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Production of Antileukemic Enzyme L-asparaginase from Marine Bacteria Associated with Coral *Siderastrea stellate* (Verrill, 1868), Brazil

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Abstract

L-asparaginase, an antileukemic drughas been approved for clinical use for many years in the treatment of childhood Acute Lymphoblastic Leukaemia (ALL), is obtained originally from bacteria *Escherichia coli* and *Erwinia carotovora*. The L-Asparaginase (LA) is an enzyme capable of selective hydrolysing of the amino acid L-asparagine (E.C. 3.5.1.1.), it presents several technological applications. L-asparaginase from bacteria can cause anaphylaxis and other abnormal sensitive reactions due to the low specificity of the enzyme. Toxicity and repression caused by bacterial L-asparaginase has intensified new research aimed to obtain bacterial strains producing this enzymatic type with a more effective enzyme without adverse reactions. In the present study, L-asparaginase producing bacteria were isolated from colonies of *Siderastrea stellata* (Verrill, 1868) Cnidaria, Scleractinia. Sixty-nine bacterial strains were tested for L-asparaginase production using a fast plaque technique, in which six were positive in screening for L-asparaginase. Positive isolates in qualitative screening demonstrated capacity to produce halotolerant and thermostable L-asparaginase, presenting growth and enzymatic production in medium containing 1.0 M NaCl. The isolates showed total enzymatic activity varying from 170.0 to 300.5 IU/mL. The producers were identified as *Bacillus subtilis* based on morphological and molecular characteristics of the *16S* rRNA gene. It was found that they have the ability to secrete this enzyme in the extracellular environment. These isolates presents an enormous potential for application in several areas of biotechnology.

Key words: bacteria, L-asparaginase, antileukemic, enzyme, potential.

INTRODUCTION

Covering more than 75% of the Earth's surface, the marine environment is a rich source of biological and chemical diversity, containing endless habitats that may present adverse survival conditions. However, these conditions may favour the establishment of microorganisms with promising characteristics on the prospecting of enzymes that have extraordinary properties (Hu et al., 2015; Dumorne et al., 2017; Izadpanah Qeshmi et al., 2018). The use of enzymes in the treatment of malignant tumors has driven great interest on replacing conventional chemotherapy, through the capacity of tumor suppression by nutritional deficiency duethe enzymatic catalytic action, being a promising approach due to the high specificity in detriment to the conventional chemotherapeutic agents (Cocco et al., 2011; Hensley et al., 2013).

The enzyme L-Asparaginase (LA), catalyzes the hydrolysis of the amino acid L-asparagine (Asn) in L-aspartic acid (Asp) and ammonia (EC 3.5.1.1), and can be produced by various organisms such as plants, bacteria and fungi (Michalska and Jaskolski, 2006; Srivastava et al., 2018). The enzyme produced by bacteria can be found in two subtypes depending on the microbial species. The subtype I is found in the cytoplasm acting on amino acid metabolism and exhibiting a high Michaelis constant (Km) due to its low specificity for the substrate, while subtype II is found in the periplasmic or extracellular region and plays an important role in the nitrogen cycle, presenting a low Km value, indicating a high substrate specificity (Campbell et al., 1967; Wriston and Yellin, 1973).

The FDA and WHO organizations have approved L-asparaginase for the effective treatment of Acute Lymphoblastic Leukaemia (ALL), Myelomonocytic Leukaemia (AML), Hodgkin's disease, lymphosarcoma treatment, reticulosarboma and melanosarcoma (Chen et al., 2014; Shrivastava et al., 2015). Among the several types of leukemia that have the greatest use of L-asparaginase is Acute Lymphoblastic Leukemia (ALL), which has nutritional enhancement of tumor cells, where the leukemic cells present a serious need for exogenous supplementation of the amino acid asparagine due to the depletion of the activity Asparagine

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Synthase. Given the high requirement of this amino acid, deprivation results in inhibition of protein synthesis and subsequent death of tumor cells (Gallagher et al., 1989; Narta et al., 2007).

