

## Bacterial Isolates from Ethiopian Soda Lake Producers of Alkaline-Active $\beta$ -Glucanases Resistant to Chelating and Surfactant Compounds

<sup>1</sup>M. Minig, <sup>2</sup>D. Walker, <sup>2</sup>P. Ledesma,

<sup>2</sup>M. Alejandra Martínez and <sup>1</sup>Javier D. Breccia

<sup>1</sup>Depto. de Química-CONICET, FCEyN, Universidad Nacional de La Pampa,  
Av. Uruguay 151, (6300) Santa Rosa, La Pampa, Argentina

<sup>2</sup>PROIMI-CONICET (Planta Piloto de Procesos Industriales Microbiológicos),  
Av. Belgrano y Caseros, 4000 Tucumán, Argentina

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**Abstract:**  $\beta$ -glucanase activities were screened from isolated bacteria of the Ethiopian Shala Lake. Five isolates were selected according to the highest production of alkaline-active  $\beta$ -glucanase. By sequence analysis of 16S rDNA and physiological tests, four strains (SES01, SES22, SES4 and SES05) were related to *Bacillus halodurans* specie and the strain SES33 was identified up to genus level as *Bacillus* sp. Intergenic spacer regions fingerprinting showed different patterns among selected strains, having DNA amplicons of high molecular weight characteristic of alkaliphilic *Bacillus*. Herein, *B. halodurans* SES01 produced a highly stable  $\beta$ -glucanase in presence of surfactant and chelating compounds (sodium lauryl sulphate, Triton X-100 and EDTA) indicating its potential as additive for laundry technologies.

**Key words:** *Bacillus*,  $\beta$ -glucanase, *Bacillus halodurans*, surfactants, EDTA

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## INTRODUCTION

Cellulose is the main structural component of plant cell walls and the most abundant carbohydrate polymer in nature. In plants, cellulose occurs mostly as a lignocellulose complex in association with lignin and hemicellulose. The distinctive structural characteristics of lignocellulosic materials make them resistant to enzymatic as well as chemical treatments, reason that encourages the search for new and more efficient lignocellulose degrading enzymes (Zhang *et al.*, 2006; Rees *et al.*, 2003; Walker *et al.*, 2006).

The main industrial applications for cellulase ( $\beta$ -glucanase) preparations consist in cloth washing and treatment of fabrics to reach a soft appearance (Horikoshi, 2004; Pyc *et al.*, 2003). Moreover, new applications are emerging like improving the extraction of vegetal compounds located in the cells and even more ambitious, generating sustainable energy through fermenting raw timber to ethanol (Navarrete-Bolanos *et al.*, 2004).

Since, glucanases for laundry use must have various properties, such as a neutral or alkaline optimum pH, resistance to anionic surfactants and oxidizing agents (main components in detergents) and high defibrillation activity, all-purpose glucanases have not been obtained yet (Koga *et al.*, 2008). The studies of extremophile microorganisms and their abilities to cleave cellulose in such harsh conditions are still attracting from the technical as well as the biochemical point of view. This study deals with the isolation, molecular and phenotypic identification and cultivation of alkaliphilic bacteria producers of  $\beta$ -1,4-glucanase activity with high stability in presence of chelating and surfactant compounds.

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**Corresponding Author:** Javier D. Breccia, Depto. de Química-CONICET, FCEyN,  
Universidad Nacional de La Pampa (UNLPam), Av. Uruguay 151, (6300) Santa Rosa,  
La Pampa, Argentina Tel: +54 2954 436787/26 Fax: +54 2954 432535

## MATERIALS AND METHODS

### Microbial Sources

Sediment and water samples from Shala Lake, Ethiopia (located 7°25' N; 38°24' E) were kindly gifted by Dr. Amare Gessesse. One gram sample was suspended in 5 mL of 30 g L<sup>-1</sup> NaCl sterile solution and used as microbial source. Reference strain *B. halodurans* DSM 497<sup>T</sup> was purchased from DSMZ GmbH (Germany) and *B. subtilis* 1A1 obtained from *Bacillus* Genetic Stock Center (BGSC). This study was conducted during 2006-07.

