Mycopath (2014) 12(1): 17-23 **Research Article**

Screening for extracellular hydrolytic enzymes production by different halophilic bacteria

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Abstract

At the present time, some industrial processes require enzymes able to act under extreme physicochemical and environmental conditions. Typical enzymes may be degraded when are acting under high temperature, extreme acidic or alkaline pH, high pressure, etc. Halophilic microorganisms have capacity to support high salt concentration in the medium, allowing live under high ionic strength, low aqueous activity, organic solvents, etc., and their enzymes also have these properties. This study describes the extracellular hydrolytic enzyme production of eight moderately halophilic bacteria, isolated from the semi-desert region of Coahuila Mexico. The evaluated enzymes were amylases, proteases, cellulases, xylanases, DNAases, pectinases, chitinases and lipases using culture media with 14% NaCl (w/v). Halo formation was the method used to detect the positive extracellular enzyme production. Most of the strains were able to produce amylases, cellulases, pectinases and xylanases. Where, xylanases were those with the greatest intensity halo formation, and may have potential to be used for bio-catalytic processes.

Keywords: Biotechnological applications, halophilic hydrolases, hydrolytic halos, xylanases.

Introduction

the microbial enzymes are very important; because some products can be obtain in minor time, with better quality, but specially generating eco-friendly processes, reducing the toxic sub-products (Bhalla *et al.,* 2011). According to BCC Research (http://www.bccresearch.com/), the global enzyme market was about $3.9 dollar billions at 2011, and it is estimated about $6.1 dollar billions at 2016. These huge sales of industrial enzymes are attributed to different causes being a one of them, the enzymatic instability to extreme operational conditions, which may generate low processes productivity (Kumar, 1998; Rozzell, 1999; Schoemaker *et al.,* 2003). For this reason, search for new enzymes able to support diverse extreme conditions is needed. Some studies have focused on microorganism's extreme enzymes. One characteristic of extreme-enzymes is the capacity for supporting more than one extreme condition (high temperature, high pressure, high salt concentration, acid or alkaline pH, low aqueous activity etc) (Gomes and Steiner, 2004; Bowers *et al.,* 2009; Marhuenda-Egea and Bonete, 2002; Karan *et al.,* 2012).

Thermophilic are one of the most studied feature of extreme microorganisms, however recently, scientists have focused on other extreme microorganisms such as halophiles. These microorganisms support high ionic strength. Their enzymes have a structure and catalytic properties that improve its capacity under extreme conditions, these features are very important for their biotechnological potential and industrial process applications (Karan *et al.,* 2012). Moderately and extreme halophilic bacteria and Archaea are the best halophilic enzyme producers. However, although Archaea are able to support more salt concentration (>10 %), these are not widely studied because their enzymes lose easily their activity under low ionic strength, and separation methods are ineffective to high salt concentration, preventing their characterization and purification (Madern *et al.,* 2000; Ventosa *et al.,* 2005). In contrast, moderately halophilic bacteria are able to produce enzymes which are more active under medium and high salt concentrations, but also are stable and active under absence of salt. For this reason, the moderately halophilic enzymes are more studied for their biotechnological potential (Ventosa *et al.,* 1998).

Some halophilic enzymes have been studied for their diverse industrial applications, e.g. *Micrococcus varians* subs. *halophilus* nuclease is used to produce guanilic-5´acid and inosinic-5´acid as flavor additives (Kamekura and Onishi, 1974). a halophilic enzymes group (proteases, lipases, ribonucleases) used under sauce fermentation which elevate nitrogen content and volatile fat acids has been evaluated (Kanlayakrit

et al., 2009). Other halophilic enzymes with potential applications are *Virgibacillus* sp. chitinase which is used for chitin hydrolysis for shrimp and crab bioconversion, lipase from *Marinobacter* sp. for fish oil hydrolysis eicosapentanoic acid free, and amylase from *Pseudomonas* sp. for marine microalgae saccharification (Karan *et al.,* 2012). In this work, screening of eight (amylases, proteases, cellulases, xylanases, DNAses, pectinases, chitinases and lipases) extracellular hydrolytic enzymes production by diverse moderately halophilic bacteria was done, these microorganisms were isolated from Coahuila, México soils.

