CHITINOLYTIC BACTERIAL ISOLATION FROM SOLAR SALTWORKS

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EXTENDED ABSTRACT

In the nature, chitin is an extremely abundant material with a high molecular weight. This material requires be degraded to be transported through the cellular membrane of the microorganisms. Halophiles bacteria carried out this process in two ways: adsorption at the surface of the chitinous material and degrading the sustrate by means of chitinolytic enzymes located in cellular membrane or using extracellular enzymes.
At the moment exists a great interest in chitinolytic microorganisms that growth in saline and hypersaline saline environments, since is considered that they can be organisms able to carry out the degradation of the chitinous materials efficiently.
The objective of this work was the isolation of chitinolytic bacterials from the solar saltworks of the company Industria Salinera of Yucatan, S.A, located in “Las Coloradas” in Yucatan, México.
The system of solar saltwork subject of study settled down six sampling stations that covered a range of salinity from the 6.88 to 32.98% of salinity. The isolation process was carried out at 30 and 40°C achieving you to isolate a total of 30 chitinolytic bacteria strains of which 24 strains grew at 30°C, and 7 strains grew at 40°C. Chitinolytic activity ratio (Ar) of strains was determined using a Petri dishes with solid culture media added with colloidal chitin, and was found that one bacteria isolated at 30°C had an Ar=2.13, similar to native strains of *Serratia marcescens*.

KEYWORDS: chitinolytic bacterial, chitinous wastes, solar saltworks

1. INTRODUCTION

Chitin, poli-β-(1(4)-N-acetil-D-glucosamine, is one of the most abundant biopolymers in nature, especially in marines organisms, insects, mushrooms and yeasts. Chitin and chitosan (deacetilada chitin), are produced industrially in Japan (Austin, P.R., 1981), using as raw material the solid wastes of the industry packer of crustaceans (Knorr, D., 1991). Since the content of chitin in solid wastes of crab and shrimp is of approximately 14 and 28% respectively. In Mexico the solid waste generated by the industry shrimp packer are not taken advantage of in an appropriate way, although they are a resource that could be used for the generation of products of high added value.
In Mexico, volumes of shrimp capture in the last 12 years indicate a relatively constant behavior, averaging volumes of capture superiors to the 50,000 annual tons. In this sense and taking in consideration that the non eatable portion of the shrimp (cephalothorax) represents approximately 20% of the crustacean weight, it is calculated that the quantity of generated waste is of around 10,000 tons. Most of those are deposited in clandestine

garbage dumps or in the sea and only a small portion is dedicated to the production of balanced feeds.
One of the possibilities of use of the shrimp wastes is the production of N-acetylglucosamine, monomer of chitin, which have application possibilities in food, cosmetics and pharmacy industries, table I.

Table I. N-acetylglucosamine application possibilities

Uses	Reference
Induction of insulin release	Ashcroft, 1975
Treating degenerative afflictions of the joints	Rovati, L., 1972; Speck, U., 1989; Ichicawa, Y., 2007
Treatment of food allergy	Burton, A.F., 1993
Femoral hemostatic patch	González-López, 2003
Skin care agent	Matahira, Y., 2003
Treatment of cellulite	Murad, H. , 2004
Radial hemostatic patch	Gómez-Fernandez, 2005
Sweetening agent	Wassenaar, W., 2007

However total hydrolysis of chitin, present in the solid waste of shrimp, until N-acetylglucosamine (NAcGlu) required the serial action of two hydrolases: chitinases (chitin glucanohydrolases, E. C. 3.2.1.14) and chitobiases (chitobise acetylamino desoxiglucohydrolases, E. C. 3.2.1.29) (Young, M., 1985). These enzymes are usually in microorganisms and they are present in a wide variety of plants and animals (Cody, R.M., 1991; Hendy, L. 1990; Young, M., 1985; Ulloa, C.J., 1992). In general, chitinase attacks chains of chitin polymer at random, liberating chitin oligosacharides of intermediate size. These oligosacharides can be hiydrolizated until N, N-diacetylchitobiose (chitobiose) with a small quantity of N-acetylglucosamine. Then the chitobiase hydroliyzes chitobiose in two molecules of NAcGlu, although it is capable from hydrolyzes chitotetraoses to falling speeds as the residuals of NAcGlu are increased.
In this sense the solar saltworks are a potential ecological systems source of chitinolytic microorganisms, because in them the degradation of the organic matter are developed as natural processes, among them the chitinous material provided by crustaceans. These solar salt ponds are willing of such a form that the present NaCl in the seawater goes concentrating conforms brines are pump through the whole system of ponds. In this lagoons the elimination of organic matter, as well as the formation of the glasses of NaCl, includes a biological process that involves the action of microorganisms, mainly bacteria and the enzymes produced by them. The process of solar evaporation also generates ecosystems with increasing salinity in which, with different capacity of adaptation to the concentration of salt, are present. For this reasons is attractive the possibility to isolate halophilic bacteria from evaporation solar salt ponds used for the production of sea salt (solar saltworks);
The objective of this work was the isolation and preliminary screening of chitynolytic bacterial adapted to different saline conditions, from solar salworks of the company Industria Salinera de Yucatán S.A., which are located in Las coloradas, municipality of Rio Lagartos in Yucatan, Mexico.

