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Haloalkalophilic Cellulose-Degrading Bacteria Isolated from an Alkaline Saline Soil

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Microorganisms that degrade cellulose in extreme environments have an enormous biotechnological potential. Seven cellulolytic microorganisms were isolated from soil of the former lake Texcoco (pH 9.6 and electrolytic conductivity 115 dS m^{-1}) in Ar medium (Congo red agar medium) and the enzyme activity was determined semi-quantitatively by measuring the hydrolysis halo around the colony, which varied from 0.8 to 5.0 cm. Of the seven isolated bacteria, two bacteria showed cellulase activity. Phylogenetic analysis based on the 16S rRNA gene, showed that one isolate was closely related to *Cellulomonas bogoriensis* with a similarity of 99.7% and the other to *Nocardiopsis dassonvillei* with 99.69% similarity. The *C. bogoriensis* strain had a maximum cellulolytic activity of 177 mU ml^{-1} and the *N. dassonvillei* strain 136 mU ml^{-1} at pH 9 and 37 °C. Celluloytic activity of the two strains isolated from the saline alkaline soil was not reported previously.

Key words: Actinobacteria, Carboxymethylcellulose (CMC), Cellulases, Extremophiles.

Cellulose is the main component of plants and the most abundant polysaccharide on earth[1]. Its characteristics have been investigated intensively[2]. The microorganisms involved in its breakdown are important in ecological terms, i.e. their importance in the global carbon cycle, and in economic terms, i.e. their role in biofuel production[3].

Cellulose is a polysaccharide composed of anhydroglucose units, which are held together by β-1,4 glucosidic bonds. In addition, the β configuration allows long cellulose chains to be formed linearly, which are not isolated, but bonded together by intramolecular hydrogen bonds forming a crystalline supramolecular structure resistant to hydrolysis[4].

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The enzymatic hydrolysis of cellulose involves the sequential functioning and synergistic action of a group of cellulases, which have different binding sites, due to the complex nature of the cellulose molecule[1]. Cellulases belong to a large family of glycosyl hydrolases (GH) and are common in some bacteria, fungi, plants and animals[6]. These enzymes include cellobiohydrolases (EC 3.2.1.91), endo-1,4-β-glucanases (3.2.1.4), β-glucosidases (EC 3.2.1.21), endo-1,4-β-xylanases (EC 3.2.1.8), β-xylosidases (EC 3.2.1.37), α-L-arabinofuranosidases (EC 3.2.1.55), acetyl xylan esterase (EC 3.1.1.6), β-glucuronidase (EC 3.2.1.131), pectatelyase (EC 4.2.2.2) and endo- β -1,4-D-mannanase (EC 3.2.1.78).

Cellulases are enzymes produced by a variety of bacteria and fungi under aerobic, anaerobic, mesophilic or thermophilic conditions. However, only some of them produce extracellular cellulase enzymes capable of hydrolyzing cellulose

in extreme pH conditions[6]. For instance, β-1,4-endoglucanase is produced by the bacteria *Cellulomonas pachnodae* found in the intestines of *Pachnoda marginata* larvae. These larvae are found in acidic environment with pH ranging from pH 4.8 to 6.0[7] and an alkalophilic *Bacillus* produces an endoglucanase with a pH range of 7.0 to 12.0[8]. An alkaline *Nocardiopsis* is another genus that produces an endo β-1-4 D-glucanase[9]. These enzymes have great economic potential in many industrial processes, i.e. agriculture, food, feed and drinks, detergents, textile, leather, pulp and paper[10].

Microorganisms that produce cellulases in extreme alkaline conditions or extremoenzymes have rarely been studied. Jones et al., [11] for instance found cellulolytic activity in genus *Cellulomonas* with optimal growth between pH 9.0 and 10.0. In a previous study, soil of former lake Texcoco with pH 9.8 and electrolytic conductivity (EC) 32.7 dS m^{-1} showed cellulolytic activity. Emission of $^{14}CO_2$ occurred when the hemicellulose fraction of ^{14}C labelled-maize was applied to the soil[12]. It is unknown, however, which microorganisms were involved in this process. Consequently, the objective of this study was to isolate, identify and characterize microorganisms involved in the degradation of cellulose in this extreme alkaline saline environment.

