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Chitosanase Production by a New Bacterial Sources

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Abstract: Microbial marine enzymes can offer novel biocatalysts with properties such as high salt tolerance, hypoerthermostability, barophilicity and a more simple approach in large scale cultivation. Chitosanases are enzymes with importance as low molecular weight chitosans and chitosan-oligomers producers, which show innumerable applications in various fields, such as the biomedical, pharmaceutical, agricultural, biotechnological and food industry fields. However, the use of chitosanase is limited due to of its high-cost implications and potential unavailability in bulk quantities. In this study the production of bacterial chitosanases was evaluated in new isolated sources from Valley of Cuatrocienegas, Coahuila, Mexico. Specific culture media using chitosan-oligomers was used as a single source of carbon. Under these conditions, *Aeromonas* sp. produced 501 U L^{-1} of chitosanase activity and 220 U L^{-1} for *Bacillus cereus*, respectively. The crude chitosanase activity of culture supernatant was maximal on the 150 h of culture. Below 40°C, viscosity kinetics have indicated that chitosan is efficiently hydrolyzed into chitosan-oligosaccharides by *Aeromonas sp* and *Bacillus cereus* in a more rapid manner. SDS-PAGE of enzymatic extract of *Aeromonas* sp. has indicated a single band of ~ 20 kDa.

Key words: Chitosanolytic activity, chitosan-oligosaccharides, *Aeromonas* sp.

INTRODUCTION

The biological and chemical diversity of the marine environment has been the source of chemical compounds with a potential for industrial development as enzymes, pharmaceuticals, cosmetics, nutritional supplements, molecular probes, etc. Marine microorganisms are relatively easy to isolate, cultivate, identify and bioprocess. They are of major interest; besides properties like high salt tolerance, hyperthermostability and cold adaptability (Debashis, 2005).

Cuatrocienegas Coahuila, Mexico, is a valley rich in fauna and flora, with a hundred of endemic species, characterized for diversity aquatic environments. This location has desert sands with high concentrations of salts mainly calcium sulfate and temperatures ranging from -10 to 50°C, these conditions make possible the presence of some extremophilic microorganisms. Also, it has been reported low phosphorus concentration and a range of water pH (7.0-9.7) (Souza, 2004).

In recent years, important studies have shown that the microorganisms isolated from this valley (*Bacillus*, *Halomonas*, *Vibrio*, *Oceanomonas*, *Iialobacterium*, *Calothrix*) are an important source of future researches with biotechnological applications (Souza, 2004; Mauricio-Benavides *et al*., 2007).

In a previous work, *Aeromonas* sp. was isolated from La Poza Azul and characterized as a Gram negative bacteria. This genus consists in straight cells of 1.0-4.4 μm growing in pairs or chains, with polar flagella for mobility and they are anaerobic facultative, oxidase positive, resistant to vibriostatic agent 0/129 and do not require NaCl for growth (Mauricio-Benavides, 2007).

Chitinolytic enzymes have been found in a variety of organisms, including bacteria, fungi and higher plants. Many chitinases produced from *Aspergillus* sp. (Rattanakit *et al*., 2003), *Bacillus* sp. (Thamthiankul *et al*., 2001; Wang *et al*., 2001), *Clostridium* sp. (Li *et al*., 2002), *Serratia* sp. (Brurberg *et al*., 1994), *Vibrio* sp. *Enterobacter*, *Pseudomonas* and *Aeromonas* sp. (Ueda *et al*., 2003; Choi *et al*., 2003a) also have been studied; however, only a few industrial applications have been reported.

These enzymes are also useful for obtaining chitosan-oligosaccharides which have interesting biological properties, such as eliciting pathogenesis-

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related proteins in higher plants (Kendra, 1984) and immunopotentiating effectors (Suzuki, 1984). New sources of chitinolytic enzymes from *Aeromonas* sp. (Huang, 1996; Choi, 2003a, b; Kojima, 2005; Kuk, 2005 and 2006), *Aeromonas hydrophila* (Mitsutomi, 1990) and chitosanases (Wu, 2003) have been published. On the other hand, information on chitosanases from *Aeromomas* sp. is quite limited.