Materials and Methods

Microorganisms

The bacterial strains used to determine the halophilic enzymes production were isolated from Coahuila Soils. This Mexican State is located in the Northern semi-desert part of Mexico (Fig. 1). Specifically, these bacteria were isolated from soils of Ramos Arizpe, Arteaga, Monclova and Cuatro Ciénegas counties. Bacterial strains were identified by sequencing of the 16S DNAr region. In this study *Halobacillus* sp. AS-04, *Halobacillus trueperi* CT2-03, *Oceanobacillus* sp. ES1-03, *Bacillus pumilus* CP-01, *Salinicoccus roseus* EC-01, *Bacillus atrophaeus* SY-01, *Bacillus atrophaeus* PN-01, and *Bacillus subtilis* AS-09 were used . These microorganisms belong to the DIA-UAdeC (Food Research Department/ Universidad Autónoma de Coahuila) microbial collection. Bacteria were cryo-conserved in milk skim and glycerol, so they were activated in nutritive media with 8% NaCl (w/v) and incubated at 37 °C for 48 h (Delgado-García, 2011).

Culture media

MH basal medium was used (Ventosa *et al.,* 1998) (g L^{-1}), which contained: yeast extract (10), $CaCl_2$ $2H_2O$ (0.36), $MgSO_4$ $7H_2O$ (1), $NaHCO_3$ (0.06), casein peptone (5), glucose (10), KCl (5), NaCl 14% (p/v) (Delgado-García, 2011), carbon source was replaced depending on which enzyme was evaluated (Table 1). In the case of DNase production, commercial agar DNA (Becton Dickinson ®) was used. Finally, the pH was adjusted to 7.0 ± 0.2 and medium was sterilized.
The colloidal chitin was prepared according to Wu *et al*. (2009), but casein peptone was not added. Also each carbon source was sterilized using a sterile nylon filter (0.2 µm) (MILLIPORE®). Skim milk was sterilized at 110 °C for 10 min (Farkas *et al.,* 1985; Wejse and Ingvorsen, 2003).

Screening for extracellular hydrolytic enzymes by bacterial strains

The culture medium with each carbon source was placed into a microplate (96 well). In each well were added 200 µL of culture medium. Using this methodology, enzyme detection is faster and cheaper. Each strain was inoculated into a specific well, and then microplate was incubated at 37 °C for 24 h and 48 h. After that, bacterial growth was estimated and each well was revealed with a special solution for hydrolysis halo detection (Table 1). The hydrolysis halo formation was observed using a stereoscope (Delgado-García, 2013).

Statistical analysis

Experiments were established under a complete block design with factorial arrangement of treatments with three replications. The factors were: type enzyme and bacteria strain. The experimental unit was each well, where the response variable was the hydrolysis halo formation (presence +, absence –). Data were analyzed using a categorical analysis for *sx*2 tables, where was evaluated if the enzyme was or was not produced by each strain (eight strains), and the number of producer and not producer strains for each enzyme (eight enzymes). Columns and files were classified nominally. Software SAS version 1997 was using for analysis.

Results and Discussion

In this study, five halophilic strains, *Oceanobacillus* sp. (ES1-03), *Halobacillus* sp. (AS-04), *H. trueperi* (CT2-03), *B. subtilis* (AS-09) and *S. roseus* (EC-01) were evaluated. These strains were grew at 24 h in the culture media, while *B. atrophaeus* (PN-01 and SY-01) and *B. pumilus* (CP-01) showing a growth until 48 h. Table 2 showed the enzymes produced by each strain.

