RESEARCH ARTICLE

Screening of protease, cellulase, amylase and xylanase from the salt-tolerant and thermostable marine *Bacillus subtilis* strain SR60 [version 1; referees: awaiting peer review]

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Abstract

**Background:** The marine environment harbours different microorganisms that inhabit niches with adverse conditions, such as temperature variation, pressure and salinity. To survive these particular conditions, marine bacteria use unique metabolic and biochemical features, producing enzymes that may have industrial value.

**Methods:** The aim of this study was to observe the production of multiple thermoenzymes and haloenzymes, including protease, cellulase, amylase and xylanase, from bacterial strains isolated from coral reefs Cabo Branco, Paraiba State, Brazil. Strain SR60 was identified by the phylogenetic analysis to be *Bacillus subtilis* through a 16S ribosomal RNA assay. To screening of multiples enzymes *B. subtilis* SR60 was inoculated in differential media to elicit the production of extracellular enzymes with the addition of a range of salt concentrations (0, 0.25, 0.50, 1.0, 1.25 and 1.5 M NaCl).

**Results:** The screening showed a capacity of production of halotolerant protease, cellulase, amylase and xylanase and thermostable by the isolate (identified as *B. subtilis* SR60). Protease, cellulase, amylase and xylanase production were limited to 1.5, 1.5, 1.0 and 1.25 M NaCl, respectively.

**Conclusions:** *Bacillus subtilis* SR60 was shown in this study be capable of producing protease, cellulase, amylase and xylanase when submitted to a high salinity environment. These data demonstrate the halophytic nature of SR60 and its ability to produce multiple enzymes.

**Keywords**

Bacteria, Thermoenzymes, Haloenzyme, Enzymes, Industrial Applications.
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Introduction
Covering large surface of the Earth’s surface, the marine environment is a rich source of biological and chemical diversity; it contains endless habitats that may present adverse conditions of survival. However, these conditions favour the establishment of microorganisms able to produce enzymes that have extraordinary properties, such as salt tolerance, thermostability, pH and temperature variations. These enzymes have many industrial applications, such as the production of detergents, food, feed, pharmaceuticals, leather and biofuel.\(^2\)

The conditions of the industrial scale activities are related to the maintenance of enzymatic activity in environments with variations in temperature (55°C to 121°C and -2°C to 20°C), pressure (> 500 atmospheres), pH (pH > 8, pH <4) and salinity (1–5 M NaCl or KCl).\(^1\) The production of enzymes of bacterial origin is a frequent application of industrial biotechnology; the enzymes produced include hydrolytic thermostable enzymes such as amylases, cellulases, proteases and xylanases for the production of biofuel.\(^1\) Use of the genus Bacillus is promising for the production of biomolecules, because it is classified by the FDA as being generally recognized as safe and research has revealed the ability of this genus to produce and secrete enzymes with infinite applications.\(^3\)

This study aimed to produce multiple thermoenzymes and haloenzymes (protease, cellulase, amylase and xylanase) expressed by Bacillus subtilis strain SR60, a bacterial symbiont isolated from Siderastrea stellate (Verrill, 1868) in a Brazilian coral reefs ecosystem 7°08’50” S; 34°47’51” W.

Methods
Isolation of thermophilic bacterial strain
The bacterial strains were obtained from aseptically collected tissues of Siderastrea stellate Verrill, 1868 (Cnidaria, Scleractinia) colonies at Cabo Branco coral reefs, Paraiba State, Brazil (7°08’50” S; 34°47’51” W). For bacterial isolation from the anthozoan, samples were suspended in sterile saline solution, agitated until homogenization was achieved and then spread over marine agar plates (pH 8.0±0.3) containing: (10 g/l xylan; 0.005 g/l NaNO; 1 g/l K,HPO\(_4\); 0.5 g/l MgSO\(_4\)∙7H\(_2\)O; 0.001 g/l FeSO\(_4\)∙7H\(_2\)O; 1 g/l yeast extract; 15 g/l agar) in increasing molarities of NaCl (0, 0.25, 0.50, 1.0, 1.25 and 1.5 M) pH 8.0±0.3. The inoculated plates were incubated at 48 h at 55°C and observed for the formation of zone of hydrolysis.\(^4\)