Chitosanolytic enzymes are increasingly gaining importance as low molecular weight chitosans (LMWC) and chitosan-oligomers producers, which show innumerable applications in various fields, such as the biomedical, pharmaceutical, agricultural, biotechnological and food industry fields (Tokoro, 1988; Tsai, 1997). But the utility of chitosanase in hydrolysis is limited because of its cost and unavailability in bulk quantities. A number of non-specific enzymes such as lysozyme, papain, lipase, cellulase, pectin lyase (Jeon, 2000; Muraki, 1993) have been used in free or immobilized forms for hydrolysis of chitosan and preparation of chitosan hydrolyzate with different molecular weights.

The objective of this study was to investigate new bacterial sources of quitosanase activity isolated from extreme conditions of the Valley of Cuatrocienegas, Coahuila, Mexico.

MATERIALS AND METHODS

Microorganisms: *Aeromonas* sp. strain, originally isolated in nutritive agar from water (Cuatrocienegas, Coahuila, Mexico) was characterized by API 20NE system (BioMerieux, France) and MicroScan Combo 20 E (Renton, WA, USA). *Pseudomonas aeruginosa* and *Bacillus cereus* were obtained from the Microbial Collection Culture Biotechnology Department (Universidad Autonoma de Coahuila). The microorganisms were maintained in two different stocks: specific medium using chitosan-oligosaccharides as a carbon source and nutritive agar.

Culture media: Bacterial strains were grown using chitosan-oligosaccharides-agar plates (chitosan-oligosaccharides 1%, NaCl 0.5 %, $NaNO_3$, 0.3%, KCl 0.5%, KH_2PO_4 0.2%, $MgSO_4$ .01 %, bacteriologic agar 2%), their homogenous grown was evaluated after 48 h at 37°C. In terms of proliferation, strains were kept in nutritive agar at 4°C. Bacteria was cultivated in 250 mL Erlenmeyer flasks containing chitooligosaccharides 1%, NaCl 0.5 %, $NaNO_3$ 0.3%, KCl 0.5 %, KH_2PO_4 0.2%, $MgSO_4$ 0.01% media, for 0, 24, 48, 72, 96, 120, 144, 168, 192 h at 37°C on a rotatory shaker (New Brunswick).

Production of enzyme extract: Bacterial biomass was removed by centrifugation at 10,000 rpm for 25 min and the supernatant was used as enzyme source. Protein concentration was done analyzed by the modificated Peterson method (Gorina, 1980). Cellular growth was measured by turbidimetry at 590 nm using a Sintra 10 UV-Vis Spectrofotometer (USA) in 1:10 dilutions. Viscosity kinetics were performed using an Oswald viscosimetry (Kimax 350 AJ 46460, USA) at 40°C.

Enzymatic assay: Chitosanase activity was assayed by reduction on viscosity by depolymerization of chitosan solution (Industrias Poseidon, Mexico) as a substrate. The reaction mixture consisted of 9.5 mL of 1.32% (w/v) chitosan disolved in 50 mM sodium acetate buffer (pH 5.5) and 0.5 mL of enzymatic extract solution. The mixture was incubated at 40°C for 180 min. One unit (U) of chitosanase activity was defined as the amount of enzyme to reduce 1% the viscosity of chitosan solution at 1.32% at 40°C.

Enzymatic activity in plate: An aliquot of 10 μL of enzymatic extract was added to a Petri dish containing 1% (w/v) agarose and chitosan-oligosaccharides (3 and 20 kDa). Sample was discarded in the center of the plate and incubated at room temperature for 24 and 48 h. Cup-plate gel was washed (4 times, during 20 min) and stained with 2 mL of blue brilliant remazol (0.2%, w/v) stain, 10 min later the gel was washed again and the halos were detected (Rangel-Rodríguez and Contreras-Esquivel, 2008).

SDS-PAGE: SDS-PAGE analysis of proteins was carried out by using 12.5% polyacrilamide gel at 120 V for 90 min. Solutions for preparing 12.5 % resolving gel included H_2O (6.4 mL), 30% acrylamide mix (8.3 mL), 1.5 M Tris (pH 8.8, 5 mL), 10% SDS (0.2 mL), 10% ammonium persulfate (0.1%) and N, N, N, N tetramethylenediamine (0.008 mL). The gel separation was performed using a traditional protocol. SDS-PAGE gel was stained by silver stain method (Sambrook *et al*., 2001).

RESULTS AND DISCUSSION

Pseudomonas aeruginosa and *Bacillus cereus* belong to the Biotechnology Department. *Aeromonas* sp. was isolated from La Poza Azul, Cuatrocienegas, Coahuila (Mauricio-Benavides *et al.,* 2007) at 120°7'26''799'' of west longitude and 26°55'20''767'' of north latitude. Biochemical methods were used to characterize the microorganisms by API and MicroScan protocols. Table 1 shows the fermentative activity of the microorganism identified. According to the API 20 E method, this isolated strain was identified as *Aeromonas* sp.