The most produced enzymes were: amylases (by six strains: *S. roseus* (EC-01), *Halobacillus* sp. (AS-04), *Oceanobacillus* sp. (ES1-03), *B. pumilus* (PN-01), *Bacillus atrophaeus* (PN-01 and SY-01) and xylanases (by six strains: *S. roseus* (EC-01), *Halobacillus* sp. (AS-04), *Oceanobacillus* sp. (ES1-03), *Bacillus pumilus* (PN-01), *Bacillus atrophaeus* (PN-01 and SY-01). On the other hand, pectinases and cellulases (by four strains: *S. roseus* (EC-01), *B. pumilus* (PN-01), *B. atrophaeus* (PN-01 and SY-01) also were

produced by most of the strains. However, chitinases (1 strain: *S. roseus* (EC-01)) and DNases (1 strain: *H. trueperi* (CT2-03) were produced with minor frequency, while lipases and proteases were not produced by the tested strains (Fig. 2). During hydrolytic halos evaluation, halos with more intensity were observed for xylan hydrolysis (Fig. 3) also, most of the strains were able to produce xylanases, for this reason, these enzymes were selected for further studies.

According to the statistical analysis, where it was tested if the strain influences the enzyme type produced. The chi-square (Qp) had a value of 3.0476 and P = 0.8806, while, Mantel-Haenszel chi-square (Qs) had a value of 0.4675 and P = 0.4941. These results suggest that there is not an association between number of enzymes produced and not produced for each strain. On the other hand, it was evaluated if there is an association between number of strains producer and no producer of each enzyme, the Qp had a value of 25.2121 and P = 0.0007, while Qs of Mantel-Haenszel had a value of 12.48 and P=0.0004, for instance, there is an significant association between number of strains producer and no producers of each enzyme. In this case, the QMH value was 12.4805 with one degree of freedom, which clearly indicates significant differences, confirming that not all strains produce the same number of enzymes.

Some studies about determination of halophilic extracellular enzymes have been reported that about 70% of the moderate halophilic bacteria have the best hydrolytic capacity (Moreno *et al.,* 2009). Actually, there are reported only few genera of halophilic bacteria producers of hydrolytic enzymes (Moreno *et al*., 2013). The positive or negative hydrolysis of some carbon source is given mainly by the strain specie (strain genus and specie, metabolic, taxonomic and genetic characteristics) and the environment from the strain was isolated (Flores *et al.,* 2010). Principally, amylases are the most produced enzymes by different genera such as *Salinicoccus* sp. (Sánchez-Porro *et al*., 2003), while lipases are the most common in *Oceanobacillus* sp., *Halobacillus* sp. and *Salinicoccus* sp. genera (Rohban *et al.,* 2009).

The tested strains were isolated from soil close to trees rhizosphere from the Coahuila semidesert region (Delgado-García, 2011). For this reason, xylanases, cellulases and amylases were the major enzymes produced because of bark residues were the largest source of available carbon. Specifically, xylan and pectin are one major components of cellular wall (Lodish, 2006), while starch are reserve carbohydrate in medullar rays, bark and roots of plants (Salama, 2005). Therefore, these substrates were more available for the tested strains.

There are reports that *Halobacillus* sp. and *Oceanobacillus* sp. are able to produce amylases, xylanases and cellulases, while DNases and pectinases are weakly produced (Rohban *et al.,* 2009; Kumar *et al.,* 2012). It was reported that *S. roseus* does not produce hydrolytic enzymes (Sánchez-Porro *et al.,* 2009). In contrast, in the present study, *S. roseus* showed hydrolytic capacity specially xylanases and chitinases. Chitinase had been reported as agent against *Botrytis cinerea* a strawberry phytopathogen, *B. cinerea* growth was inhibited in a 70% by chitinases (Sadfi-Zouaoui *et al.,* 2007). On the other hand, *S. roseus* chitinase can be applied in diverse processes as chitin bioconversion, for example in shrimp and fish waste or for waste water treatment of chitin, generating more sustainable processes (Karan *et al*., 2012). *H. salinarum* NRC-1 chitinase has been sequenced and cloned and can be used in oligosaccharides synthesis using organic solvents (Enache and Kamekura, 2010), or as control agent of phyto-pathogens.

