isolated from different microorganisms, including other *Bacillus* strains (like *B. flexus*) with optimum pHs of 7.0 and 10.0, respectively, showed similar properties of conserving their own activity under pH variations [26–29].

Fig. 5. Effects of pH and temperature on Bc22Cel activity.

(A) For the optimal temperature (closed circles), Bc22Cel was incubated at different temperatures (20–80°C) and its stability (opened circles) determined by pre-incubating it for 60 min and then allowing it to react with substrate within standard conditions. (B) For optimal pH (closed circles) estimation, the enzyme was reacted with 1% of CMC at different buffers for 60 min, and to assay the pH stability (opened circles), the enzyme was pre-incubated at different pHs for 1 h and then its residual activity was determined by the standard assay method. Relative activity (the mean $\pm$ SEM) was determined by three replicate assays.

Effect of NaCl on Bc22Cel Activity and Stability

The purified Bc22Cel was stable at higher salt molarities, retaining more than 80% of activity at 1.5 M NaCl (Fig. 6). The enzymatic activity was slightly improved in the presence of low salt concentrations (NaCl 0.5 M), differing from other cellulases described from the *Bacillus* genus [6, 9], and decreased when incubated with more than 1.0 M NaCl. The enzyme activity did not suffer considerable variation when pre-incubated with 0.5–1.5 M NaCl for 1 h, and even with 2.0 M NaCl retained considerable residual activity (more than 80%), showing it to be effective against denaturation by high-saline content solutions.

Kinetic Analysis

To determine the enzyme's kinetic parameters, Bc22Cel

Fig. 6. Activity (closed circles) of Bc22Cel under increasing concentrations of NaCl in the reaction buffer and its stability (opened circles) after 60 min in different NaCl concentrations against 1% (w:v) of CMC.

Relative activity (the mean $\pm$ SEM) was determined by three replicate assays.

Fig. 7. Bc22Cel activity at increasing concentrations of substrate (CMC) showing (A) characteristic hyperbolic behavior corresponding to a Michaelian enzymatic pattern and (B) its corresponding Lineweaver-Burk plot.

was incubated with different concentrations of CMC. The K_m and V_{max} were estimated to be 0.704 mg/ml (a lower value and therefore a high affinity for CMC (Fig. 7) as substrate than other described cellulases) and 29.85 $\mu\text{mol}\cdot\text{ml}^{-1}\cdot\text{min}^{-1}$, respectively [23, 30].

In summary, this study described the isolation of an endo-β-1,4-glucanase, Bc22Cel, from a marine *Bacillus* sp. strain with approximate molecular mass of 37 kDa, characterized by its large pH range and temperature stability as well as a considerable halophilic property even when compared with other previously described endo-β-1,4-glucanases, which makes the enzyme a potential and useful candidate for industrial applications.

The current cutting-edge methodology concerning the 2nd generation of ethanol still requires a technical breakthrough in the biochemical supplies being able to be accessed from natural cellulose polymer aggregates, in order to significantly lower the production costs and accelerate investment and deployment [31].

Further studies are necessary to optimize the large-scale production of this enzyme in order to develop a robust and cost-effective process for the bioprocessing of cellulosic biomass capable of positively impacting the industry of biofuels and value-added bioproducts.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

1. Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, *et al.* 2006. The path forward for biofuels and biomaterials. *Science* **311**: 484-489.
2. Jia L, Gonçalves G, Takasugi Y, Mori Y, Noda S, Tanaka T, *et al.* 2015. Effect of pretreatment methods on the synergism of cellulase and xylanase during the hydrolysis of bagasse. *Bioresour. Technol.* **185**.
3. Dantur KI, Enrique R, Welin B, Castagnaro AP. 2015. Isolation of cellulolytic bacteria from the intestine of *Diatraea saccharalis* larvae and evaluation of their capacity to degrade sugarcane biomass. *AMB Exp.* **5**: 15.
4. Yin L-J, Lin H-H, Xiao Z-R. 2010. Purification and characterization of a cellulase from *Bacillus subtilis*. *J. Mar. Sci. Technol.* **18**: 466-471.
5. Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. 2002. Microbial cellulose utilization: fundamentals and biotechnology. *Bioresour. Technol.* **66**: 506-577.
6. Rastogia G, Bhalla A, Adhikari A, Bischoff KM, Hughes SR, Christopher LP, *et al.* 2010. Characterization of thermostable cellulases produced by *Bacillus* and *Geobacillus* strains. *Bioresour. Technol.* **101**: 8798-8806.
7. Sun Y, Cheng J. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresour. Technol.* **83**: 1-11.
8. An T, Dong Z, Lv J, Liu Y, Wang M, Wei S, *et al.* 2015. Purification and characterization of a salt-tolerant cellulase from the mangrove oyster, *Crassostrea rivularis*. *Acta Biochim. Biophys. Sin. (Shanghai)* **47**: 299-305.
9. Gaur R, Tiwari S. 2015. Isolation, production, purification and characterization of an organic-solvent-thermostable alkalophilic cellulase from *Bacillus vallismortis* RG-07. *BMC Biotechnol.* **15**: 19.
10. Saratale GD, Saratale RG. 2012. Production and characterization of multiple cellulolytic enzymes by isolated *Streptomyces*