Table 1: Comparative test of identification isolated bacteria

Test	Active component	Reaction/Enzymes	API 20 E	MicroScan
ONPG	2-nitro-phenyl-βD-galactopyranoside	β-galactosidase	+	+
ADH	L-arginine	Arginina-dihidrolase	+	
LDC	L-lysine	Lisin descarboxilase	+	+
ODC	L-ornithine	Ornitin descarboxilase	-	+
CIT	Trisodic citrate	Citrate use	+	+
H_2S	Sodium thiosulfate	H_2S production	-	-
URE	Urea	Urease	-	-
TDA	L-triptofan	Triptofano desaminase	-	
IND	L-triptofan	Indol production	-	
VP	Sodium pyruvate	Acetoine production	+	
GEL	Gelatin	Gelatinase	+	
GLU	D-glucose	Fermentation/oxidation	-	+
MAN	D-manitol	Fermentation/oxidation	+	
INO	Inositol	Fermentation/oxidation	-	-
SOR	D-sorbitol	Fermentation/oxidation	+	+
RHA	L-rhamnose	Fermentation/oxidation	+	

We have evaluated the accuracy of these two methods by the identification of *Aeromonas* and *Bacillus* strains. The isolates were grown on nutritive agar at 37±2°C for 24 h. This culture was used to inoculate the API 20 E and Microscan negative combo 20 type panels. Oxidase testing was performed as a complement for both systems. The panel reading gave a 10-digit number that was compared to the corresponding database.

Vivas *et al*. (2000) reported a poor accuracy of MicroScan identifying *Aeromonas* isolates to the sp. where 19.3% were confirmed and identified. An explanation of this discrepancy could be the fact that those authors compared the results obtained with Micro Scan with those obtained with other biochemical procedures and in general, it has been demonstrated that biochemical identification are not reliable for this propose. It is well known that the genus *Aeromonas* is frequently confused with *Vibrio* (Abbortt, 1992).

Figure 1 shows the morphology presented by the microorganisms studied in this study. *Pseudomonas aeruginosa* plate growth was not homogeneous in the specific chitooligosaccharides agar after 72 h at 37°C; this factor was limited to be used for the proliferation. *Bacillus cereus* and *Aeromonas* sp. presented cellular growth after 48 and 96 h at 37°C, respectivly. Figure 2 show the cellular growth of *Aeromonas* sp. and *Bacillus cereus* with a rate of growth (μ) of 0.0018 OD/h and 0.007 OD/h, respectively. Both microorganisms presented a stationary phase after 96 h. Several scientific papers have reported the use of a culture media with colloidal chitin using acetic acid as a solvent, showing promissory results in enzyme production. This work proposes the use of a culture medium with chitosan-oligosaccharides of 30 kDa as the only source of carbon with satisfactory results. The use of this carbohydrate on a medium makes the isolation and growth of this kind of microorganisms easier due to the chitooligomers water solubility as well as the production of the extracellular enzyme (Charles-Rodríguez, 2007).

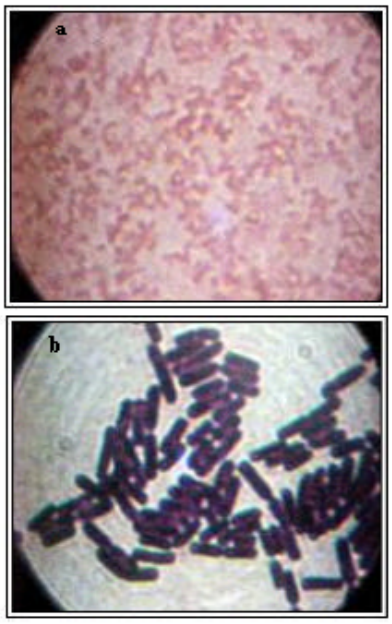

Fig. 1: Microscopical morphology (100 ×) of *Aeromonas* sp. (a) and *Bacillus cereus* (b)