B. pumilus has been reported as a xylanase producer (Menon *et al.,* 2010; Nagar *et al.,* 2010). In other studies, *Bacillus* sp. is reported as the best producer of extracellular enzymes to industrial level (Schallmey *et al.,* 2004). In this study, the weakly growth of *B. pumilus, B. atrophaeus* and *B. subtilis*, can be attributed principally to salt concentration, which can inhibit these bacterial strains growth. Ionic and hydrophobic effects are essentials for protein stability. For this reason, salt concentration is very important in enzymatic activity because influence stability and structure of halophilic proteins. During hydrolytic halos evaluation, halos with more intensity were observed for xylan hydrolysis (Fig. 3), the most of the strains were able to produce xylanases. Although, cellulose was used as carbon source, one advantage is that xylanase is produced more than cellulases (Salama, 2005). In comparison with other studies where amylases (Sánchez-Porro *et al.,* 2003; Dang *et al.,* 2009; Moreno *et al.,* 2013; Makhdoumi *et al.,* 2011), lipases (Cojoc *et al.,* 2009; Rohban *et al.,* 2009) and DNases (Moreno *et al.,* 2012) were produced by halophilic strains but in this case, xylanases were more produced and showed the influence of the isolation environment on hydrolysis capacity.

Specifically, xylanases are very important in diverse industries. It has been reported that

halophilic xylanases showed stability and activity at 0-5 M of NaCl concentration (Enache and Kamnekura, 2010). Xylanases are used in bakery to improve dough properties, also have great potential in the paper and pulp industry where are required alkaline and thermostable enzymes, properties showed by halophilic enzymes, allowing the chloride in pulp bleaching. Actually, information about halophilic xylanases from halophilic or halotolerant microorganisms is scarce. Some scientists are using *Glaciecola mesophila* (Guo *et al.,* 2009), *Chromohalobacter* sp. (Prakask *et al.,* 2009) and *Halorhabdus uthaensis* (Sarma *et al.,* 2010). Some applications of halophilic xylanases include lignocellulosic material and agro-waste bio-conversion using fermentations, juice clarification, coffee manufacturer, dough, etc (Subramaniyan and Prema, 2002; DasSarma *et al.,* 2010).

Actually, our research group is searching new application for halophilic enzymes, so, these results provide information about xylanases, amylases, pectinases and cellulases from extreme microorganisms, which may have major advantage in some industrial processes, where the operational conditions are hash (Sánchez-Porro *et al.,* 2003; Flores *et al.,* 2010). It is important to remark, that studies about characterization of production conditions, purification and gene sequencing will allow to obtain knowledge about physiology of these microorganisms and its protein adaptations to function in front extreme conditions (Enache and Kamekura, 2010; Ozcan *et al.,* 2009).

Table 1: Culture media used for each hydrolytic enzyme production.

Enzyme	Carbon source	Halo formation solution	Reference
Amylases	Soluble starch 0.2%	0.1 % I_2- 0.2% KI	González *et al.,* 1978
Proteases	Skim milk 2 %	Halo formation	Collins and Lyne, 1989
Lipases	Olive oil 1% and Rhodamine B (0.001 %)	Orange halo under UV light	Ozcan *et al.,* 2008
Xylanases	Xylan 1%	Congo red 0.1 %	Wejse and Ingvorsen, 2003
Cellulases	Carboxymethyl cellulose 0.5 %	Red Congo 0.2 % y 1N NaCl	Farkas *et al.,* 1985
Pectinases	Pectin 0.5%	0.1% I_2-0.2 % KI	Soares *et al.,* 1999
Chitinases	Colloidal chitin 1%	0.1% I_2-0.2 % KI	Makhdouni *et al.,* 2011
DNases	DNase agar: DNA (2 g/L)	Yellow halo formation	Becton Dickson ®

Table 2: Hydrolytic extracellular enzymes produced by each halophile bacterial strain.

	Enzymes							
	Am	**Pec**	**Qui**	**Xin**	**Cel**	**Lip**	**DNAs**	**Pr**
Salinicoccus roseus (EC-01)	+	+	+	++	–	–	–	–
Halobacillus sp. (AS-04)	+	–	–	++	+	–	–	–
Oceanobacillus sp. (ES1-03)	+	–	–	++	+	–	–	–
Halobacillus trueperi (CT2-03)	–	–	–	–	+	–	+	–
Bacillus pumilus (CP-01)	+	+	–	++	–	–	–	–
Bacillus subtilis (AS-09)	–	–	–	–	+	–	–	–
Bacillus atrophaeus (PN-01)	+	+	–	++	–	–	–	–
Bacillus atrophaeus (SY-01)	+	+	–	++	–	–	–	–