### Culture Media and Fermentation Conditions

Enrichment medium contained (g L<sup>-1</sup>): carboxymethyl cellulose medium viscosity 10; yeast extract 1; casein peptone 1; NaCl 30 (or indicated in the text), pH 10. Selection medium was supplemented with 15 g L<sup>-1</sup> agar. The media pH was adjusted to 10 supplementing them with 100 mM Na<sub>2</sub>CO<sub>3</sub>. Fermentation medium for *B. halodurans* SES01 (g L<sup>-1</sup>): milk peptone 10, yeast extract 5; glucose 10 and NaCl 35. A 2 L fermentor (Discovery 210, New Brunswick, USA) was employed for culturing at different pH values, adjusted between 6.0 and 10.5 using 2 M NaOH (sodium carbonate was replaced in order to decrease the dilution effect). It was operated at 30°C with an agitation speed of 400 rpm and aeration of 1 vvm. The working volume was 1.0 L. Reference strain *B. halodurans* DSM 497<sup>T</sup> was grown according to DSMZ GmbH instructions (<http://www.dsmz.de>).

### Isolation of Microorganisms

One milliliter of each sample suspension was added to 5 mL of enrichment medium and incubated at 30°C. After 2 days, serial dilutions in 30 g L<sup>-1</sup> NaCl solution were streaked out on selection medium and the plates were incubated at 30°C during 16-24 h. Carboxymethyl cellulose hydrolysis zone on the Petri dishes was detected as described by Breccia *et al.* (1995). The strains were cultured for 48 h in an orbital shaker (250 rpm, 25°C), in 20 mL selection medium, centrifuged for 10 min at 12,000 g. The supernatants were frozen at -20°C until processing.

### Stability Assays

The supernatants of 48 h old cultures containing 0.2 mM PMSF, were incubated with 1 and 10 mM EDTA and 1 and 10% (w/v) SDS and Triton X-100 during 3 h at 37°C. Afterwards, samples were dialyzed overnight against 5 mM sodium phosphate buffer pH 8 at 5°C, before measuring enzymatic activity.

### Carboxymethyl cellulase Activity

For screening, semi-quantification of activity was performed measuring ratio between: diameter of the hydrolysis zone/diameter of the bacterial colony, in solid culture medium. Activity quantification, briefly: samples (400 µL) were incubated with 400 µL of 10 g L<sup>-1</sup> carboxymethyl cellulose (CMC) low viscosity in 50 mM sodium phosphate buffer pH 8, at 40°C for 1 h. The reaction was stopped by adding 400 µL of 3,5-dinitro salicylic acid, placed in a boiling water bath for 10 min and subsequently cooled before measuring absorbance at 540 nm (Miller, 1959). One unit of carboxymethyl cellulase activity was defined as the amount of enzyme required to release 1 µmol of reducing sugars (as glucose) per hour.

### Analytical Assays

Protein concentration was determined by the method of Bradford, using bovine serum albumin as standard. Concentration of organic acids were determined by HPLC using a Rezex Organic Acid column (300×7.8 mm) fitted with a pre-column and a Gilson R32 refractive index detector (Gilson,

Villers le Bel, France). Sulphuric acid (25 mM) was used as mobile phase, with a flow rate of 0.6 mL min<sup>-1</sup> at 55°C. DNA extraction, sequence and PCR assays were carried out as previously described by Martinez *et al.* (2002).

## RESULTS

β-glucanase producing bacteria were isolated using sediment and water samples from Shala Lake (Ethiopia). Approximately, 200 isolates were assayed for carboxymethyl cellulose (CMC) hydrolysis at pH 10. For most isolates, in solid medium, the colonies had to be removed to see the subtle CMC hydrolysis directly beneath the cell-biomass. Few strains produced prominent hydrolysis and among them 5 isolates were selected with ratios ranging from 1.3-2.5.

Selected alkaliphilic strains were gram positive; rod shaped and developed cream white colored colonies with a slightly filamentous margin, except for isolate SES33 that presented a creamy brown colony with smooth margin. By sequence analysis of 16S rDNA four strains (SES01, EU808046; SES22, EU808047; SES4, AY144582 and SES05, FJ147199) were related to *Bacillus halodurans* specie and strain SES33 (EU808048) was identified up to genus level as *Bacillus* sp. The strain *B. halodurans* SES4 carried out a cryptic plasmid with unknown function (data not shown). PCR fingerprint of polymorphic intergenic rDNA spacers was performed, showing that four *B. halodurans* strains were different variants of the specie and high molecular weight DNA amplicons were observed (Fig. 1).

Biochemical tests were carried out for phenotypic characterization. Nitrate reduction was positive for most strains except for *B. halodurans* SES01 and the reference strain *B. halodurans* DSM 497<sup>T</sup> (Table 1). All of them were positive for the hydrolysis of Tween 20, 40 and 60, although *Bacillus* sp. SES33 developed the faster and more prominent tween(s) hydrolysis. Particularly, it was unable to grow with inositol as sole carbon source. The strains were citrate positive and negative for phenylalanine and Vogor Proskauer tests. Regarding growth temperature dependence, *Bacillus* sp.