Clinical trials indicate that the treatment of Acute Lymphoblastic Leukaemia (ALL) using LA causes complete remission of tumour cells in more than 90% of the affected children by neoplasia within 4 weeks, a major advance in modern oncology (Cocco et al., 2011; Shrivastava et al., 2015). Despite the enormous importance of LA in antineoplastic therapy, there are few microorganisms used in the production of the enzyme, the forms coming from *Escherichia coli* and *Erwinia carotovora* (Narta et al., 2007). Several reports of undesirable side effects of the drug from these microorganisms, such as the development of diabetes, dysfunction of liver and pancreas, skin rashes, fever, leukopenia, neurological seizures, mild allergic reactions, anaphylaxis, haemorrhage (Mahajan et al., 2014; Alqasim et al., 2018).

To overcome the negative side effects of LA using the enzyme that shows toxicity and immunosuppression, new research is being carried out in the hope of finding new enzymes with new and differentiated biochemical propertiesfor application in antitumor therapy. Among the infinite environments and LA producing microorganisms, bacteria of marine origin have demonstrated great production capacity with great potential of application with new chemical structures, high specificity, low molecular weight and low toxicity (Prihanto et al., 2016; Prabhu et al., 2017; Lee et al., 2018)

This study aimed to evaluate the production capacity of L-asparaginase and to identify coral symbiotic bacteria from *Siderastrea stellata* (Verrill, 1868) in a Brazilian coral reefs ecosystem 7°08'50" S; 34° 47'51" W.

MATERIALS AND METHODS

Isolation of bacteria

The bacterial strains were aseptically obtained from collected tissues of *Siderastrea stellata* 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 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 achieved (Dustan, 1973).

Bacterial identification

To identify the L-asparaginase-producing isolates, morphophysiological and molecular data were evaluated (Hogg, 1999). The sequence of gene 16S rRNA was amplified from extracted DNA. Bacterial universal primers 26F (5'- GAGTTTGATCMTGGCTCAG- 3') and 1492R (5' -ACGGCTACCTTGTTACGACTT-3') were used to amplify the 16S rDNA gene by Polymerase Chain Reaction (PCR) performed in MWG-Biotech Primus 96 Plus Thermal Cycler (Primus, USA). Each reaction mixture (50 µL) contained reaction buffer, 2 mM $MgCl_2$, 0.2 µM of each primer, 0.2 mM of dNTPs, 1U of DNA polymerase and 40 ng of genomic DNA. The amplification products were directly purified from the PCR reaction using the Wizard® SV Genomic DNA Purification System. The obtained 16S rRNA gene was sequenced by ATCGene (UFRGS, Porto Alegre, RS, Brazil) using the automated sequencer ABI-PRISM 3100 Genetic Analyzer. The isolated sequence was compared to sequences deposited in the Genbank database (NCBI). For the local alignment, the BLASTn tool (NCBI) was used. The MEGA 6.0 software was used for monitoring multiple sequences and for construction of a dendrogram by the Neighbor-Joining method.

Screening of L-asparaginase producing bacteria

The detection of LA- producing bacteria was performed according to the modified methodology of Mahajan et al. (2013), using the Czapek Dox agar medium having the following composition: Na_2HPO_4 – 6,0 g / l; KH_2PO_4 – 2,0 g /l; L-asparagine – 10,0 g / l; Glycerol – 2,0 g /l; $MgSO_4$-$7H_2O$ − 0,5 g /l; $CaCl_2$-$2H_2O$ − 0,005 g / l; Agar–20,0 g / l, at different pHs (4.5, 5.5, 6.5, 7.5). The media was supplemented separately with the indicators Bromocresol Green, Bromothymol Blue, Phenol Red, Bromocresol Purple, Neutral Red. Plates with the bacterial cultures were incubated at 55°C for 48 hours, with the positive result of forming a halo around the colonies.

The positive isolates in the screening were evaluated for the capacity of producing halotolerant L-asparaginase, being the isolates grown in medium Czapek Dox agar medium with the red phenol indicator, in increasing NaCl molarities (0, 0.25, 0.50, 1.0, 1.25 and 1.5 M).

