CAPÍTULO 11

SCREENING OF L-ASPARAGINASE THE SALT-TOLERANT AND THERMOSTABLE MARINE BACILLUS SUBTILIS STRAIN SR61

Bruno Oliveira de Veras
Universidade Federal de Pernambuco, Departamento de Antióbioticos, Recife-Pernambuco.

Yago Queiroz dos Santos
Universidade Federal do Rio Grande do Norte, Departamento Bioquímica, Natal-Rio Grande do Norte.

Anderson Felipe Jácome de França
Universidade Federal do Rio Grande do Norte, Departamento Bioquímica, Natal-Rio Grande do Norte.

Penha Patricia Cabral Ribeiro
Universidade Federal do Rio Grande do Norte, Departamento de Nutrição, Recife-Pernambuco.

Elaine Costa Almeida Barbosa
Universidade Federal da Paraíba, Departamento de Energias Renováveis, João Pessoa-Paraíba.

Krystyna Gorlach-Lira
Universidade Federal da Paraíba, Departamento de Biologia Celular e Molecular, João Pessoa-Paraíba.

RESUMO: O ambiente marinho abriga diferentes microorganismos que habitam nichos com condições adversas, como variação de temperatura, pressão e salinidade. Para sobreviver a essas condições particulares, as bactérias marinhas usam características metabólicas e bioquímicas únicas, produzindo enzimas que possuem elevado valor industrial. O objetivo deste estudo foi observar a produção de L-asparaginase termoestável e halotolerante por bacterias marinhas isolado dos recifes de coral do Cabo Branco, Estado da Paraíba, Brasil. Foi obtido um isolado bacteriano produtor de L-asparaginase, sendo identificado identificada por análise filogenética como sendo *Bacillus subtilis* SR61, através de um ensaio de RNA ribossómico 16S. Para a triagem de L-asparaginase por SR61, foi inoculado em meio diferencial para induzir a produção de enzima termostável extracelular tolerante ao sal com a adição de concentrações crescentes de sal (0, 0.5, 1.0, 1.5 e 2.0 M NaCl) em 55 ° C por 24 horas. A triagem mostrou capacidade de produção de L-asparaginase halotolerante e termoestável pelo isolado identificado como Bacillus subtilis, sendo a produção limitada a 1,0 M de sal, tendo como atividade total (UI / mL) 231,4 ± 3,57 e especifica IU/µg 8,39. *Bacillus subtilis* SR61 mostrou-se capaz de produzir L-asparaginase quando submetido a um ambiente de alta salinidade, demonstrando a natureza halofítica do isolado, tendo diversas aplicações em diversos ramos industriais.

PALAVRAS-CHAVE: L-Asparaginase, Bactérias, Atividade enzimática.

ABSTRACT: The marine environment harbors different microorganisms that inhabit niches with adverse conditions, such as variation

of temperature, pressure and salinity. To survive these particular conditions, marine bacteria use unique metabolic and biochemical characteristics, producing enzymes that have high industrial value. The objective of this study was to observe the production of thermostable and halotolerant L-asparaginase by marine bacteria isolated from the coral reefs of Cabo Branco, State of Paraíba, Brazil. An L-asparaginase-producing bacterial isolate was identified and identified by phylogenetic analysis as *Bacillus subtilis* SR61, via a 16S ribosomal RNA assay. For screening of L-asparaginase by SR61, it was inoculated in a differential medium to induce the production of salt tolerant extracellular thermostable enzyme with the addition of increasing salt concentrations (0, 0.5, 1.0, 1.5 e 2.0 M NaCl) by 55 ^{0}C for 24 hours. The screening showed a capacity of halotolerant and thermostable L-asparaginase production by the isolate identified as *Bacillus subtilis*, the production being limited to 1.0 M salt, having a total activity (IU / mL) 231.4 ± 3.57 and specifying IU / µg 8.39. Bacillus subtilis SR61 was able to produce L-asparaginase when submitted to a high salinity environment, demonstrating the halophytic nature of the isolate, having several applications in several industrial branches.
KEYWORDS: L-Asparaginase, Bactéria, Enzyme activity.

1 I 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 (HU ET AL., 2015; FATEMEH ET AL., 2018).

The conditions of the industrial scale activities are related to the maintenance of its activity in environments with temperature variation (55°C to 121°C and -2°C to 20°C), pressure (> 500 atmospheres), alkalinity or acidity pH (pH> 8, pH <4), salinity (1-5 M NaCl or KCl) (DUMORNÉ et al., 2017).

