Title: A new salt–tolerant thermostable cellulase from a marine Bacillus sp. Strain

Keywords: Sugarcane bagasse–degrading bacteria, thermostable enzymes, bioprocessing
A new salt-tolerant thermostable cellulase from a marine *Bacillus sp.*

**Strain**

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**Running Title:**

A new cellulase for bioconversion of biofuels
ABSTRACT

A salt-tolerant cellulase secreted by a marine *Bacillus* sp. SR22 strain with wide resistance to temperature and pH was purified and characterized. The approximate mass was 37 kDa endoglucanase named as Bc22Cel was purified by ammonium sulphate precipitation, gel filtration chromatography and extraction from the gel after non-reducing sodium dodecylsuflate-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 hour. Kinetic analysis of purified enzyme showed the $K_m$ and $V_{max}$ to be 0.704 mg·mL$^{-1}$ and 29.85 µmol·mL$^{-1}$·min$^{-1}$, respectively. Taking together, the present data indicates the Bc22Cel as a potential and useful candidate for industrial applications such as the bioconversion of sugarcane bagasse on its derivatives.

**Keywords:** Sugarcane bagasse-degrading bacteria; thermostable enzymes; bioprocessing.
INTRODUCTION

On the early 1970s, an energy crisis has renewed the interest worldwide for bioresources aiming a sustainable alternative for the synthesis of fuels [1]. Some countries, i.e. Brazil, have managed to provide new manufacturing concepts for converting sugar cane to valuable fuels and products. The followed decade has been featured by a notable increase of the bioethanol car industries in Brazil, emerging a different perspective on the energy challenges and inspiring new researches 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 (B2G) produced from lignocellulosic material ordinarily discarded at agrindustrial processes [2, 3] and specialists foreseen 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, the average annual production is estimated on 4 x 10^9 tons [4]. Nevertheless only a small portion of organisms are able to carry out its complete decomposition due 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 (EC: 3.2.1.4) are able to cleave the cellulosic structure in internal sites generating a large number of ends where exo-β-glucanases (EC: 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 β- glycosidases
(EC: 3.2.1.21) [8].

Enhancing the cost structure of bioethanol has lately moved research attention into
new sources of cellulase as an important tool in depolymerization pretreatment of
lignocellulosics. 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 genetic engineering 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 *Bacillus* genera includes a wide spectra and
ubiquitous group of bacteria that can be found from the forest soil until marine ecosystems
occurring in association with a variety of aquatic organisms as scleractinian corals present in
the intertidal boulders. Therefore, these microorganisms are exposed to various abiotic
stresses which cause an 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 to scleractinian coral and evaluated its
availability for industrial purposes.

**MATERIAL 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 mesoderm 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; 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 screened for its cellulolytic activity. The strains were grown for 48 h at 40 °C on pH 7.0 in 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, 16S rRNA gene sequence was amplified from previous extracted DNA. Bacterial universal primers 26F (5′-GAGTTTGATCMTGGCTCAG) 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 μL) contained reaction buffer, 2 mM MgCl2, 0.2 μM 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 reaction using the AxyPrep™ DNA Gel Extraction (AxiGen Biosciences, USA) according to the manufacturer’s instructions. Purified reactions were sequenced by the ACTGene (UFRGS, Porto Alegre, RS, Brazil) using an automated sequencer **ABI-PRISM 3100 Genetic Analyzer** (Applied
The obtained 16S rRNA gene sequence for the SR22 isolate was compared to deposited sequences in the Genbank database (NCBI). For the local alignment, which was based on a BLASTn search and bootstrapping of 1000 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 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 sulphate; 2.0% (w:v) sugarcane bagasse supplemented with crescent molarity of NaCl (0 to 2 M) for 48 hour at 40 °C and 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 firstly grown in 100 mL of CMC broth. After the exponential phase of growth, the cells from seed cultures were re-inoculated (aliquot of 500 µL 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 1118 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 DNS [14]. Briefly, 500 µL of substrate (1% soluble CMC in 0.05 M phosphate buffer pH 7.0) and 500 µL of crude enzyme were incubated for 60 min at 40 °C and the reaction stopped by adding 1 mL of 3,5-dinitrosalicylic acid followed by boiling for 5 min. 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 min.mL⁻¹.