Enzymatic Production

The isolates were grown in Czapek Dox (Na_2HPO_4 – 6,0 g / l; KH_2PO_4 – 2,0 g / l; L-asparagine – 10,0 g / l; Glycerol – 2,0 g /l; $MgSO_4$-$7H_2O$ − 0,5 g /l; $CaCl_2$-$2H_2O$ − 0,005 g / l) pH 7.0± 0.3, under stirring at 150 rpm for 48 hours at 55°C (Mahajan et al., 2013). Subsequently the cultures were centrifuged at 12.000 g for 15 min and the supernatant used for quantification of the activity and evaluation of extracellular L-asparaginase production.

Extracellular L-asparaginase activity

To confirm the production of extracellular L-asparaginase, the cultures were then filtered and the proteins precipitated in 1:10 (w/w%) acetone. The resulting proteins were transferred into tubes containing 1.0 mL of L-asparagine solution (100 mM) in Tris-HCl (50 mM) pH 7.0. The mixture was incubated in a water bath for 30 minutes and the secretion of extracellular LA evaluated by thin layer chromatography (TLC) using silica gel plates, using standards aspartate, asparagine and mix (asparagine + aspartate) (Hendriksen et al., 2009).

L-asparaginase assay

The activity was measured by Direct Nesslerization of using ammonia or the modified method of Mashburn and Wriston (1964). The enzyme assay mixture consisted of 100 µl L-asparagine (189 mmol / L) in Tris-HCl buffer (pH 8.6) and 100 µl crude enzyme extract. The reaction the mixture was incubated at 37°C for 30 min and the interposed by the addition of 100 µl of 15% trichloroacetic acid (TCA). The reaction mixture was centrifuged at 6.000 g for 5 min at 4°C to remove the precipitates. The ammonia released in the The supernatant was determined by adding 500 µl of Nessler reagent to the sample containing 200 µl of supernatant and 4.3 ml of distilled water. The samples were centrifuged and incubated at room temperature for 10 min., OD was measured at 396 nm against blanks that received TCA prior to the addition of crude enzyme. The ammonia produced in the reaction was determined on the basis of in the standard curve obtained with ammonium sulphate. One Unit of L-asparaginase is the amount of enzyme which liberates 1 µmol of ammonia in 1 minute at 37°C.

RESULTS AND DISCUSSION

In Czapek Dox medium containing different indicators it was possible to verify that six of the sixty-nine isolates had the capacity to produce L-asparaginase. It was possible to easily verify the presence of halo hydrolysis in the medium containing the indicators red phenol, bromocresol purple and neutral red, having better visualization using red indicators of phenol and bromocresol purple (Figure 1 and Table 1). In the detection of halotolerant L-asparaginase in medium containing phenol red indicator it was possible to verify the presence of halos up to the maximum limit of 1.0 M NaCl (Figure 2 and Table 2).

Although several microorganisms have the ability to produce L-asparaginase with application in tumor therapy, the main sources of the enzyme for therapeutic use are *E. coli* and *E. carotovora*, requiring further studies are needed to obtain new microorganisms producing this type with new biochemical characteristics (Godfrin and Bertrand, 2006). Therefore, to optimize the actual therapy, the search for new L-asparaginase with chemotherapeutic agents is urgently needed (Husain et al., 2016). The screening step is necessary for the discovery of new microorganisms capable of producing L-asparaginase with novel characteristics, and several studies are being carried out in order to demonstrate and find bacterial isolates with L-asparaginase production capacity. Thirunavukkarasu et al. (2011) in fungal screening in association with green algae, obtained 84 isolates of which 64 were positive for L-asparaginase production. Izadpanah Qeshmi et al. (2014) in the screening obtained 12 bacterial isolates from promising L-asparaginase-producing marine sediments.

