Research article

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Isolation of moderately halophilic bacteria in saline environments of Sonora State searching for proteolytic hydrolases

Abstract: The aim of the study was to isolate moderately halophilic bacteria that produce proteolytic enzymes with industrial biotechnological value. Screening of halophiles from various saline habitats, led to the isolation of 210 moderately halophilic bacteria producing industrially important hydrolases; such as proteases, which are enzymes that hydrolyze the peptide bonds of proteins, represent one of the three groups of industrial enzymes. The present study of halophilic bacteria, producing proteases and isolated from different saline soils of Sonora State, was divided in two parts: the first part included screening of moderately halophiles bacteria collected from various regions, while the second part consisted of enzyme production by fermentation in liquid medium in flask. Three strains of bacteria with potential proteolytic activity (BLRMAM1066, BLCLAM1064, PPSE3AM1053) were selected to continue the electrophoresis and zymogram tests, obtaining molecular weights from 19 to 193 kDa. One of the a priori objectives of this research is to have a collection of halophilic bacteria with high proteolytic activity.

Keywords: Isolation, Screening, Saline areas, Moderate halophiles, SDS-PAGE, Proteases

1 Introduction

Screening of new source of novel and industrially useful enzymes is a key research pursuit in enzyme biotechnology. For applications in industrial processes, the enzymes should be stable at high temperature, pH, presence of salts, solvents, toxicants etc. In this context, the halophiles have emerged as a vast repository of novel enzymes in recent years (Kumar et al. 2012). The proteases are the only class of enzymes that occupy an important position with respect to its application in the commercial and biological fields (Banik and Prakash 2004). These enzymes catalyze the breakdown of peptide bonds in other proteins, are degradative enzymes, which catalyze the complete hydrolysis of proteins and represent one of the three largest groups of industrial enzymes (Oren 2010). Microorganisms are an excellent source of enzymes, these accounts for 40% of global sales of enzymes (Litchfiel 2011). In addition to its physiological importance, this group of enzymes has an enormous industrial interest being widely used in the detergent industry, food, beverage, textile and paper (Margesin and Schinner 2011). On the other hand, although the proteases have a very specific action, it is a very diverse group of enzymes, making it very attractive for biotechnological exploitation (Nigam 2013).

Estimates of worldwide sales of industrial enzymes are very high, and of these 75% have hydrolytic activities
Proteases represent one of the three main groups of industrial enzymes (Li et al. 2013). This domain of proteases in the industrial market is expected to continue to increase (Sánchez et al. 2003). Demand for enzymes with strict requirements of various biotechnological processes has led to investigations of proteolytic enzymes that satisfy both market needs and environmental preservation. Proteases produced today have provided; years of useful biotechnological tools, not only to improve the efficiency of industrial processes, which have surpassed traditional methods (detergents, textiles, silver recovery, etc.). Therefore, there is a need for novel enzymes having properties with greater tolerance to factors such as temperature, pH, salinity; to allow them to be coupled to the extreme conditions of industrial processes (Margesin and Schinner 2011).

The objective of the present study was to isolate moderately halophilic bacteria protease producers isolated from saline environments. According to our data, no studies have been conducted to describe the diversity of halophilic bacteria in the state of Sonora in several saline systems.

2 Materials and methods

2.1 Sample Collection

For the isolation of halophilic bacteria, the present study was based on a methodology by Rohban et al. (2009). During April and May 2014, soil samples (up to 20 cm deep) were collected to isolate halophilic bacteria in Bahía de Lobos (110°27'34" W, 27°22'18" N), Yavaros (109°33'34" W, 26°44'29" N), Guaymas (110°46'32" W, 27°55'05" N) and Puerto Peñasco (113°29'28" W, 19°19'46" N). All enrichments and strains described here were isolated from twenty soil samples, collected from different saline environments from the state of Sonora. The samples were brine, solar salt, salt crust, saline soil, saline mud and rhizosphere soil. Figure 1 shows the location of these saline areas of Sonora. Samples were collected in sterile plastic containers and were kept aseptically at 4°C until analyzed.