2. MATERIALS AND METHODS

Sampling procedure.

The samples were obtained of stations located in the solar saltworks system, belonging to Industria Salinera of Yucatan, S. A., located in Las Coloradas, in the municipality of Rio Lagartos in the state of Yucatan, Mexico. The samples consisted of silts and water column. Temperature of sampling zones was determined to the moment of the collection. Samples was collected and stored using sterilized glass flasks. After gathering the samples, they were placed in ice and they were transported to the laboratory and conserved in refrigeration until their analysis.

Samples characterization.
In all the samples was determined pH and salinity. This last determination was carried out by means the cuantification of chlorides, titling with AgNO3, and was reported as molar concentration of NaCl.

Preparation of colloidal chitin.
Colloidal chitin used as substrate was produced using chitin obtained of shrimp solid waste that were gathered in a industry located in Lerma, Campeche, Mexico.
The colloidal chitin production was carried out according to method described by Monreal, J., (1969). This method consisted on shaking 10.0 g of chitin in 100 ml from H_3PO_4 to 85.0% until forming a jellied material, it was stored in refrigeration 48 hours, and then was precipited using excess of water and washed with commercial demineralized water until neutrality. Water was eliminated by means of filtration with pressed,

Enrichment culture media.
To enrich the population of chitynolytic microorganisms, present in each sample, we use liquid culture media that consisted in 0.5 yeast extract%, and 1.0% of colloidal chitin; the pH and the salinity (using NaCl) were adjusted to the existent conditions in sampling zones .

Isolation culture media.
Isolation of the chitynolytic bacteria was carried out using Petri dishes with solid culture media according to Carroad, P. (1978). The bottom layer contained 0.5% yeast extract and 2.0 % agar and the top layer included 1.0% of colloidal chitin. Conditions of pH and salinity in culture media was adjusting to the pH and the salinity (using NaCl) to the registered conditions in sampling zones.

Isolation of the microorganisms.
To isolate chitynolytic bacteria we use aliquot of 1.0 ml of each samples and they were used for inoculating 250 ml Erlenmeyer flasks with liquid enrichment culture media. Flasks were incubated at 30 and 40 °C, during 7 days. In all the cases two serial enrichment steps were made. The qualitative determination and isolation of chitinolytic bacteria was carried out based on the observation, in Petri dishes whit isolation culture media, of chitin hydrolysis zones around the microbial colonies.

Preselection system.
Best chitinolytic bacterial strain, was identified by means of activity ratio (Ar), which is defined as the ratio bettwen diameter of the chitin hydrolysis zone and diameter of the bacterial colony.

3. RESULTS
The sampling stations included areas of low, intermediate and high salinity, using this criterion the isolated bacteria were classified in two categories, moderate halofiles and extreme halófilas Table II.
Table III shows the localization of the sampling stations, conditions of temperature and pH of each one, which were used in the isolation process
Table IV and V present the results of the bacterial strains isolated in each sampling station. The temperature of 30 °C is a mean value of the temperatures found in the evaporation lagoons, and the temperature of 40 °C was chosen due to the possibility of finding thermophilic bacteria, given the interest that is manifested at the present time by this type of microorganisms: in this work were isolated 24 moderate halophiles bacteria,

Table IV and 7 extreme halophiles bacteria, Table V, corresponding to the sampling zones I, II, III and IV, those which were able to grow in colloidal chitin. The preselection was carried out based on the Activity ratio (Ar), Table IV and V.

Table II. Isolated bacteria classification in function of salinity in the sampling stations

Sampling station	Salinity M NaCl	Classification
COL I	1.177	Moderate halophile
COL II	1.481	Moderate halophile
COL III	2.622	Extreme halophile
COL IV	2.203	Moderate halophile
COL V	5.625	Extreme halophile
COL VI	5.643	Extreme halophile

Five strains isolated in station COL II have particular interest because they presents Ar 1.754, 2.134, 2.047, 1.90 and 1.788, similar to Ar reported for different strains of Serratia marcescens that fluctuate between 2.0 and 2.8 (Carroad, P., 1978).
In two of the samples obtained in hypersaline environments, COL V and COL VI the enrichment process for obtain chitinolytic bacteria was not achieved. This is very interesting, since few reports exist on the isolation of chitinolytic bacteria, in a solar salt pond with salinity of 250 ‰ ((Liaw, H.J., 1992) During the enrichment in liquid culture media, the growth of the red bacteria halophiles was observed, however these bacterias only use yeast extract but colloidal chitn was intact. Also, the appearance of big granules of salt was observed. This behaviour shows that one compound produced by this bacteria are essential in the NaCl crystallization process in the last stages of the production of salt in the solar salt ponds.