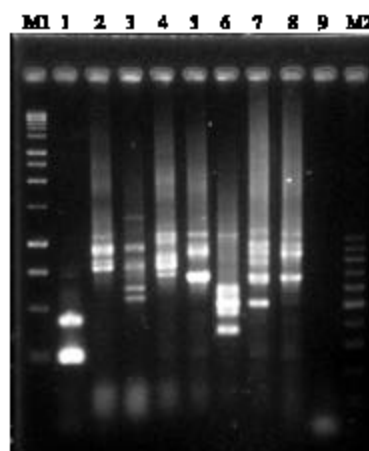


Fig. 1: ISR-PCR from *Bacillus* sp. strains. Lane , M1: 1 kb DNA ladder, 1: *B. subtilis* 1A1, 2 and 3: *B. halodurans* DSM497<sup>T</sup> and MIR32, respectively. Lanes 4 -5 and 7-8: *B. halodurans*, strains: SES01, SES22, SES4, SES05; Lane 6: *Bacillus* sp. SES33; 9: Control reaction without DNA template, Lane M2: 100 bp DNA ladder

Table 1: Physiological and biochemical characteristics of alkaliphilic strains

Assay	<i>B. halodurans</i>					<i>Bacillus</i> sp.
	SES01	SES22	SES4	SES05	DSM 497 <sup>T</sup>	SES33
Growth on inositol	+	+	+	+	+	-
NaCl (0 M)	+++	+	++++	++++	++	+++
NaCl (0.14 M)	++++	+++	+++	+++	++	++
NaCl (0.85 M)	+++	++	+++	+++	+	-
NaCl (1.3 M)	++	++	++	+++	+	-
NaCl (1.7 M)	++	+	+	++	+	-
NaCl (2.1 M)	++	+	-	-	-	-
NaCl (3 M)	-	-	-	-	-	-
Opt. Temp. (°C)	45	45	37	45	45	30
Growth at 50°C	+	+	+	+	-	-
Growth at 55°C	+	+	-	-	-	-
Growth at 60°C	-	-	-	-	-	-

+: Growth; -: Not growth. Growing on NaCl; -: Not growth, Final optical density at 560 nm: + 0.1-0.2, ++ 0.3-0.5, +++ 0.5-0.7, ++++: >0.7

SES33 exhibited an optimum at 30°C and it did not show detectable growth at 50°C. On the other hand, *B. halodurans* supported higher temperatures, being the strains SES01 and SES22 capable to grow up to 55°C at pH 10 (Table 1).

These strains were able to grow at pH 7 only with a previous adaptation and characteristic stressed cells as grouping and pellets formation were observed. Regarding ionic strength of the media, *B. halodurans* strains were shown to grow at sodium chloride concentration up to 1.7-2.1 M, while *Bacillus* sp. SES33 was unable to grow in presence of 0.85 M NaCl or higher (Table 1). When the strains were cultured in a medium with xylene as carbon source, all of them produced high levels of  $\beta$ -1,4-xylanase, comparable to values previously reported (data not shown) (Breccia *et al.*, 1998). Production of  $\beta$ -glucanase, measured as the hydrolysis of CMC, was rather low in comparison with xylanase activity. Similar levels of cellulase and xylanase production are rare in bacteria, although they were reported for the unrelated genus *Fibrobacter* (Bera-Maillet *et al.*, 2004).

In order to assess  $\beta$ -glucanase stability, residual activities after exposing extracellular  $\beta$ -glucanases to surfactant (SDS, Triton X-100) and chelating (EDTA) compounds were measured (Fig. 2a-c). Even when EDTA diminished the activity for all the strains, *B. halodurans* SES01 and SES4  $\beta$ -glucanases were found to have remarkable high residual activity: 60 and 80% in presence of 1 mM EDTA, respectively. The  $\beta$ -glucanase from *B. halodurans* SES01 was not significantly affected by 1% (w/v) Triton X-100, while around 60% residual activity was observed for all the strains when its concentration was augmented to 10% w/v. On the other hand, SES01  $\beta$ -glucanase in presence of sodium lauryl sulphate, one of the most used tensioactive compounds in laundry powder preparations, showed the highest stability displaying 70 and 62% with 1 and 10% (w/v) SDS, respectively (Fig. 2).