*__Am__ (amylases), __Pec__ (pectinases), __Qui (__chitinases), __Xin__ (xylanases), __Cel__ (cellulases), __Lip__ (lipases), __DNAs__ (DNases), __Pr__ (proteases), (+) production, (_) no production

Conclusions

The tested strains showed a varied hydrolytic profile, associated principally to the location where the strains were isolated, showing their capacity to produce enzyme in high salt concentration. All strains had a hydrolytic capacity on starch; pectin, cellulose and xylan front 14% of NaCl (p/v). According to the obtained results, the best hydrolytic capacity of the tested strains was front xylan obtaining intense hydrolysis halos.

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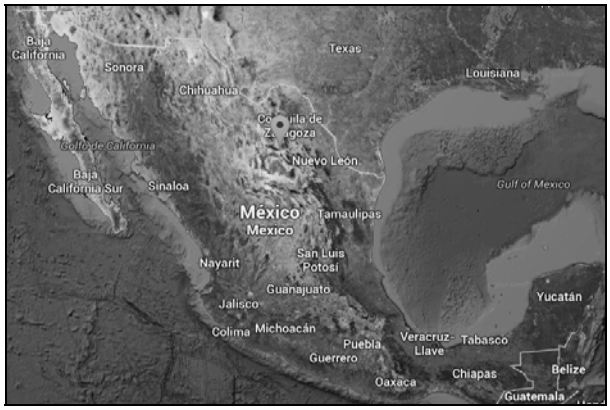

Fig 1: Coahuila state located in the Northern semi-desert part of Mexico with a red point. Geographical coordinates : 27°18'08"N°102°02'41"O

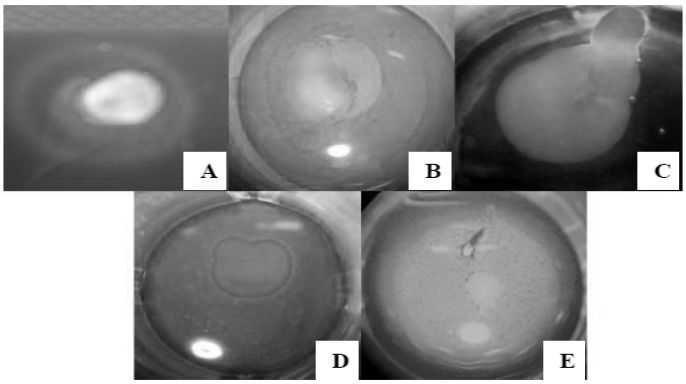

Fig 2: Hydrolysis halos. **A:** cellulases (*Halobacillus* sp.) **B:** xylanases (*Salinicoccus roseus*) **C:** pectinases (*Bacillus pumilus*) **D:** chitinases (*Salinicoccus roseus*) **E:** xylanases (*Halobacillus* sp.).

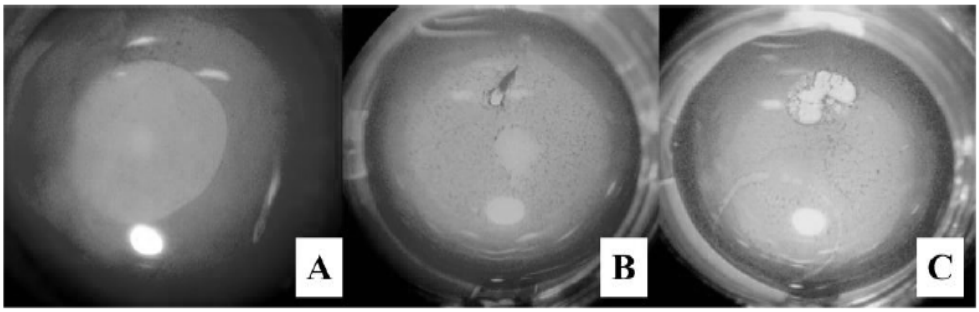

Fig 3: Xylanase activity A: *Salinicoccus roseus* (EC-01) B: *Halobacillus sp*. (AS-04) C: *Oceanobacillus sp*. (ES1-03).

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