Table III. Characteristics of sampling zones

Sample zone	Localization	Depth and silt type	Temp. °C	pH
COL I	Area of pumping, pond of water reception of the estuary 21° 34' 18'' N 87° 51' 53.9'' W	40 cm Muds	25	7.71
COL II	Estuary adjacent to the area of pumping 21° 34' 18'' N 87° 51' 53.9'' W	40 cm Muds	27	7.59
COL III	Chel evaporation pond with intermediate salinity 21° 36' 17.3'' N 87° 58' 17.1'' W	30 cm Red mud	29	7.84
COL IV	Chel evaporation pond with intermediate salinity 21° 36' 17.3'' N 87° 58' 17.1'' W	65 cm Red mud	29	7.84
COL V	Pond of evaporation of high salinity 21° 35' 55.4'' N 88° 02' 40.7'' W	70 cm Mud	30	7.40
COL VI	Pond of evaporation of high salinity 21° 35' 55.4'' N 88° 02' 40.7'' W	120 Mud	30	7.40

Table IV. Chitinolytic bacterial strains isolated at 30°C in “Las Coloradas” Yucatán, México

Sample	Sampling station	Strain nomenclature	Colony diameter (mm)	Clear zone diameter (mm)	Activity ratio (Ar)
42	COL I	GBRM30C01	5.8	6.4	1.10
43	COL I	GBRM30C02	5.0	NC	ND
44	COL I	GBRM30C03	9.2	9.7	1.05
45	COL I	GBRM30C04	5.2	5.7	1.10
46	COL I	GBRM30C05	7.7	8.4	1.09
47	COL II	GBRM30C06	6.6	7.0	1.06
48	COL II	GBRM30C07	14.9	15.5	1.04
49	COL II	GBRM30C08	5.7	10	1.75
50	COL II	GBRM30C09	4.2	8.6	2.05
51	COL II	GBRM30C10	12.9	17.4	1.35
52	COL II	GBRM30C11	4.7	5.1	1.09
53	COL II	GBRM30C12	6.7	14.3	2.13
54	COL II	GBRM30C13	5.2	9.3	1.79
55	COL II	GBRM30C14	5.0	9.5	1.90
56	COL II	GBRM30C15	3.2	4.0	1.25
57	COL II	GBRM30C16	14.1	15.2	1.08
58	COL III	GBRM30C17	3.9	4.3	1.10
59	COL III	GBRM30C18	6.9	7.2	1.04
60	COL III	GBRM30C19	8.0	8.7	1.09
61	COL III	GBRM30C20	5.9	6.5	1.10
62	COL III	GBRM30C21	4.5	5.2	1.16
63	COL IV	GBRM30C22	4.7	5.3	1.13
64	COL IV	GBRM30C23	6.0	6.6	1.10
65	COL IV	GBRM30C24	7.3	7.9	1.08

Table V. Chitinolytic bacterial strains isolated at 40° C in “Las Coloradas” Yucatán, México

Sample	Sampling station	Strain nomenclature	Colony diameter (mm)	Clear zone diameter (mm)	Activity ratio (ar)
66	COL II	GBRM40C01	3.5	5.0	1.43
67	COL II	GBRM40C02	3.1	4.1	1.32
68	COL II	GBRM40C03	4.5	6.4	1.42
69	COL III	GBRM40C04	3.5	4.5	1.29
70	COL III	GBRM40C05	5.5	6.1	1.11
71	COL IV	GBRM40C06	4.0	4.5	1.13
72	COL IV	GBRM40C07	5.8	6.4	1.10

4. CONCLUSIONS

Results obtained in this study reveal the possibility to isolate chitinolytic bacterial strains whit capacity to produce high Activity ratio, similar or bigger to those that have been obtained with native strains of *Serratia marcescens*. In the different sampling stations was possible to isolate bacteria able to produce chitinolytic activity, besides growing at superior levels of salinity that most of the marine bacteria that grow at salinity of around 35.0 %. This suggests that those habitats are an important potential source of chitinolytic microorganisms. Of the isolated bacterial strains were of particular interest two strains isolated of the sampling zone COL II at 30° C, with capacity to produce activity ratios of 2.0476 and 2.1343 respectivaly

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