MATERIALS AND METHODS

Site description

Lake Texcoco is located to the north-east of Mexico city at 2240 m above sea level, with an average annual temperature of 16 ºC and precipitation of 705 mm[13]. A soil sample was taken from the 5 to 20 cm layer (19°26'45'' N, 97°56'16'' W). The alkaline saline soil had pH 9.5 and electrolytic conductivity (EC) 115 dS m^{-1}. Sampled soil was taken to the laboratory on ice immediately.

Isolated strain

A soil extract used was prepared by adding 10 g soil to 25 ml distilled water and the solution was centrifuged at 6,000 rpm for 10 min. The microorganisms that degrade cellulose were isolated on Petri dishes with Congo red agar medium[14] that contained (l^{-1}): soil extract, 250 ml; $MgSO_4 \cdot 7H_20$, 0.25 g; K_2HPO_4, 0.5 g; Congo red, 0.18 g; gelatine, 1.8 g; carboxymethylcellulose (CMC), 2.0 g; agar 20 g. An aliquot of 100 µl soil suspension was diluted (10^{-1} to 10^{-6}) and incubated on the culture medium mentioned above at 30 ºC for 24 to 48 h in triplicate.

DNA extraction, PCR amplification and phylogenetic analysis

The extraction of genomic DNA for molecular characterization of each of the isolates was done using the QIAcube automatic system with the DNeasy Blood & Tissue Kit (Qiagen, Valencia, USA). Its integrity was assessed by visualization on agarose gel electrophoresis in 0.8%. Different aliquots of genomic DNA were stored at ⁻20 °C until processing. Amplification of DNA from each of the strains was done using the amplification protocol described by Rusznyák *et al.*,[15]. The PCR reaction used a 25 µl mixture with (µl): Buffer, 2.5, 25 mM; $MgCl_2$, 1.5; Oligonucleotides, 1.25, 60 mM; dNTPs, 0.5; genomic DNA, 1; Taq Polymerase, 0.125; BSA (bovine serum albumin), 7.5; H_2O, 9.125; Dimethylsulfoxide, 1.5. The primers used were 27F (5'-AGA GTT TGA TCM TGG CTC AG-3 ') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3'). The samples were placed in a thermocycler under the following conditions: initial heating at 94 °C for 4 min (initial denaturation), 35 cycles of heating at 94 °C for 1 min to denature, 1 min at 57 °C for alignment and 2 min at 72 °C for polymerization, and a final polymerization step at end of 72 °C for 10 min. Finally, the reaction was kept at 4 °C until the amplification reaction. The size of the amplicons was approximately 1465 bp.

The PCR product was purified and sequenced by MACROGEN (http://dna.macrogen.com/eng/). The sequences obtained were compared with reference 16S rRNA gene sequences retrieved from GenBank/EMBL by means of a BLAST search[16]. Multiple alignments with sequences of the most closely related bacteria and calculations of sequence similarity were done using CLUSTAL X[17]. Maximum likelihood phylogenetic analysis was performed using the online program PhyML 3.0 (http://www.atgc-montpellier.fr/phyml/) [18]; with the data sets using the general time reversible model substitution model[19].

Enzyme assays

Strains were inoculated an Ar culture medium. The optical density of the medium was adjusted to 0.05 units in a Spectrophotometer 3000 SmartSpecTM flow, Catalogue Number 170–2501

at 600 nm. Cultures were incubated at 30 °C and 120 rpm for 60 days. Cellulose activity of the above mentioned isolates was confirmed by measuring the amount of reducing sugars in Ar culture medium (22). One ml aliquot was taken approximately every 5 days for 2 months, centrifuged at 13,000 rpm for 3 min. A 500 µl aliquot of the supernatant was taken and added to 500 µl glycine buffer with 1% low viscosity CMC. The samples were incubated at 25 °C, 37 °C, 50 °C, 70 °C and 90 °C at pH 9, and at pH 7, 8, 9, 10 and 11 at 50 °C for 5 min. The temperatures were varied around the optimum, which was 50 °C, and around the optimum pH of 9.0[20]. The mixture was boiled for 5 min and then cooled on ice for 5 min. The sample was read in a spectrophotometer at 540 nm to measure the reduced sugars. The amount of reducing sugars was calculated using a standard glucose curve[21]. A milliunit is considered the amount of enzyme needed to obtain 2.5 micromoles of reducing sugar per min and divided by thousand.