Bacterial identification
In order to identify the isolate, morphophysiological and molecular data were evaluated.\(^1\) The obtained 16S rRNA gene was sequenced by ATC Gene (UFRGS, Porto Alegre, RS, Brazil) using the automated sequencer ABI-PRISM 3100 Genetic Analyzer. The SR60 isolate sequence was compared to sequences deposited in the Genbank database (NCBI). For the local alignment, the BLASTn tool (NCBI) was used. MEGA 6.0 software was used for monitoring multiple sequences and for construction of a dendrogram by the Neighbor-Joining method.

Screening of protease
The isolated bacterial strains were screened production for protease on agar medium comprising 10 g/l gelatine and 20 g/l agar in increasing concentrations of NaCl (0, 0.25, 0.50, 1.0, 1.25 and 1.5 M) pH 8.0±0.3. The inoculated plates were incubated at 48 h at 55°C and observed for the formation of zone of hydrolysis.\(^5\)

Screening of cellulase
The ability of isolate on produce cellulose was tested a plate containing 1 g/l carboxymethylcellulose (CMC); 0.5 g/l NaNO; 1 g/l K,HPO\(_4\); 0.5 g/l MgSO\(_4\)∙7H\(_2\)O; 0.001 g/l FeSO\(_4\)∙7H\(_2\)O; 1 g/l yeast extract; 15 g/l agar) in increasing molarities of NaCl (0, 0.25, 0.50, 1.0, 1.25 and 1.5 M) for 48 h at 55°C on pH 8.0±0.3 and then overlaid with 0.2 g/l potassium iodide for 5 min, bacterial colonies showing clear zones were considered to be cellulase producers.\(^6\)

Screening of amylase
Amylolytic activity of culture was screened on starch nutrient agar plates containing: 10 g/l starch; 0.05 g/l NaNO; 1 g/l K,HPO\(_4\); 0.5 g/l MgSO\(_4\)∙7H\(_2\)O; 0.001 g/l FeSO\(_4\)∙7H\(_2\)O; 1 g/l yeast extract; 15 g/l agar, in increasing molarities of NaCl (0, 0.25, 0.50, 1.0, 1.25 and 1.5 M). After incubation at 55°C pH 8.0±0.3 for 48 h, the zone of clearance was determined by flooding the plates with 0.2 g/l potassium iodide for 5 min.\(^7\)

Screening of xylanase
Xylanase activity was detected using a saline medium containing: (10 g/l xylan; 0.005 g/l NaNO; 1 g/l K,HPO\(_4\); 0.5 g/l MgSO\(_4\)∙7H\(_2\)O; 0.001 g/l FeSO\(_4\)∙7H\(_2\)O; 1 g/l yeast extract; 15 g/l agar) in increasing molarities of NaCl (0, 0.25, 0.50, 1.0 and 1.5 M) on pH 8.0±0.3. After incubation at 55°C for 48 h, the plates were with 0.2 g/l potassium iodide for 5 min. The clear zones around colonies indicated qualitative xylanase activity.\(^8\)

Results and discussion
Bacterial identification
The SR60 isolate was revealed to be a Gram-positive spore-forming bacillus, facultative anaerobe, catalase-positive; it was negative for indole, H\(_2\)S production and citrate utilization bacterium (Table 1). 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 SR60 strain formed a clade with Bacillus subtilis (Figure 1). The nucleotide sequence was deposited in GenBank under accession number MH698455.1.