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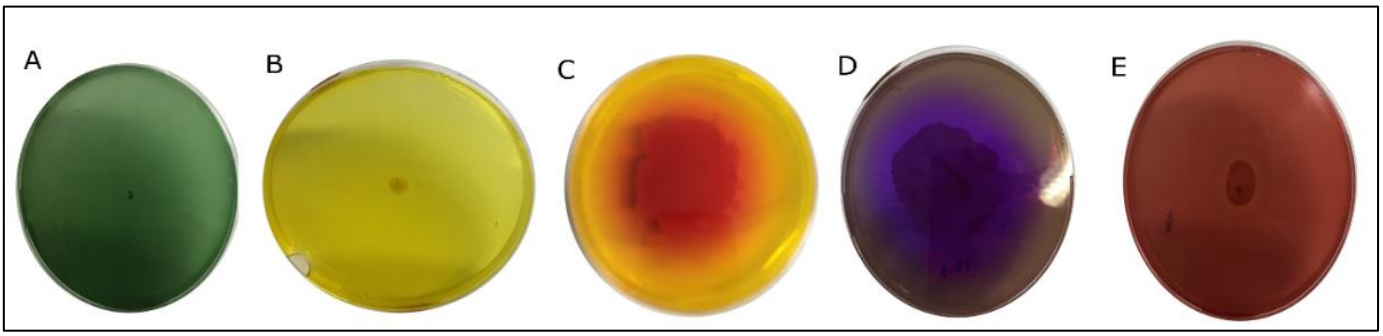

Figure 1. Screening of L-asparaginase by bacterial isolates using the Czapek Dox agar and different indicators.
Legend: Halos around bacterial colonies are indicative of L-asparagine hydrolysis; Indicators: (A) Bromocresol Green; (B) Bromothymol Blue; (C) Phenol Red; (D) Bromocresol Purple; (E) Neutral Red.

Table 1. Screening of L-asparaginase by bacterial isolates using different indicators.

Isolated	Bromocresol Green	Bromothymol Blue	Phenol Red	Bromocresol Purple	Neutral Red
SR22	—	—	+	+	+
SR41	—	—	+	+	+
SR44	—	—	+	+	+
SR60	—	—	++	++	+
SR61	—	—	+	+	—
SS03	—	—	++	++	+

Legend: (—), Non observable zone; (+) positive with halo less than 3 cm; (++) positive with halo greater than 3 cm.

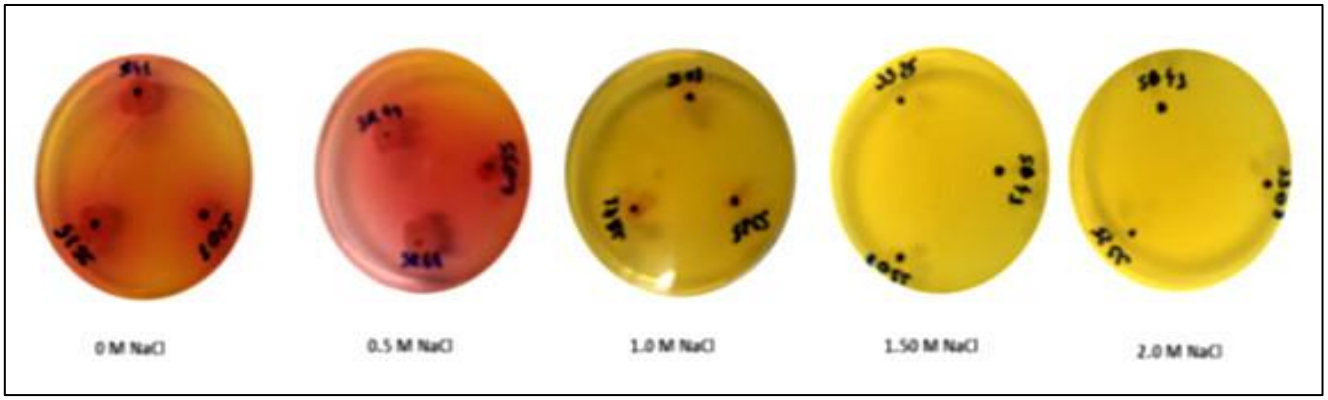

Figure 2. Screening of L-asparaginase by bacterial isolates using Czapek Dox agar with red phenol indicator at different molarities of NaCl.
Legend: Halos around bacterial colonies are indicative of L-asparagine hydrolysis.

