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RESEARCH ARTICLE

Screening of protease, cellulase, amylase and xylanase from the salt-tolerant and thermostable marine *Bacillus subtilis* strain SR60 [version 1; peer review: 1 approved with reservations, 1 not approved]

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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, Paraíba 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 multiples enzymes.

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Approval Status ?

	1	2
version 1		
26 Oct 2018	view	view

1. **Jorge Olmos-Soto**, Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE), Ensenada, Mexico

2. **Shohreh Ariaenejad**, Agricultural Biotechnology Research Institute of Iran (ABRII), Agricultural Research Education and Extension Organization (AREEO), Karaj, Iran

Any reports and responses or comments on the article can be found at the end of the article.

Keywords

Bacteria, Thermoenzymes, Haloenzyme, Enzymes, Industrial Applications.

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Author roles: **de Veras BO:** Conceptualization, Data Curation, Formal Analysis, Validation, Writing – Review & Editing; **dos Santos YQ:** Formal Analysis, Funding Acquisition, Investigation, Methodology; **Diniz KM:** Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Carelli GSC:** Investigation, Methodology, Resources; **dos Santos EA:** Methodology, Software, Supervision, Validation, Visualization

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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^{1,2}.

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)³. 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 biofuel⁴. 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 applications⁵.

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, Paraíba 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 5 g/l peptone; 1 g/l yeast extract; 15 g/l agar diluted in sterile marine water and incubated at 55°C until adequate growth was achieved⁶. A total of 12 bacterial isolates were obtained, which were analysed for protease, cellulase, amylase and xylanase production capacity, and only the one with the simultaneous production capacity of these enzymes was selected.

For further screening of enzymatic activity described below, two bacterial colonies, isolated using the above culturing conditions, were inoculated onto each plate. A total of three replicates were performed for each salt molarity.

Bacterial identification

In order to identify the isolate, morphophysiological and molecular data were evaluated⁷. The obtained 16S rRNA gene was sequenced by ATCGene (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 hydrolysis⁸.

Screening of cellulase

The ability of isolate on produce cellulose was tested a plate containing 1 g/l carboxymethylcellulose (CMC); 0.5 g/l NaNO₃; 1 g/l K₂HPO₄; 0.5 g/l MgSO₄·7H₂O; 0.001 g/l FeSO₄·7H₂O; 1 g/l yeast extract; 15 g/l agar) in increasing molarities 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 producers⁹.

Screening of amylase

Amylolytic activity of culture was screened on starch nutrient agar plates containing: 10 g/l starch; 0.05 g/l NaNO₃; 1 g/l K₂HPO₄; 0.5 g/l MgSO₄·7H₂O; 0.001 g/l FeSO₄·7H₂O; 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 min¹⁰.

Screening of xylanase

Xylanase activity was detected using a saline medium containing: (10 g/l xylan; 0.005 g/l NaNO₃; 1 g/l K₂HPO₄; 0.5 g/l MgSO₄·7H₂O; 0.001 g/l FeSO₄·7H₂O; 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 activity¹¹.

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, H₂S 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, aminolytic 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.

Parameter	Result
Gram staining	Positive
Morphology	Bacillus
Arrangement	Absent
Endospore	Positive
Catalase	Positive
Urease	Negative
Citrate Utilization	Negative
H ₂ S Production	Negative
Indole Production	Negative

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 production¹². 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.

Molarity NaCl	Zone Formation			
	Pro	Cel	Amy	Xyl
0 M	+	+	+	+
0.25 M	+	+	+	+
0.50 M	+	+	+	+
1.0 M	+	+	+	+
1.25 M	+	+	-	+
1.5 M	+	+	-	-

Screening of Protease

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

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 environment¹⁵. 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

Screening of Cellulase

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

Screening of Amylase

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

Screening of Xylanase

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

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).

Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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<http://www.doi.org/10.7910/DVN/J5JCC0>

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Current Peer Review Status: ? ✖

Version 1

Reviewer Report 21 April 2021

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Shohreh Ariaeenejad

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The main idea of this work is 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 screen 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).

Comments to the author(s):

Comment #1:

The introduction is very poorly written. No history of similar work has been mentioned in this regard.

Comment #2:

At the end of the introduction: "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." - What does this mean?

You expressed some enzyme genes to *Bacillus subtilis* strain SR60?

Or

You found a novel bacterial symbiont isolated from *Siderastrea stellate* and this bacteria had some enzyme genes?

The Authors wrote in materials "A total of 12 bacterial isolates were obtained, which were analysed for protease, cellulase, amylase and xylanase production capacity, and only the one with the simultaneous production capacity of these enzymes was selected." - This is very different from the last sentence of the introduction.

Comment #3:

At the end of the isolation of the thermophilic bacterial strain: "For further screening of enzymatic activity described below, two bacterial colonies, isolated using the above culturing conditions, were inoculated onto each plate." - Why did you choose the two bacterial colonies?

Comment #4:**In the part of Bacterial identification:**

Why are there no comparisons of the phylogenetic tree of these 12 identified strains? Why is their access number not in the article?

Comment #5:

The results are very vague. Which strains showed what enzymatic activity? The whole article is written as a short story.

Comment #6:**In the Table 2. Screening of enzyme production culture in different molarities NaCl.**

The results should be expressed as a comparative percentage (Relative activity).

Please see my related article about effect of salt on enzymes¹.

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Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Enzyme biochemical characterization

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Reviewer Report 16 May 2019

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Jorge Olmos-Soto

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- The isolated *B. subtilis* strain only has the capacity to degrade cellulose at high saline concentrations (1.25-1.5 M) and its xylanase activity is only developed at medium salt concentration (1 M). However, protease and amylase activity cannot be considered as halotolerant because these enzymes only have good activity at 0.25-0.5 M. In this sense, I believe the conclusion must be reoriented to point out these results.
- All the figures contain the same legend, please correct this issue.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

No

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of

expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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