The enzyme L-Asparaginase (ASNase) catalyses 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; JASKOLSKI, 2006). L-Asparaginase (ASNase) is an important therapeutic agent used in the treatment of acute lymphoblastic leukemia (ALL) and other hematopoietic disorders. Unlike normal cells, leukemic cells have a serious depletion in the activity of the enzyme Asparagine Synthase, being unable to perform asparagine synthesis by de novo pathways. Given the high requirement of exogenous asparagine, a deprivation of this amino acid for leukemic cells results in inhibition of protein synthesis and subsequent death of tumor cells (CHEN, 2015). Although several microorganisms have the capacity

to produce L-asparaginase, the main sources of the enzyme for therapeutic use are *E. coli* and *E. carotovora* (KUMAR; SOBHA, 2012)

Besides this, the food industry has been showing increasing attention by L-asparaginase as a promising agent for acrylamide mitigation, considering that the thermal treatment of foods rich in carbohydrates mainly the amino acid asparagine culminates in several chemical reactions, among them the Maillard Reaction (PEDRESCHI et al., 2006; HENDRIKSEN et al., 2013). Various microorganisms of L-asparaginase producers have been described as potential candidates for use in foods as a mitigation of acrylamide formation since up to 99% reduction in the formation of acrylamide in cold potatoes was obtained using the enzyme from microbial sources (ONISHI et al., 2015).

However, the production of L-asparaginase in the various microorganisms can be influenced by several factors, and studies are needed to optimize production, besides the search for new microorganisms producing L-asparaginase with different biochemical characteristics (SILVA et al., 2016).

The work aimed at the production of L-asparaginase thermostable produced by bacteria symbiont isolated of *Siderastrea stellate* (Verrill, 1868) in a Brazilian coral reefs ecosystem (7°08’50” S; 34°47’51” W).

2 I MATERIALS AND METHODS

2.1 Isolation of thermophilic bacterial strains

The bacterial strains were obtained from aseptically collected tissue 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, sample 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, 1969). Twelve bacterial isolates were obtained, which were analysed for L-asparaginase production capacity, only the one with the production capacity of enzyme was selected.

2.2 Qualitative Screening of L-asparaginase Bacteria

The detection of L-asparaginase-producing bacteria was performed using the Czapek Dox agar medium at different pHs (4.5, 5.5, 6.5, 7.5) (GULATI et al. 1997). The media will be supplemented separately with the indicators Bromocresol Green, Bromothymol Blue, Phenol Red, Bromocresol Purple, Neutral Red, plates with the bacterial cultures will be incubated at 55°C for 48 hours. For analysis of the production

of halotolerant L-asparaginase, the positive isolates in the qualitative analysis were grown on plates containing the Czapek Dox agar medium and the red phenol indicator in increasing molarities NaCl (0, 0.5, 1.0, 1.5 e 2.0 M).

2.3 Quantitative Screening of L-asparaginase Bacteria

An enzymatic assay was performed using the positive isolates in qualitative analysis using Czapek Dox containing 1.0 M NaCl, cultures being maintained at 55°C for 24 hours at 150 rpm. The enzyme activity of L-asparaginase was determined by measuring the amount of ammonia formed, using direct nesslerization method based (MASHBURN; WRISTON 1963). The quantification of total proteins was performed using a standard protein curve constructed using dilutions and quantification of bovine serum albumin (BSA) (BRADFORD, 1976; LOWRY et al.1951).

2.4 Bacterial identification

In order to identify the isolate, morphophysiological and molecular data were evaluated (HOGG, 1999). The obtained 16S rRNA gene was sequenced by ATCGene (UFRGS, Porto Alegre, RS, Brazil) using the automated sequencer ABI-PRISM 3100 Genetic Analyzer. The 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.

3 I RESULTS AND DISCUSSION

3.1 Qualitative and Quantative Screening

In the present study, 12 bacteria strains were investigated for the production of the enzyme L-asparaginase using the test method in plate. In Czapek Dox medium containing different indicators it was possible to verify that one of the Twelve isolates have 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 (Figure 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).

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*, for which further studies are needed to obtain new microorganisms producing this type with new biochemical characteristics (GODFRIN;

BERTRAND, 2006; HUSAIN et al. 2016).