Table 2. Qualitative screening production of L-asparaginase culture in different NaCl molarities.

Molarity NaCl	SR22	SR41	SR44	SR60	SR61	SS03
0 M	+	+	+	++	+	++
0.50 M	+	+	+	+	+	+
1.0 M	+	+	+	+	+	+
1.5 M	—	—	—	—	—	—
2.0 M	—	—	—	—	—	—

Legend: (—), Non observable zone; (+) positive with halo less than 3 cm; (++) positive with halo greater than 3 cm.

Several studies indicate that the use of some indicators may mask the production capacity of L-asparaginase in bacterial screening. Mahajan et al. (2013) states that made screening using red phenol indicator reported that this methodology would not be one of the most sensitive methods for detecting activity because the contrast zone obtained (between yellow and pink) is not very marked and distinct, and that the use of another indicator such as bromothymol blue would be more accurate, a fact not seen in this study. However, according to the obtained data it is possible to state that the use of the bromocresol purple indicator shows clearer results in the screening of LA-producing microorganisms.

The morphophysiological data showed that the six isolates producing L-asparaginase are Gram-positive spore-forming bacilli, facultative anaerobe, catalase-positive; it was negative for indole, H_2S production and citrate utilization bacterium (Table 3). 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 formed a clade with *Bacillus subtilis* (Figure 3). The nucleotide sequence was deposited in GenBank under accession numbers MH698454, MH700655, MH698455, MH700947, MH700752, MH700955, MH701772.

Table 3. Morphological and biochemical characteristics of L-asparaginase-producing isolates.

Characteristics	SR22	SR41	SR44	SR60	SR61	SS03
Gram staining	+	+	+	+	+	+
Morphology	Bacillus	Bacillus	Bacillus	Bacillus	Bacillus	Bacillus
Arrangement	—	—	—	—	—	—
Endospore	+	+	+	+	+	+
Catalase	+	+	+	+	+	+

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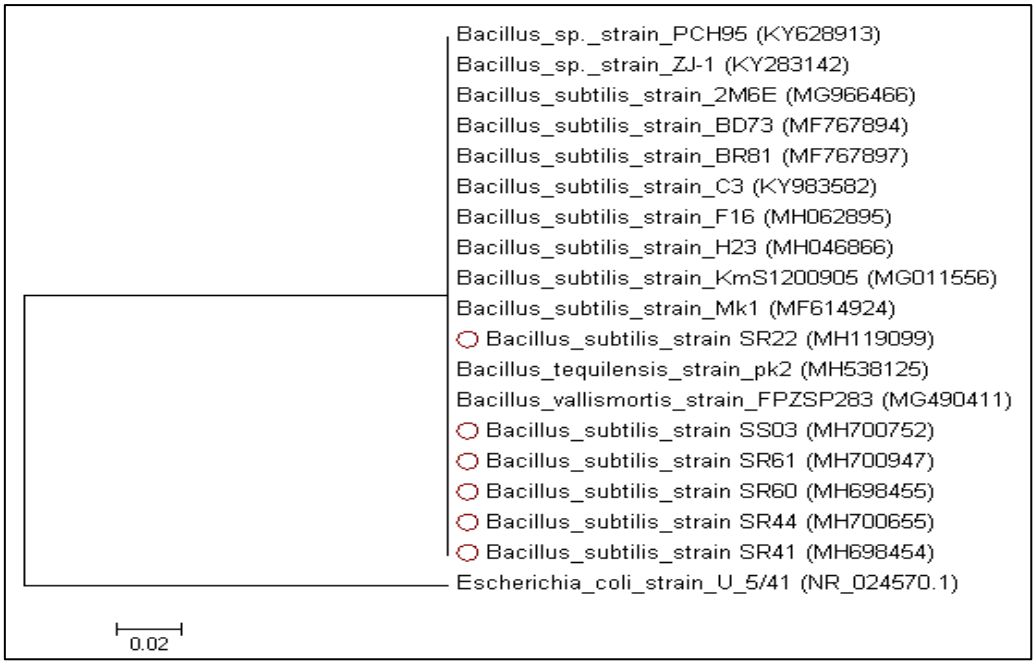
Urease	—	—	N.D.	—	—	—
Citrate Utilization	—	—	—	—	—	—
H_2S Production	N.D.	—	—	—	N.D.	—
Indole Production	—	—	—	—	—	—