Considering the promising features of  $\beta$ -glucanase activity from *B. halodurans* SES01, it was selected for further studies. Cultural characteristics were determined in a bench scale fermentor. The optimum growth was found at pH 8.0 ( $\mu = 0.31 \text{ h}^{-1}$ ) and 45°C. The efficiency of glucose consumption was high, ranging between 0.90-0.96, upto pH 10, but at pH 10.3 it significantly dropped to 0.76 (Fig. 3). The main organic acids released to the extracellular medium were acetic (30±6 mM) and lactic acids (60±10 mM). Their concentrations did not vary when growing at different pH values. In contrast, succinic acid was detected in lower concentrations, being practically absent when *B. halodurans* SES01 was grown at pH 8.0, while at higher pH values (9-10.4) it reached a concentration of 2.0±0.6 mM after 48 h cultivation (Fig. 3). To keep pH constant during cultivation, a high addition rate of NaOH (>2 mL h<sup>-1</sup> of 2 M NaOH) was observed. The data exposed above suggested that acidification promoted by *B. halodurans* SES01 was mainly caused by carbonic acid and ammonia, more than the organic acids produced by the bacterial metabolism.

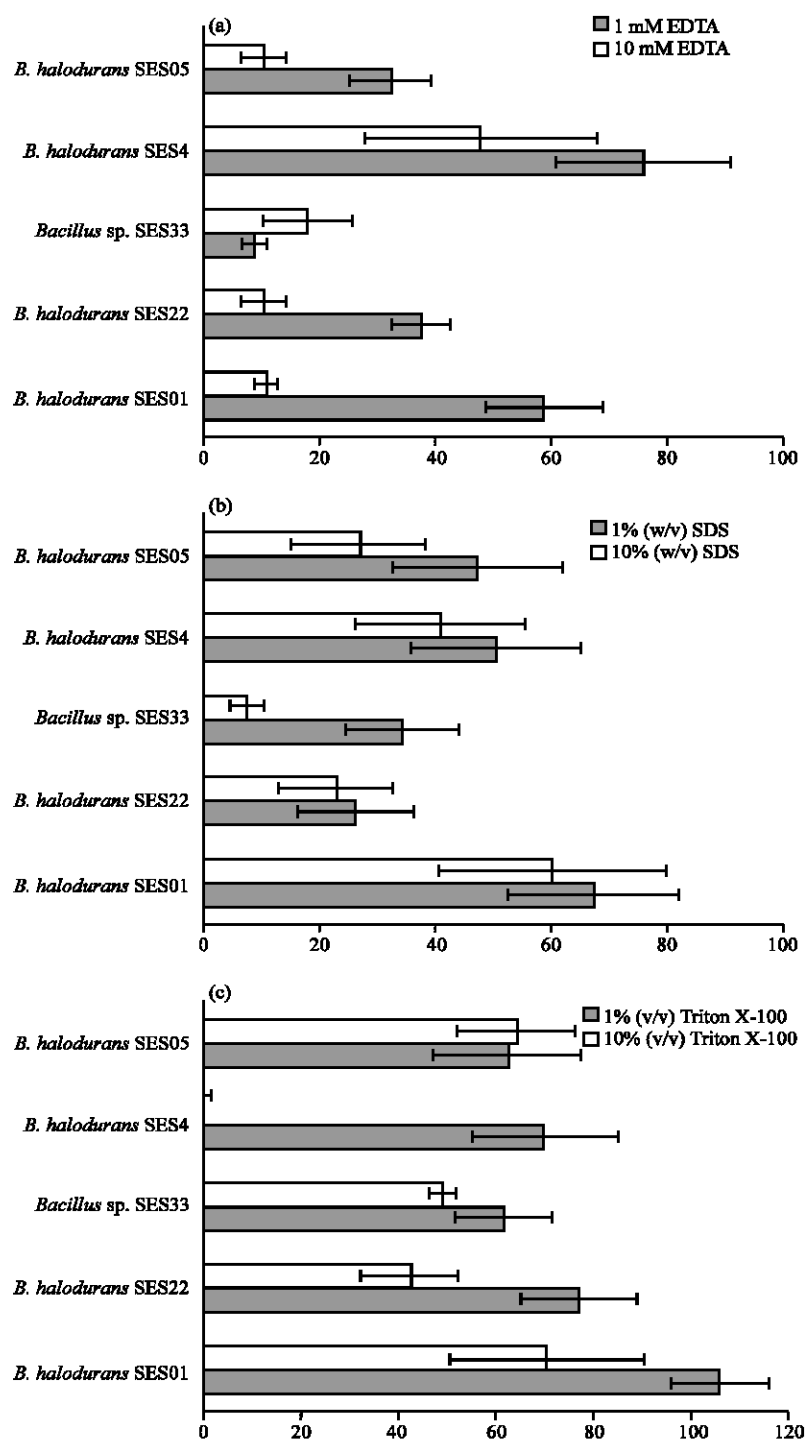


Fig. 2: Stability of the  $\beta$ -glucanase after incubation at 37°C for 3 h with: (a) EDTA, (b) Sodium lauryl sulphate and (c) Triton X-100