The activity of L-asparaginase by isolated was estimated by the Nesslerization method, presenting total enzymatic activity varying from 231,4 ± 3,57 IU/mL and specific activity from 8,39 IU/µg (Table 1), whose data indicates a high enzymatic activity in medium containing 1.0 M NaCl. Dharmaraj (2011) was able to obtain a total activity of 331.0 IU/ml in *Streptomyces noursei* MTCC 10469 marine in medium in the absence of salt.

Figure 1. Qualitative screening of L-asparaginase by bacterial isolate using the Czapek Dox agar medium and different indicators.

Legend: Halos around bacterial colonie are indicative of hydrolysis L-asparagine.

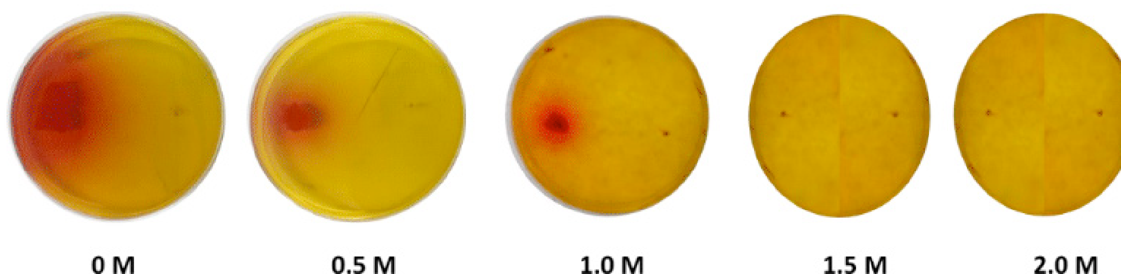

Figure 2. Qualitative screening of L-asparaginase by bacterial isolated using Czapek Dox agar medium with red phenol indicator at different molarities NaCl.

Legend: Halos around bacterial colonie are indicative of hydrolysis L-asparagine.

Isolated	Total activity (IU/mL)	Protein (µg/mL)	Specific activity (IU/µg)
SR61	231,4 ± 3,57	27,55 ± 2,98	8,39

Table 1. Quantitative screening production of L-asparaginase for isolated *Bacillus subtillis* sp. SR61.

3.2 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_2S production and citrate utilization bacterium (Table 2). 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 3). The nucleotide sequence was deposited in GenBank under accession number MH700947.1.

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

Table 2. Morphological and biochemical characteristics of isolated *Bacillus subtillis* sp. SR61.

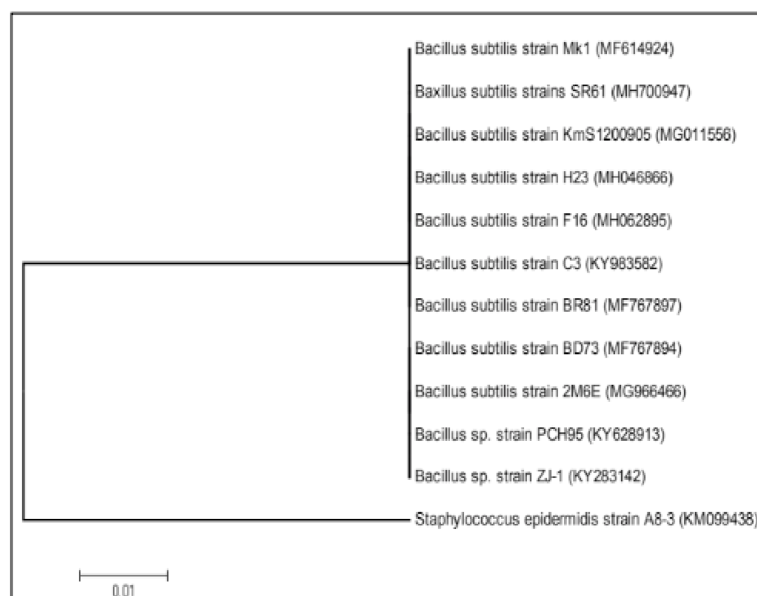

Figure 3. Phylogenetic tree of isolated SR61 and other related species based on 16S rRNA sequences. The scale bar represents 0.01 substitutions per site. GenBank accession numbers of the sequences are given in parentheses.

4 I CONCLUSIONS

The obtained data indicate that the isolate obtained in the present work has the potential to produce greater enzymatic activity after the optimization to meet the needs of pharmaceutical and other industries.

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