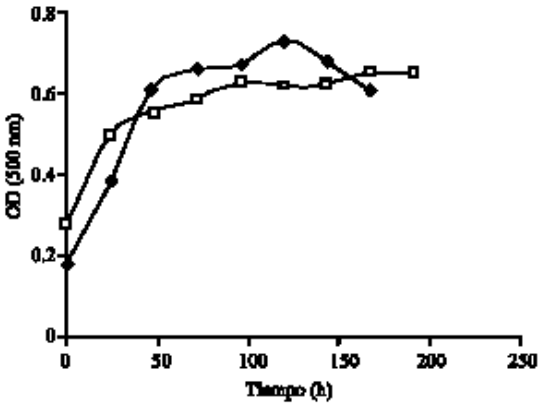

Fig. 2: Growth curve of *Aeromonas* sp. (□) and *Bacillus cereus* (■)

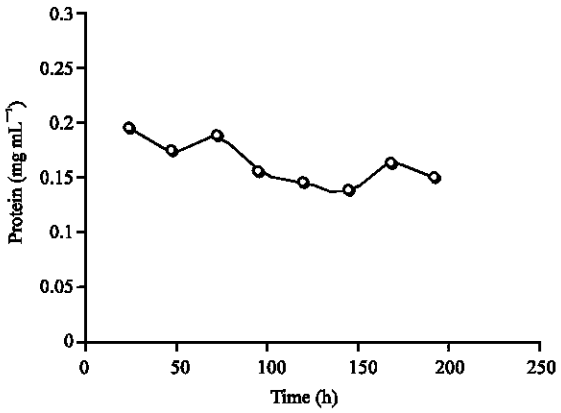

Fig. 3: Extra-cellular protein content of *Aeromonas* sp.

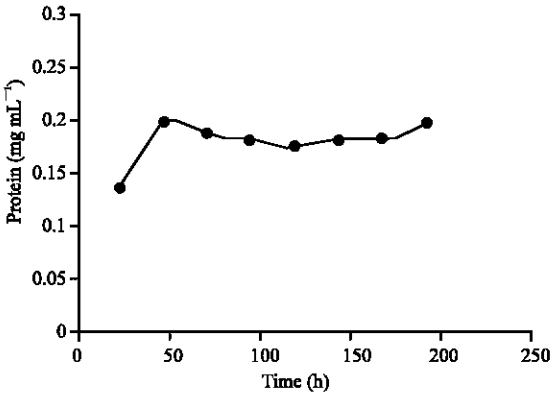

Fig. 4: Extra-cellular protein content of *Bacillus cereus*

Figure 3 and 4 show the extracellular protein content in the enzymatic extract from *Bacillus cereus* and *Aeromonas* sp., the maximum concentration of protein was at 48 h (0.1999 mg mL^{-1}) and 24 h (0.1949 mg mL^{-1}), respectively. Protein concentration was stable for 198 h of fermentation time for *Bacillus cereus* and *Aeromonas* sp.

The method using chitosan-oligosaccharides as the substrate was found to be effective for the determination of chitosanase activity. The conventional method using colloidal chitin as the substrate is based on the evaluation for reducing sugars released due to the chitinase action (Kuk *et al*., 2006). In addition to other *Aeromonas* sp. in the literature, the *Aeromonas* sp. isolated from Cuatrocienegas Coahuila, Mexico, found to be a chitosanase-secreting microorganism. As shown in Fig. 3 and 4, *Aeromonas* sp. and *Bacillus cereus* could secret a significant amount of chitosanase to the medium, with 48 h using chitosan or oligosaccharides of chitosan as inducer. The activity of oligosaccharides of chitosan-cleavage in the medium by increasing and approaching for *Aeromonas* sp. and *Bacillus cereus* after 2 and 3 days the