2.2 Growth Conditions

All samples were cultured after collection in marine agar with a final concentration of 10% (w/v) NaCl for moderately halophilic bacteria. The pH of the culture media was adjusted to 7.35 before autoclaving. Cultures were incubated at 37°C in a Yamato incubator for 3–5 days or more depending on the growth rate of the isolates. Colonies growing on the plates were selected based on morphological features, considering pigmentation and size. Each isolate was subjected to successive streak plating until a pure colony was obtained. The isolates were stored in glycerol stocks (25% v/v) at −80°C.

2.3 Screening of strains for extracellular hydrolytic activities

To detect the production of extracellular hydrolysis; different enzymatic assays were carried out in the agar plate assay. The pH of all media was 7.35 and 10% NaCl

Figure 1: a) Map of Sonora State showing the location of the sampled sites: Puerto Peñasco, Guaymas, Bahía de Lobos and Yavaros (Fuente: Imágenes ©2016 Landsat, Data SIO, NOAA, U.S. Navy, NGACO, Data LDEO-Columbia, NSF, NOAA, Datos del mapa ©2016 Google, INEGI)
was added to detect hydrolytic activities in moderately halophilic bacteria (Babavalian et al. 2014). In order to obtain the production of extracellular enzymes, isolates from a total of 210 moderately halophilic strains from different saline areas of the State of Sonora were isolated. Enzymatic assays were performed on agar plate. Only twenty-two halophilic bacteria were examined and evaluated for the production of different extracellular enzymes such as amylase, lipase, protease, cellulase, inulinase, xylanase, pectinase, chitinase, pullulanase, esterase and DNase according to the methodology of Biswas and Paul (2014), in order to choose those bacteria with interesting protease activity. Moderately halophiles were isolated by salt (100.0 g/l NaCl) and substrate enrichment as used by Amoozegar et al. (2003). The marine agar supplemented with respective substrates was used for the production of these enzymes, a clear zone around the bacterial growth indicated the positive hydrolysis of each substrate.

2.4 Isolation and identification of protease producing bacterial strain

Pure bacterial isolates were cultured at 37°C for 48 h in marine agar medium containing 10% NaCl and 2% skimmed milk. The clear zone of hydrolysis around the colony confirmed the production of protease. The bacterial strain was selected based on the hydrolysis of maximum diameter. The individual colonies were transferred to marine agar plates. All the isolates formed as obvious zone of cleaning in marine broth of 2% skimmed milk like BLRMAM1064, PPSE3AM1053 and BLCLAM1069. The cultures were incubated at 37°C with rotary agitation at 120 rpm and adjusted to pH 7.35. The protease production concentration was verified every 24 h for five days, with the proteolytic enzymatic activity determined according to the method described in the protease assay. The culture was centrifuged at 10,000 xg for 10 minutes at 4°C. The supernatant was used for further studies.

2.5 Protein determination

Protein concentration was measured by the method of Bradford (1976) using bovine serum albumin (BSA) as the standard. Protein concentration was estimated by observing the absorbance at 595 nm. All experiments were done in triplicate.

2.6 Protease assay

The activity of protease was measured by a slightly modification of Iversen and Jorgensen (1995). The crude enzyme solution was incubated with azocasein solution. The supernatant was harvested; one milliliter of supernatant was read at 366 nm. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µmol of azo group in 1 min at 37°C. The specific activity was expressed in units of enzyme activity/mg of protein.