Legend: (—), Negative; (+) Positive (N.D.) Not determined.

Figure 3. Phylogenetic tree of isolated species producing L-asparaginase 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.



Previous studies related the L-asparaginase production of *Bacillus subtilis* collected from India. Despite the isolate *Bacillus subtilis* strain hswx88 showed the enzymatic activity (23.8 UI/mL) 1.7 and 14.5 times higher than the reference organisms (*Pectobacterium carotovorum* MTTC1428 and *Bacillus sp.* BCCS 034) (Pradhan et al., 2013), the enzymatic activity of the isolates found in the present study was even higher, with values ranging from 139,6 to 300,5 UI/mL. The isolate SR22 showed the highest value, emphasizing the great potential of enzyme production from this strain (Table 4).

Table 4. Quantitative screening production of L-asparaginase for isolated bacterial.

Isolated	Total activity (IU/mL)
SR22	300,5± 3,83
SR41	222,8 ± 3,59
SR44	280,5 ± 4,06
SR60	139,6 ± 4,93
SR61	231,4 ± 3,57
SS03	170,0 ± 5,70

Aiming to confirm the production of extracellular L-asparaginase, the isolates were evaluated by TLC and this production was confirmed (Figure 4), sincethe amino acid L-asparagine was consumed in all of the samples, demonstrating that this substrate was used by the produced enzyme. The extracellular production is considered advantageous over the intracellular type because of the higher production of soluble bioactive proteins, easy extraction and relatively free from endotoxins, minimizing the possible adverse reactions. Extracellularproteins exported to the medium are probably more soluble, bioactive and relatively free from endotoxins. The extracellular enzymes are also related to have the proper folding, specially that requiring disulfide bridge formation, resulting in interesting enzymes for the development of new drugs (Pradhan et al., 2013).

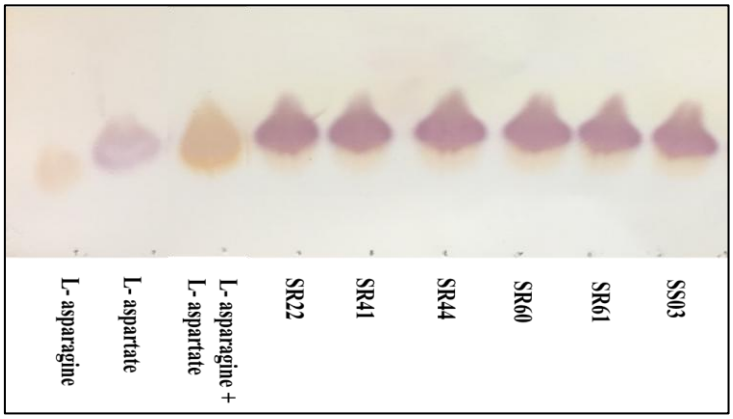


Figure 4. Thin-layer chromatography (CCD) for L-asparaginase activity by bacterial isolates.

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CONCLUSIONS

The bacteria obtained from the *Siderastrea stellata* coral have potential being capable of producing L-asparaginase with thermostable and halotolerant properties. More detailed and further studies are still needed to optimize culture conditions in order to obtain a high production of L-asparaginase among the analyzed isolates. In addition, the intrinsic characteristics of the isolates can be used in recombinant DNA technology.

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CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

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