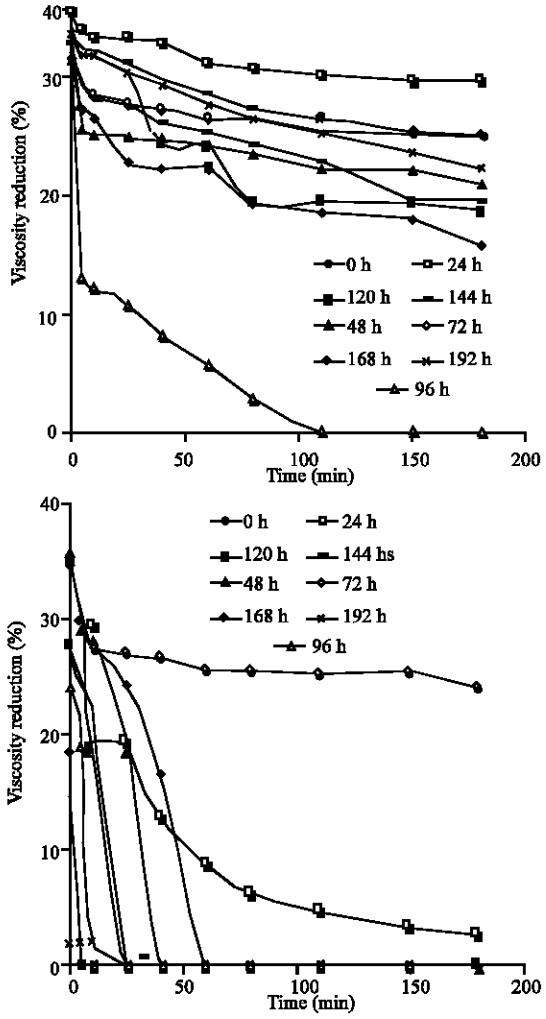

Fig. 5: Viscosity kinetic of chitosan hydrolysis by *Bacillus cereus* (a) and *Aeromonas* sp. (b) extracts

highest value, respectly. The extracellular enzyme activity remained almost the same level at the end of 8 days of culture.

Figure 5 showns the behavior of *Bacillus cereus,* the viscosity is almost constant for a long reaction time; it means that is necessary 98 h of fermentation time and 100 min to reduce the viscosity of the substrate. In the case of *Aeromonas* sp. at 48 h of fermentation time and 100 it min is enough for the total reduction of the viscosity of chitosan. These results are higher than Kurk´s publications, were chitinase activity of *Bacillus* sp. gave a 1.44 U mL^{-1} (Kurk, 2005).

In this reseach, isolated *Aeromonas sp.* was found to secret major levels of chitosanolytic activity than the *Bacillus cereus* and other chitinase-producing *Aeromonas* sp. Althought the synthesis and secretion

of chitosan oligosaccharides for extracelular chitosanases to the medium for the hydrolysis of chitosan by *Aeromonas* sp. and *Bacillus cereus* was observed. Further studies are needed to optimized the culture conditions. Several factors such as chitosan concentration and additional carbon and nitrogen sources might influence the production of chitosanases. Also, cloning of the chitosanase gene into an industrial microorganism might be an alternative for the production of chitosanases (Chen, 2006).

A rapid method to determinate chitosanase activity in Petri plates was performed (Rangel-Rodriguez and Contreras-Esquivel, 2008). The procedure indicate that the colorant with negative charge had an important role in the interaction with the chitosan-oligosaccharides (positive charge) immobilized into agar. Several washes are required, the chitooligosaccharides with low molecular weight will be released and a white halo will be observed if the enzyme acts over the substrate. This semi-quantitative technique is useful for the determination of different activities in several microorganisms. As shown in Fig. 7 higher halos were observed in *Aeromonas* sp. after 24 and 48 h of reaction than those observed for *Bacillus cereus* (Fig. 6). Oligosaccharides of 3 kDa were no usefull for this technique because of its low molecular weight.

There are some methods for quitosanase activity determination; however, the main problem is the substrate´s low solubility in water. This cause that the kinetics measurements be more difficult, uncertain, doubtful and inaccurate. The main approach has been to use soluble chitosan in sodium acetate acetic acid buffer to measure the changes in viscosity (Fenton and Eveleigh, 1981).

Figure 8 shows the *Aeromonas* sp. and *Bacillus cereus* chitosanase production. Chitosanase enzyme is quickly excreting to the culture media, the activity was stable for 150 h of fermentation; also the maximum activity value (501 U L^{-1}) is 2.3 time greater than the maximum chitosanase activity of *Bacillus cereus*. The extracellular enzyme of *Bacillus cereus* is in addition unstable because almost a total activity reduction is observed after 100 h of culture fermentation. It is well known that the extracellular enzyme production in submerged cultures is highly sensible to the production of proteases, generated by stress conditions, causing proteolysis (Aguilar *et al*., 2002). Moreover, it is important to know the mode of enzymatic hydrolysis action on the substrate that depends of the degree of acetilation and polymerization of the chitosan.

Most of the enzymes are endo-acting releasing mixture of chitosan dimmers, trimmers and oligomers.