RESULTS AND DISCUSSION

Fifty isolates were obtained with the Congo red agar culture medium with CMC. Only seven strains (1r, 3r, 5r, 6r, 7r, 8r, and 16r) showed hydrolytic activity on Congo red agar medium with CMC as C source (Table 1). These strains showed a hydrolysis area ranging from 0.8 to 5.0 cm and an HC value (area compensation colony diameter) from 1.6 to 10.0; similar as those reported by Lu *et al.*,[22]. These results demonstrated that all strains have hydrolytic capacity in this medium and a possible production of extracellular enzymes or cellulases within 10 days.

The seven microorganisms that showed hydrolytic activity on Congo red agar media were isolated and characterized based on their 16S rRNA gene sequence. All seven isolates belonged to the phylum Actinobacteria (Table 1). *Arthrobacter globiformis* (strain 5r) is a well studied marine bacteria with resistance to low temperatures[23], although it is not clear if it can degrade cellulose. *Nesterenkonia halotolerans* (strain 16r) was first isolated from a hypersaline soil, but little is known about its metabolic pathways[24]. Different strains belonging to the genus *Streptomyces* are known to contain cellulases[25], but it is not clear if *S. aurantiacus* (strains 3r and 6r) shows cellulolytic activity. *Cellulomonas cellasea* (strain 7r) is known to possess cellulolytic activity[26]. Little information exists about *C. bogoriensis* (strain 1r)[11, 27]. It was described by Jones *et al.*,[11] as *'an alkaliphilic, slightly halotolerant, chemo-organotrophic, Gram-positive, rod-shaped bacterium'* and was isolated from the sediment of the littoral zone of Lake Bogoria, Kenya'. They stated that its optimal growth occurred between pH 9.0 and 10.0. *Nocardiopsis dassonvillei* (strain 8r) belongs to the genus *Nocardiopsis*, a widespread group, which is versatile and pathogenic that produces a wide range of bioactive metabolites[28, 29]. It is known to show cellulolytic activity[30].

Cellulose alone can not be degraded completely by physical methods and microbial enzymes are required for its complete mineralization; such as those produced by cellulolytic microorganisms isolated in this study. While there are many reports on the isolation of cellulose degrading microorganisms from different ecosystems[31], few studies have isolated or identified cellulolytic bacteria from saline-alkaline soils. Grant *et al.*,[32] reported on a wide range of halophilic bacteria isolated from saline environments, such as seas with dissolved salts (> 30%). They found phylotypes belonging to the genus *Marinococcus* and *Sporosarcina*, and strains of *Bacillus salinicoccus*[33, 34, 35]. Ramirez *et al.*,[36] reported on halo-alkaliphilic bacteria in different geographical regions of Mexico belonging to the genera *Bacillus* and *Halomonas*.

In previous studies of Valenzuela-Encinas *et al.*,[37] and Soto-Padilla *et al.*,[38], phylotypes were identified in the alkaline saline Texcoco soil belonging to the genera *Kocuria, Micrococcus, Salinicoccus, Kurthia, Gracilibacillus, Bacillus, Halomonas, Arthrobacter, Nesterenkonia, Cellulomonas, Streptomyces* and *Nocardiopsis*; all belonging to the order Actinomycetales or Firmicutes. Some strains belonging to these genera are known to show cellulolytic activity. In the study reported here, some of the above-mentioned genera, i.e. *Arthrobacter, Nesterenkonia, Cellulomonas, Streptomyces* and *Nocardiopsis*, were isolated.

Of the seven strains that showed hydrolytic activity on Congo red, only two showed

cellulolytic enzyme activity. Their cellulolytic activity was further investigated using the DNS method.

Strain 1r had a 99.7% similarity with *Cellulomonas bogoriensis* and strain 8r 99.69% with *Nocardiopsis dassonvillei* (Figure 1). These two strains were tested at different pH (7 to 11) and temperatures (from 25 to 90 °C) (Figures 2, 3, 4, 5). Strain 1r (*Cellulomonas bogoriensis*) had a maximum activity of 177 mU ml^{-1} at pH 9 and 37 °C and a minimum of activity 19.6 mU ml^{-1} at pH 8 and 50 °C. Strain 8r (*Nocardiopsis dassonvillei*) showed a maximum activity of 136 mU ml^{-1} at pH 9 and 37 °C and a minimum of activity 14.6 mU ml^{-1} at pH 7 and 50 °C.