**Purification procedure**

The crude enzyme supernatant from *Bacillus sp.* SR22 culture was precipitated overnight with 60-90% (NH₄)₂SO₄ and the pellet recovered by centrifugation at 10000 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 salt before storing the enzyme lyophilized for the next experiments. The concentrated sample was applied to a G-200 Superdex column (GE Healthcare, Wisconsin, USA) on a FPLC system equilibrated with borax buffer (200 mM, pH 7.0) and the flow rate 15 mL.h⁻¹. Tubes containing high cellulosic activity within the same chromatographic peak were jointed, lyophilized and re-chromatographed at the same conditions. In order to concentrate the purified enzyme, the re-chromatographed sample was submitted to a 12.5% SDS polyacrylamide gel electrophoresis (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. Protein concentration was determined by 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 on polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli method [16]. The proteic bands also were visualized by silver revealing [17]. Approximate molecular weight 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 half an hour in order to remove SDS. After that, the sample was washed in distilled water for equal time followed by soaking the gel for 3 h at 60 °C on 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 removed by 1 M NaCl until the appearance of clear bands demonstrating at where CMC was hydrolyzed as previously described [8].

**Effect of pH, temperature and halophilic properties of Bc22Cel**

The relative activity and stability of purified Bc22Cel was assayed by measuring the enzymatic activity at varying pH (ranging from 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), glycine-NaOH buffer (pH 8.0-9.0). To evaluate the pH stability, Bc22Cel was pre-incubated in different pH for 60 min at 4 °C and the residual activity measured in sodium acetate buffer (pH 7.0) for 60 min at 40 °C.

**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 its stability verified by pre-incubating for 30 min at above specified temperatures following by the measurement of the residual activity. The effect of NaCl was evaluated incubating the Bc22Cel in (pH 7.0) with 1% CMC in a crescent saline molarity (0 to 2 M) for 60 min at 40 °C. All enzymatic activity was determined by Miller’s method using 3,5-dinitrosalicylic acid (DNS) [14].
Enzymatic kinetics

The influence of substrate concentration on reaction velocity was determined by incubating the purified Bc22Cel in a crescent concentration of CMC ranging from 0.25 to 4.0 mg.mL\(^{-1}\). 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 Lineweaver-Burk.

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 ten strains were positive at cellulolytic screening on CMC agar plates with one strain (named after SR22) showing the best growth and cellulolytic activity even at high salt concentrations was chosen for further studies. The SR22 isolate showed to be Gram-positive spore-forming bacilli, facultative anaerobe, catalase positive as well as negative for indole, H\(_2\)S production and citrate utilization bacterium (table 1). Same morphological characteristics were found by previously studies with cellulolytic *Bacillus* sp. strains [6, 18, 19]. Those findings led us to consider the isolate belonging to the genus *Bacillus* which was posteriorly confirmed by the phylogenetic analysis, which revealed that the SR22 strain formed a clade with *Bacillus subtilis* (Fig. 1). Its nucleotide sequence deposited in GenBank as MH119099 accession number and the degree of sequence similarity for the 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 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 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. Since those microorganisms plays an important role in the biogeochemical cycle of carbon as a decomposers in marine ecosystems [21].

**Purification of Bc22Cel from Bacillus sp.**

The extracellular proteins from Bacillus sp. culture were fractioned and the cellulase activity detected in the 60-90% ammonium sulphate fraction. After a successive dialysis, the sample was concentrated and loaded into a gel filtration chromatography where the cellulolytic activity was detected in a single chromatographic peak (Fig. 3A). The sample was re-chromatographed and also a single protein signal was detected with cellulolytic activity (Fig. 3B), SDS-PAGE showed a single band (Fig. 4) with an approximate molecular weight of 37 kDa similar to a previously found cellulase from soil bacterium B. licheniformis [22] but differing at saline resistance and kinetic parameters and possessing the ability to degrade CMC as visualized after a zymographic staining of the sample. The protein showed to be a monomer which is consistent with molecular pattern previously described of bacterial cellulases ranging from monomers with 185 kDa from B. subtilis until 36 kDa in Pseudomonas fluorescens strains [19, 23].

**Determination of 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 hour at different temperatures the enzyme showed more than 40% of residual activity until 70 °C demonstrating a considerable thermal stability when compared to other purified cellulases [8].