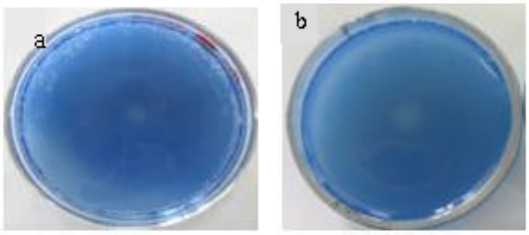

Fig. 6: Hab formation on cup-plate assay by supernantant of *Bacillus cereus* after incubation of a) 24 h and b) 48 h

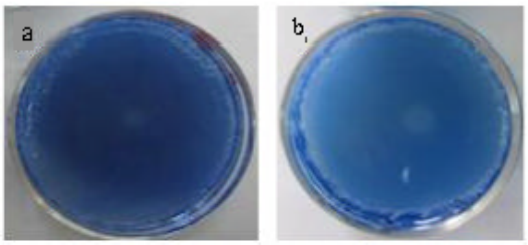

Fig. 7: Hab formation on cup-plate assay by supernantant of *Aeromonas* sp. after incubation of a) 24 h and b) 48 h

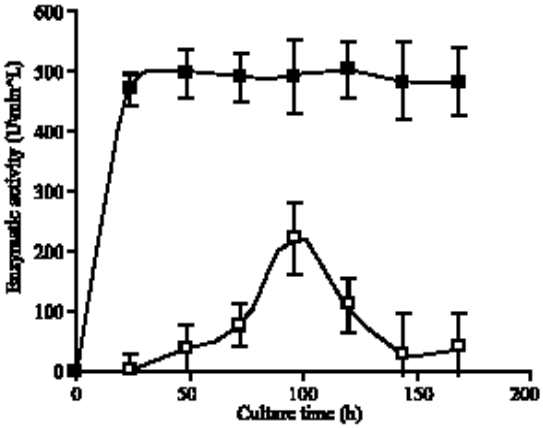

Fig. 8: Kinetic comparision of quitosanase extracellular production activity by *Bacillus cereus* (□) and *Aeromonas* sp. (■)

For *Bacillus cereus* is important the presence of N-acetylglucosamine residues in the substrate to maintain the enzymatic activity. The action mode of *Bacillus cereus* suggests that the exo-chitosanase requires a substrate with three or more remainders of glucosamine or N-acetylglucosamine to express its activity. A chitosanase from *Bacillus* P1-7S hydrolyzed chitotetrose to glucosamine, *Bacillus licheniformis* UTK and *Bacillus*

circulans hydrolyzed chitotriose and chitotetrose to chitobiose. Chitopentose or chitohexose does not release glucosamine; it suggests that the active site of each chitosanase can recognize more than two glucosamine residues in both sides of the chain against the separation point of glucosamine polymer causing a decrease in activity (Kurakake, 2000).

Several microorganisms have been used for the chitinase and chitosanase production using different carbon sources. Chitosanase activity of 42-47 U mg^{-1} of protein is observed using chitosan as a substrate by *Mucor rouxii*; this fungi increases the activity to 102 U mg^{-1} of protein when colloidal chitin is used as substrate. *Bacillus megaterium* P1 displays an activity of 154-158 U mg^{-1} of protein using chitosan, chitin or carboximetilchitosan as a substrate (Somasheakar, 1996). Chitosanase activity of *Bacillus* sp. KCTC 0377BP increased from 1.2 U mL^{-1} in a minimal chitosan culture to 100 U mL^{-1} in an optimized media (Choi, 2004).

Bacillus alvei reached chitosanase activity levels of 2.3 U mL^{-1} (Abdel-Aziz, 1999); 53 U mg^{-1} for *Bacillus cereus* S1 (Kurakake, 2000); *Aeromonas* sp. GJ-18 presented 10 U of chitinolytic activity (Kurk, 2005). In this study SDS-PAGE of *Aeromonas sp* was performed and a unique band in the range of 15-20 kDa was obtained (data not shown).

CONCLUSION

New bacterial chitosanases sources were identified using microorganisms isolated and characterizated from Valley of Cuatrocienegas, Coahuila, Mexico. *Aeromonas* sp. showed higher chitosanase activity than others microorganisms reported in literature. The use of chitosanoligosaccharides as only source of carbon had a positive effect to produce an extracellular chitosanase. A methodology for chitosanase activity in Petri plate was validated as a new qualitative and quantitative technique to determine chitosanase activity. Further studies are needed to define the extreme isolation conditions of *Aeromonas* sp., optimized the culture media, kinetic parameters and molecular biology as a tool for sequencing *Aeromonas* sp.

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