2.7 Molecular weight determination by SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was determined to determine the molecular weight of the crude extract, according to Laemmli (1970). For the zymogram analysis of alkaline protease enzyme, SDS-PAGE was performed according to a method modified by Garcia-Carreno et al. (1993). Electrophoresis of the enzyme extract samples were performed for samples taken every 24 hours. Protein electrophoresis was carried out in 12% polyacrylamide gels using a Mini Protein Tetra Cell Plus (Bio-Rad, USA). The molecular weight of protease was estimated by comparing the relative mobility of proteins of different molecular size using a Precision Plus Protein Standards molecular weight marker (Bio-Rad USA). The protein bands were determined through the silver-staining process as described by Blum et al. (1987).

Ethical approval: The conducted research is not related to either human or animals use.

3 Results and discussion

3.1 Qualitative evaluation of production of proteases

To test the proteolytic activity of the 22 moderately halophilic strains isolated, a plate assay was performed according to the protocol described in the Materials and Methods section. In this study, we investigated hydrolytic enzyme patterns, which are produced by strains from saline soils (PPSE-3 and BLCL), and rhizosphere (YR, GR, BLRM). In these environments, the number of moderately halophilic isolates was remarkably higher than light and extremely halophilic bacteria. The strains producing proteolytic enzymes showed a distinct extracellular form.
These used the marine agar medium supplemented with skim milk 2% and incubated at 37°C (Figure 2). The release of proteases and the activity of these were evidenced by the method of Alquicira (2003). Studies by Sánchez et al. (2004) reported that 26 strains isolated from fish effluent were grown in culture medium containing 1% casein. Sharmin et al. (2005) isolated Bacillus amovovirus, which exhibited proteolytic capacity for the hydrolysis of casein agar skim milk. Vishwanatha et al. (2010) while searching substrates for the production of proteases by Bacillus licheniformis, observed hydrolysis of the medium casein (1% w/v) milk powder. Similarly, Hindhumathi et al. (2011) identified Bacillus sp GPA4 agar with proteolytic activity when incubated with skim milk provided a clear zone of hydrolysis after 24 hours of incubation.

![Image](https://via.placeholder.com/150)

Figure 2: Halo hydrolysis presented by the strains after 24 hours of incubation at 37°C in marine agar with 2% skim milk

The aim of the present study was to isolate moderate halophilic bacteria to produce proteases from different saline areas of the State of Sonora; and ten different extracellular hydrolases were evaluated. Strains were isolated with significant ability to produce protease, amylase, lipase, inulinase, pectinase, DNAase, chitinase, pullulanase, esterase, cellulase and xylanase. Clearly, sources of carbon and nitrogen are transformed as microorganisms metabolize them, and in any case the nutrients contain all the molecules that a cell requires, so the breakdown of nutrients in compounds useful for the maintenance of the microorganism is carried out by performing biochemical reaction enzymes. In microorganisms, nitrogen (both organic and inorganic forms) is metabolized to produce primarily amino acids, nucleic acids, proteins and cell wall components. Alkaline protease production heavily depends on the availability of both carbon and nitrogen sources in the medium. Both have regulatory effects on the enzyme synthesis (Patel et al. 2005). The organic nitrogen sources used in our study allowed the bacterial growth, due to the ability of these strains to produce proteases such as BLRMAM1066, PPSE3AM1053, and BLCLAM1064. In earlier reports, it was found that other organic nitrogen sources supported protease production in other microorganisms including skim milk, peptone, casamino acids, beef extract, and others, depending on the source organism (Ibrahim et al. 2015). Screening bacteria from saline soil and mud of Bahia de Lobos, Yavaros, Guaymas and Puerto Peñasco led to the isolation of 22 moderately halophilic bacteria from where 7 were Gram-positive (5 rods and 2 cocci) and 15 Gram-negative (7 rods, 6 coccobacilli, 1 coccus and 1 vibrio). Combined hydrolytic activities have been detected in a number of strains. Only four strains presented the eleven hydrolytic activities tested (amylase, DNase, protease, pectinase and xylanase). Besides these, 14 strains showed three combined hydrolase activities and 5 strains were able to produce two extracellular enzymes (Table 1).