- sp. MDS. *Biomass Bioenergy* **47**: 302-315.
11. Kasana RC, Salwan R, Dhar H, Dutt S, Gulati A. 2008. A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. *Curr. Microbiol.* **57**: 503-507.
 12. Hogg JC, Lehane MJ. 1999. Identification of bacterial species associated with the sheep scab mite (*Psoroptes ovis*) by using amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **65**: 4227-4229.
 13. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, et al. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**: 3389-3402.
 14. Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31**: 426-428.
 15. Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-254.
 16. Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.
 17. Blum H, Beier H, Gross HJ. 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8**: 93-99.
 18. Singh S, Moholkar VS, Goyal A. 2013. Isolation, identification, and characterization of a cellulolytic *Bacillus amyloliquefaciens* strain SS35 from rhinoceros dung. *ISRN Microbiol.* **2013**: 728134.
 19. Rawat R, Tewari L. 2012. Purification and characterization of an acidothermophilic cellulase enzyme produced by *Bacillus subtilis* strain LFS3. *Extremophiles* **16**: 637-644.
 20. Sadhu S, Saha P, Sen SK, Mayilraj S, Maiti TK. 2013. Production, purification and characterization of a novel thermotolerant endoglucanase (CMCase) from *Bacillus* strain isolated from cow dung. *Springerplus* **2**: 10.
 21. Lee B-H, Kim B-K, Lee Y-J, Chung C-H, Lee J-W. 2010. Industrial scale of optimization for the production of carboxymethylcellulase from rice bran by a marine bacterium, *Bacillus subtilis* subsp. *subtilis* A-53. *Enzyme Microb. Technol.* **46**: 38-42.
 22. Bischoff KM, Rooney AP, Li X-L, Liu S, Hughes SR. 2006. Purification and characterization of a family 5 endoglucanase from a moderately thermophilic strain of *Bacillus licheniformis*. *Biotechnol. Lett.* **28**: 1761-1765.
 23. Bakare MK, Adewale IO, Ajayi A, Shonukan OO. 2005. Purification and characterization of cellulase from the wild-type and two improved mutants of *Pseudomonas fluorescens*. *Afr. J. Biotechnol.* **4**: 898-904.
 24. Doney SC, Fabry VJ, Feely RA, Kleypas JA. 2009. Ocean acidification: the other CO₂ problem. *Annu. Rev. Mar. Sci.* **1**: 169-192.
 25. Parker LM, Scanes E, O'Connor WA, Coleman RA, Byrne M, Pörtner HO, et al. 2017. Ocean acidification narrows the acute thermal and salinity tolerance of the Sydney rock oyster *Saccostrea glomerata*. *Mar. Pollut. Bull.* **122**: 263-271.
 26. Ko C-H, Chen W-L, Tsai C-H, Jane W-N, Liu C-C, Tu J. 2007. *Paenibacillus campinasensis* BL11: a wood material-utilizing bacterial strain isolated from black liquor. *Bioresour. Technol.* **98**: 2727-2733.
 27. Pason P, Kyu KL, Ratanakhanokchai K. 2006. *Paenibacillus curdlanolyticus* strain B-6 xylanolytic-cellulolytic enzyme system that degrades insoluble polysaccharides. *Appl. Environ. Microbiol.* **72**: 2483-2490.
 28. Lee Y-J, Kim B-K, Lee B-H, Jo K-I, Lee N-K, Chung C-H, et al. 2008. Purification and characterization of cellulase produced by *Bacillus amyloliquefaciens* DL-3 utilizing rice hull. *Bioresour. Technol.* **99**: 378-386.
 29. Trivedi N, Gupta V, Kumar M, Kumari P, Reddy CRK, Jha B. 2011. An alkali-halotolerant cellulase from *Bacillus flexus* isolated from green seaweed *Ulva lactuca*. *Carbohydr. Polym.* **83**: 891-897.
 30. Raddadi N, Cherif A, Daffonchio D, Fava F. 2013. Halo-alkalitolerant and thermostable cellulases with improved tolerance to ionic liquids and organic solvents from *Paenibacillus tarimensis* isolated from the Chott El Fejej, Sahara desert, Tunisia. *Bioresour. Technol.* **150**: 121-128.
 31. Sims REH, Mabee W, Saddler JN, Taylor M. 2010. An overview of second generation biofuel technologies. *Bioresour. Technol.* **101**: 1570-1580.