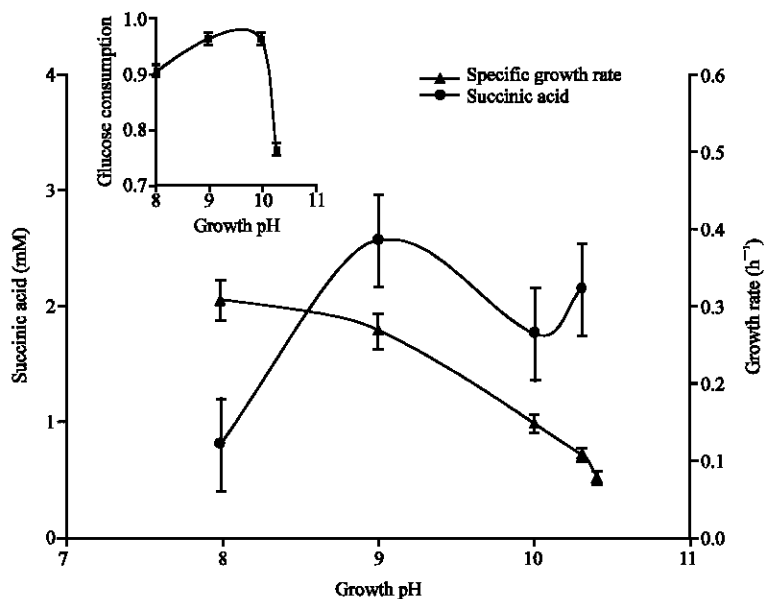


Fig. 3: Batch cultures of *B. halodurans* SES01 in bench scale fermentor at different pH values. Specific growth rate (h<sup>-1</sup>) and succinic acid in cell-free supernatant after 48 h cultivation. The insert corresponds to glucose consumption at different pH values

## DISCUSSION

Cellulolytic enzymes from alkaliphilic organisms attract scientific attention through the speculation of catalytic independence from divalent metals ions (Hagihara *et al.*, 2002). Alkaline environments, such as the lakes of central Africa, offer interesting systems for ecological and biochemical studies (Kebede *et al.*, 1994). With this perspective, several microbial strains were isolated from the Shala Lake (Ethiopia) to evaluate their capacity of  $\beta$ -glucanase production. Among the five strains presenting the larger CMC-hydrolysis at pH 10, four of them were related to *B. halodurans*, suggesting the relevancy of the specie for the turnover of  $\beta$ -polymers in this alkaline environment.

Polymorphic intergenic rDNA spacers were demonstrated to be an important supplement to 16S rDNA sequencing for differentiating related bacterial species (Orrillo *et al.*, 2007). Concerning alkaliphilic *Bacillus* strains, eight *rrm* loci have been sequenced and analyzed on the chromosome of the alkaliphilic strain *B. halodurans* C-125, where six of them were also observed in *B. subtilis* and the other were specific for the alkaliphilic strain (Nakasone *et al.*, 2000). The IRS patterns of the strains isolated in the present study showed differences among them, with the common attribute of having DNA amplicons of high molecular weight characteristic of alkaliphilic *Bacillus* (Martínez and Sifleriz, 2004).

A genetically modified amylase which lacks calcium in its chemical structure, from *Bacillus* sp. KSM-K38, was resistant to chelators and oxidative reagents, although it was more sensitive to thermodynamic denaturation than the parental protein (Hagihara *et al.*, 2002). More recently, an endoglucanase CMC hydrolyzing enzyme, having an optimum pH of 4.5, was reported as a resistant enzyme to anionic surfactant and oxidizing agents (Baba *et al.*, 2005). Extreme environments are still an interesting source of biocatalysts naturally exposed to harsh conditions. *Bacillus halodurans* SES01 is a wild type strain which carried an interesting enzymatic system for cellulose depolymerization at high pH values. It also showed considerable stability in presence of compounds used for laundry commercial preparations.

## CONCLUSION

The description of several alkaliphilic bacteria belonging to *Bacillus* genus that produced CMC-hydrolysis at high pH values was performed. The most common isolated specie for CMC-depolymerization in Shala Lake samples was *B. halodurans*. Amongst them, *B. halodurans* SES01 was able to grow in extreme conditions (pH 10.4 and 55°C) and its  $\beta$ -1,4-glucanase system showed the highest stability in presence of chelating and surfactant compounds. Such features showed the potential of this enzymatic system as a laundry additive.

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