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

**Salt Effect of NaCl on Bc22Cel activity and stability**

The purified Bc22Cel showed to be 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 to other cellulases described from Bacillus genera [6, 9] and decreased when incubated with more than NaCl 1.0 M. The enzyme activity did not suffer considerable variation when pre-incubated with 0.5 to 1.5 M NaCl for one hour and even with NaCl 2.0 M retained a considerable residual activity (more than 80%), showing to be effective against denaturation by high saline content solutions.

**Kinetics analysis**

To determine kinetic parameters, Bc22Cel were incubated with different concentrations of CMC, the $K_m$ and $V_{max}$ was 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 µmol·mL$^{-1}·$min$^{-1}$, respectively [23, 30].

This study describes the isolation of an endo-β-1,4-glucanase called Bc22Cel from a marine Bacillus sp. strain with approximate molecular weight 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 that makes the enzyme a potential and useful candidate for industrial applications.

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

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

AUTHORS’ CONTRIBUTIONS

Conceptualization: YQS LM EAS; Data curation: YQS JV LM EAS; Formal analysis: YQS BOV AFJF KG-L JV LM EAS; Funding acquisition: LM EAS; Methodology: YQS BOV AFJF KG-L EAS; Project administration: LM EAS; Resources: LM EAS; Software: YQS BOV AFJF KG-L; Writing – original draft: YQS EAS; Writing – review& editing: YQS JV LM.

CONFLICT OF INTEREST
Authors declare that there is no conflict of interest.

ACKNOWLEDGEMENTS

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**FIGURE LEGEND**

Figure 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.
Figure 2. *Bacillus sp.* SR22 culture containing bagasse (A line) and carboxymethylcellulose (B line) as sole carbon sources in a crescent saline molarity. Halos around bacterial colonies are indicative of cellulose degradation.

Figure 3. 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 were protein concentration (closed circles) and endo-β-1,4-glucanase activity (opened circles) were measured.

Figure 4. Electrophoretic profile and activity staining of purified Bc22Cel. SDS-PAGE revealed with silver showing purified cellulase (B) with an approximate molecular weight of 37 kDa (A corresponds to an empty lane). (C) Zimography stained with congo red 0.1% showing enzymatic activity as a single degradation band even after treatment with SDS and Triton X-100. WM= Weight Markers.

Figure 5. (A) For the optimal temperature (closed circles) the enzyme was incubated at different temperatures (20-80 °C) and its stability (opened circles) determined by pre-incubating Bc22Cel for 60 min and then allowed 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 pH for one hour and then its residual activity determined by the standard assay method. Relative activity (the mean ± SEM) was determined by three replicate assays.
Figure 6. The activity (closed circles) of Bc22Cel on increasing concentrations of NaCl in the reaction buffer was estimated as well as its stability (opened circles) after 60 min in different NaCl concentrations against 1% (w:v) of CMC. Relative activity (the mean ± SEM) was determined by three replicate assays.

Figure 7. Bc22Cel activity at increasing concentrations of substrate (CMC) showing (A) a characteristic hyperbolic behavior corresponding to a Michaelian enzymatic pattern and (B) its correspondent Lineweaver-Burk plot.
Table 1. Morphological and biochemical characteristics of isolated *Bacillus sp.* SR22

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Result</th>
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<td>Gram staining</td>
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<tr>
<td>Morphology</td>
<td>Bacillus</td>
</tr>
<tr>
<td>Arrangement</td>
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<tr>
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<td>Urease</td>
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</tr>
<tr>
<td>Citrate Utilization</td>
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<tr>
<td>H₂S Production</td>
<td>Negative</td>
</tr>
<tr>
<td>Indole Production</td>
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Table 2. Purification summary of the cellulase produced by *Bacillus* sp. SR22

<table>
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<th>Volume (mL)</th>
<th>Protein Concentration (mg.mL⁻¹)</th>
<th>Total activity (IU)</th>
<th>Specific activity (IU.mg⁻¹)</th>
<th>Yeld (%)</th>
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<td>G-75 Superdex</td>
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<td>0.276</td>
<td>59.38</td>
<td>43.02</td>
<td>20.10</td>
</tr>
</tbody>
</table>
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