Screening for protease, cellulase, amylase and xylanase
In differential media for the production of different extracellular enzymes, it was observed that conditions of high salinity from 0 to 1.5 M NaCl, a SR60 strain showed proteolytic, cellulolytic, amylolytic and xylanolytic activity, these productions being observed by zones of enzymatic hydrolysis (Table 2). The halo detection for protease and cellulase was observed up to the maximum salinity, 1.5 M NaCl (Figure 2 and Figure 3). Cellulolytic enzymes comprise a group of glycosidic hydrolases,
Table 1. Morphological and biochemical characteristics of isolated *Bacillus subtilis* sp. SR60.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram staining</td>
<td>Positive</td>
</tr>
<tr>
<td>Morphology</td>
<td>Bacillus</td>
</tr>
<tr>
<td>Arrangement</td>
<td>Absent</td>
</tr>
<tr>
<td>Endospore</td>
<td>Positive</td>
</tr>
<tr>
<td>Catalase</td>
<td>Positive</td>
</tr>
<tr>
<td>Urease</td>
<td>Negative</td>
</tr>
<tr>
<td>Citrate Utilization</td>
<td>Negative</td>
</tr>
<tr>
<td>H₂S Production</td>
<td>Negative</td>
</tr>
<tr>
<td>Indole Production</td>
<td>Negative</td>
</tr>
</tbody>
</table>

**Figure 1.** Phylogenetic tree of isolated SR60 and other related species based on 16S rRNA sequences. The scale bar represents 0.01 substitutions per site. GenBank accession numbers of the sequences are given in parentheses.

including endoglucanases, exoglucanases and beta-glycosidase. In general, the production of the enzyme group is mainly observed in fungi, actinomycetes and some other bacteria. The use of fungi to produce cellulases has been practiced in the food, textile, fuel and chemical industry, but the growth period for the microorganism does not match the high demand from the industries for production. In an attempt to solve this problem bacteria present rapid growth and high enzymatic production. Bacterial isolates produced from different environments, such as bovine ruminants, soil and in isolation, were found to
Table 2. Screening of enzyme production culture in different molarities NaCl.

<table>
<thead>
<tr>
<th>Molarity NaCl</th>
<th>Zone Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pro Cel Amy Xyl</td>
</tr>
<tr>
<td>0 M</td>
<td>+ + + +</td>
</tr>
<tr>
<td>0.25 M</td>
<td>+ + + +</td>
</tr>
<tr>
<td>0.50 M</td>
<td>+ + + +</td>
</tr>
<tr>
<td>1.0 M</td>
<td>+ + + +</td>
</tr>
<tr>
<td>1.25 M</td>
<td>+ + - +</td>
</tr>
<tr>
<td>1.5 M</td>
<td>+ + - -</td>
</tr>
</tbody>
</table>

Figure 2. Screening of protease in crescent saline molarity. Halos around bacterial colonies are indicative of cellulose degradation.

Figure 3. Screening of cellulase in crescent saline molarity. Halos around bacterial colonies are indicative of cellulose degradation.

Figure 4. Screening of amylase in crescent saline molarity. Halos around bacterial colonies are indicative of cellulose degradation.

Figure 5. Screening of xylanase in crescent saline molarity. Halos around bacterial colonies are indicative of cellulose degradation.

Conclusions
The *Bacillus* sp. isolate identified in this study, *Bacillus subtilis* SR60, has the capacity for proteases, cellulases, amylases produce hydrolases\(^{12,13}\). Biofuel industries that use lignocellulose as the first raw material pre-treatment process for the release of cellulose, making it more accessible to the enzymatic action. During the processing of the cellulose, various compounds containing salts are used, the enzymatic catalysis being reduced or inhibited in this halophilic environment\(^{15}\). The extracellular production of amylase and xylanase reached an upper NaCl concentration limit of 1.0 M and 1.25 M NaCl, respectively (Figure 4 and Figure 5); however, as a bacterial cell growth molecule at the other salt concentrations.

Conclusions
The *Bacillus* sp. isolate identified in this study, *Bacillus subtilis* SR60, has the capacity for proteases, cellulases, amylases
and xylanases with thermostable and halotolerant characteristics. These products can be used as thermostable enzymes in the production of biofuels in crucial stages of this bioprocess.

**Data availability**
The sequence of the *Bacillus subtilis* strain SR60 16s RNA gene isolated in this experiment is available from GenBank, accession number MH698455.1: https://identifiers.org/ncbigi/GI:1435753077.

Images of the repeats of the screening for enzymatic activity have been uploaded to Harvard Dataverse, DOI: https://doi.org/10.7910/DVN/J5JCC0. Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

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### References


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