The use of a second quantitative method, i.e. the DNS technique, allowed to confirm the presence of microorganisms with cellulolytic

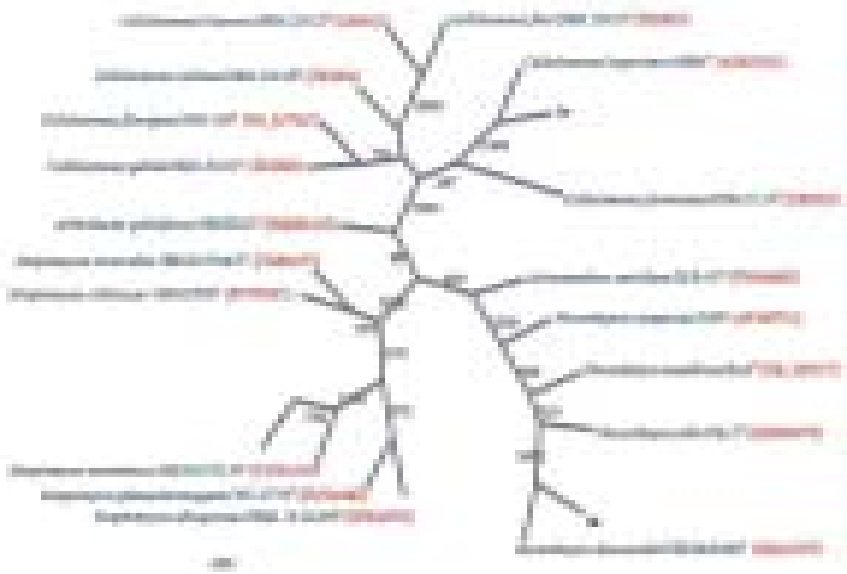

Fig. 1. Phylogenetic analysis based on 16S rRNA gene sequences of strains with cellulolytic activity (access numbers are in parenthesis). Percentages in the branching points with only values with > 50% shown

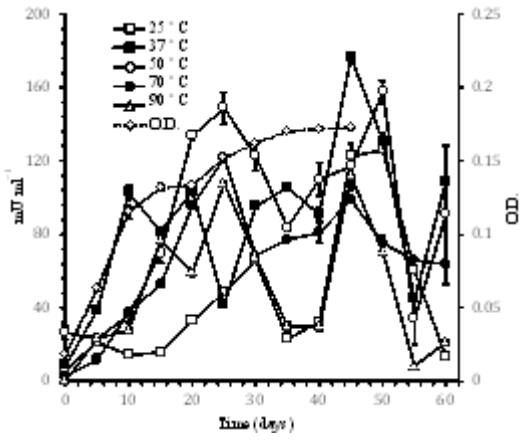

Fig. 2. Cellulolytic activity (mU ml^{-1}) and microbial growth (O.D.) of strain 1r *Cellulomonas bogoriensis* at five different temperatures and pH 9.0 incubated for 60 days

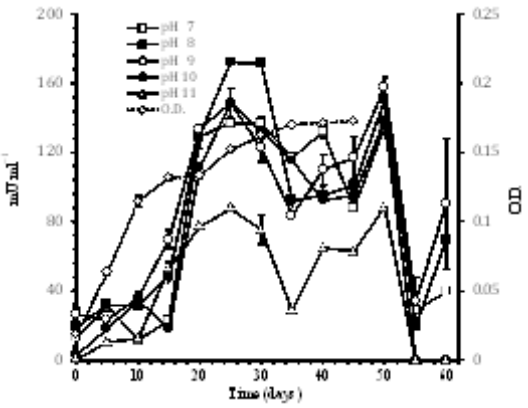

Fig. 3. Cellulolytic activity (mU ml^{-1}) and microbial growth (O.D.) of strain 1r *Cellulomonas bogoriensis* at different pH and 50 °C incubated for 60 days