### 3.2 Determination of concentration of soluble protein and proteolytic activity in marine agar with 2% skim milk

It has been established (Coolbear et al. 1991) that there is not necessarily a good correlation between zones of clearing around colonies on skim milk agar plates and levels of proteolytic activity produced. To assess proteinase production more quantitatively, the azocasein assay method was used on culture supernatant fluid of each strain (data not shown). To stimulate the enzymatic activity of these microorganisms, and in order to study the ability to metabolize protein substrates as sole carbon source and nitrogen, kinetics fermentation was carried out for five days in culture. The strains BLRMAM1066, PPSE3AM1053 and BLCLAM1064 were allowed to grow and develop their activities, should the nutrients have provided energy and materials for the biosynthesis of the culture medium.

### 3.3 Polyacrylamide gel electrophoresis and determining the molecular weight.

SDS-PAGE analysis of the molecular mass of the crude extract revealed several protein bands (Figure 3a, 3c and 3e). A zymogram revealed the high-level activity of protease (Figure 3b, 3d and 3f). The enzyme found from crude extract fermentation kinetics appeared several bands in 12% SDS-PAGE gels, some of them were obtained constantly, from 193.6 kDa to 19.15 kDa at all evaluated times and analysis zymogram, with casein as substrate proteolytic bands being the most important the 96 hours.
Table 1: Cellular morphology and hydrolytic activity of moderately halophilic strains from Bahía de Lobos, Yavaros, Guaymas and Puerto Peñasco

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<th>Gram</th>
<th>Cellular Morphology</th>
<th>Skim milk</th>
<th>Starch</th>
<th>CMC</th>
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<th>Olive oil</th>
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(*) negative, (+) HP<30%, (++) 30%<HP<50%, (+++) HP>50%; where HP= Hydrolysis percent

Figure 3: (a, c, e) Electrophoresis of enzymatic extract SDS-PAGE gel at 12%, using silver staining by Chevallet et al. (2006). MM lane molecular weight marker. Figure (b, d, e) zymogram, using 1.5% casein as substrate
The apparent molecular weight of these proteases was 25 kDa. Reports by Rao et al. (2009) describe the appearance of a single band by zymography with a molecular mass of 39.5 kDa. Hernández-Martínez et al. (2011) cited different molecular weights of proteases and serine proteases of 32 kDa and 124 kDa. Annamalaia et al. (2014) showed that purified proteases of Bacillus firmus CAS 7 have molecular masses ranging from 21 kDa to 34 kDa, and in Bacillus cereus TKU006 the weight was 33 kDa.

The molecular weight of crude extract was estimated approximately by SDS-PAGE where the bacterial strain PPSE3AM1053 presents few electrophoretic bands, as well as a clear zone of hydrolysis was observed in the gel indicating the presence of proteolytic activity and could be the best candidate to purify this enzyme extract (Figure 2d).

4 Conclusions

This researching was performed to determine the diversity of moderately halophilic bacteria and their ability to produce extracellular hydrolytic enzymes from different saline areas of Sonora State and to select the best hydrolytic enzymes producers, especially proteases. 210 strains were isolated from rhizosphere and saline soil samples, but these remarkable moderately halophilic strains: bacillus Gram (-) (BLCLAM1064, and PPSE3AM1053) and coccobacillus Gram (-) (BLRMAM1066) have been found to be able to secrete extracellular proteases. The molecular masses of proteases were found in the range of 19 to 193 kDa. Moderately halophilic bacteria constitute the most versatile group of microorganisms that could be used as a source of salt adapted enzymes. Also it is highly recommended to determine the phylogenetic position of strains, growth conditions for optimal protease activity, and therefore establish the optimal growth conditions for producing the highest extracellular protease activity, suggesting that they have potential for use as biocatalysts in industry.

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Conflict of interest: Authors state no conflict of interest.

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