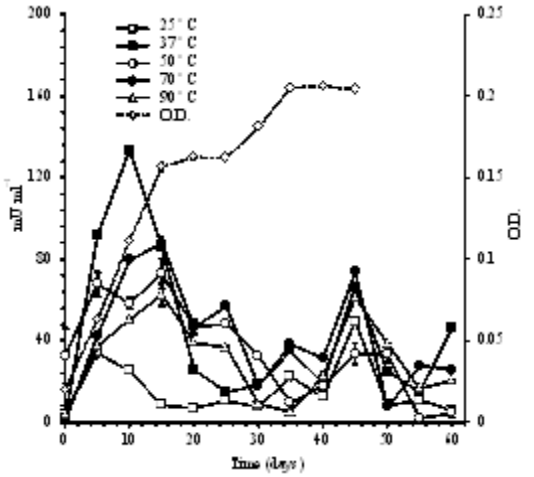

Fig. 4. Cellulolytic activity (mU ml^{-1}) and microbial growth (O.D.) of strain 8r *Nocardiopsis dassonvillei* at five different temperatures and pH 9.0 incubated for 60 days

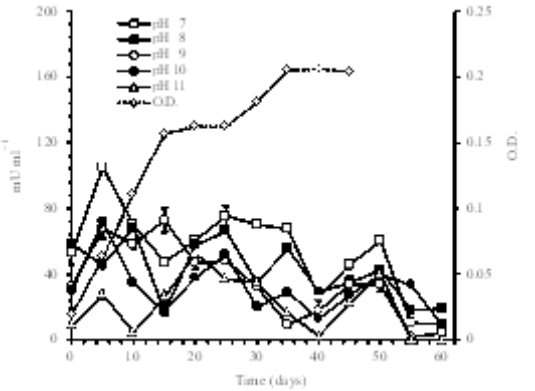

Fig. 5. Cellulolytic activity (mU ml^{-1}) and microbial growth (O.D.) of strain 8r *Nocardiopsis dassonvillei* at different pH and 50 °C incubated for 60 days

Table 1. Identification of the isolated cellulolytic strains based on the gene sequence encoding for 16S rRNA and hydrolysis halos around the colony isolates of 7 estimated by the ratio of size of maximum clearing zone and hydrolytic capacity (HC) value for 10 days.

Strain	Micro-organism	% Similitude	Maximum clearing zone size (cm)	Maximum HC value
1r	*Cellulomonas bogoriensis*	99.70	3.1	6.2
3r	*Streptomyces aurantiacus*	99.41	3.7	7.4
5r	*Arthrobacter globiformis*	98.75	1.3	2.6
6r	*Streptomyces aurantiacus*	99.31	2.8	5.6
7r	*Cellulomonas cellasea*	99.10	1.3	2.6
8r	*Nocardiopsis dassonvillei*	99.69	5.0	10.0
16r	*Nesterenkonia halotolerans*	98.68	0.8	1.6

activity producing extracellular enzymes[39, 40]. Of the seven strains that showed hydrolytic activity on Congo red, only two showed cellulolytic enzyme activity, i.e. strains 1r and 8r. The maximum cellulolytic activity of 177 mU ml^{-1} for *C. bogoriensis* and 136 mU ml^{-1} for *N. dassonvillei* was higher than values reported by Amore *et al.*,[41] ranging from 30 to 110 mU ml^{-1} for cellulolytic microorganisms in an industrial waste based compost, but lower than values reported for cellulolytic microorganisms in a lower stalks-vegetable waste co-composting system[22]. The strains 1r and 8r grew also on xylan and lignin and showed xylanase and ligninase activity (Data not shown). This study suggests that saline alkaline soil may be a source of new strains of cellulolytic bacteria, and these two species might play an important role in cellulose degradation in extreme saline environments.

CONCLUSION

Seven strains were isolated from the saline-alkaline soil that showed hydrolytic activity on Congo red agar. These strains belonged to the genera *Cellulomonas, Nocardiopsis, Streptomyces, Arthrobacter* and *Nesterenkonia*, all of them Actinomycetes. Two of those seven strains, one with 99.7% similarity with *Cellulomonas bogoriensis* and one with 99.69% similarity with *Nocardiopsis dassonvillei* showed cellulolytic activity as evidenced by the production

of reducing sugars. The *C. bogoriensis* strain had a maximum cellulolytic activity of 177 mU ml^{-1} at pH 9 and 37 °C, and the *N. dassonvillei* strain 136 mU ml^{-1}. Suggest that saline alkaline soil may be a source of novel strains and extremoenzymes of cellulolytic bacteria.

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