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ORIGINAL PAPER

Carboxyl ester hydrolases production and growth of a halophilic archaeon, *Halobacterium* sp. NRC-1

Rosa María Camacho · Juan Carlos Mateos-Díaz · Dulce María Diaz-Montaño · Orfil González-Reynoso · Jesús Córdova

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Abstract The capability of *Halobacterium* sp. NRC-1 to synthesize carboxyl ester hydrolases was investigated, and the effect of physicochemical conditions on the growth rate and production of esterases was evaluated. The haloarchaeon synthesized a carboxyl ester hydrolase, confirming the genomic prediction. This enzymatic activity was intracellularly produced as a growth-associated metabolite. Esterase activity was assayed using different *p*-nitrophenyl-esters and triacyl-glycerides, which showed a preference for hydrolyzing tributyrin. The archaeal growth rate and esterase production were significantly influenced by the pH and the NaCl concentration. An interaction effect between temperature and NaCl was also seen. The maximal growth rate and esterase production found for *Halobacterium* sp. NRC-1 were 0.136 h^{-1} (at 4.2 M NaCl, pH 6 and 44°C) and 1.64 U/l (at 4.6 M NaCl, pH 6 and 30°C), respectively. Furthermore, the effects of NaCl concentration, pH and temperature on enzyme activity were studied. Two maximal esterase activities were elucidated from the intracellular crude extract when it was incubated at different NaCl concentrations (1 M and 5 M) and at different pHs (6 and 7.5). This is the first report that shows experimentally the synthesis of carboxyl ester hydrolases by *Halobacterium* sp. NRC-1. This enzyme was found to be extremely halophilic (5 M NaCl) and thermophilic (80°C), making it very interesting for future investigations in non-aqueous biocatalysis.

Keywords Halophilic archaea · *Halobacterium* sp. NRC-1 · Carboxyl ester hydrolase

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O. González-Reynoso · J. Córdova (✉)
Departamento de Ing. Química, Universidad de Guadalajara,
Blvd. Marcelino García Barragán 1421,
C.P. 44430 Guadalajara, Jalisco, Mexico
e-mail: jesuscordovaudg@yahoo.com.mx

R. M. Camacho · J. C. Mateos-Díaz · D. M. Diaz-Montaño
Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco A.C., Av. Normalistas 800,
Guadalajara, Jalisco, Mexico
e-mail: camachros@gmail.com

Introduction

Halophilic archaea are found in natural hypersaline environments such as the Dead Sea, the Great Salt Lake, salterns and soda lakes, where concentrations of NaCl can reach saturation levels of ~6 M (Danson and Hough 1997; Sellek and Chaudhuri 1999). To proliferate in these hypersaline habitats, their cytoplasm is osmoregulated by K^+ accumulation (Oren 2002). Therefore, the intracellular and extracellular proteins have to struggle with very high salt concentrations of KCl ~4 M and NaCl ~5 M (Van den Burg 2003). Enzymes from halophilic organisms are stable and active at low water activity (as low as 0.75), making them robust biocatalysts with potential applications in synthesis using non-aqueous media (Eichler 2001; Marhuenda-Egea and Bonete 2002; Sellek and Chaudhuri 1999).

Carboxyl ester hydrolases, like lipases and esterases, constitute an important group of biocatalysts with biotechnological applications. These enzymes are essentially distinguished on the basis of their substrate specificity. Lipases preferentially hydrolyze triglycerides made up of long chain fatty acids, and esterases hydrolyze only water soluble esters, e.g., short-chain fatty acid triglycerides (Verger 1997). Both enzymes have diverse applications, including: the modification of biologically active molecules, the enhancement of flavor or nutraceutical properties

in foods and the resolution of racemic mixtures (Bornscheuer and Kaslauskas 1999; Jaeger and Eggert 2002; Schmid and Verger 1998; Sharma et al. 2001). These enzymes are also active in the presence of some organic solvents, carrying out esterification, amidation and polymerization reactions (Bornscheuer and Kaslauskas 1999; Schmid and Verger 1998; Sharma et al. 2001). Novel lipases and esterases have been isolated from thermophilic and psychrophilic extremophiles, and some of them have been overexpressed in *E. coli* (Demirjian et al. 2001; Jaeger and Eggert 2002). Nevertheless, to date, reports showing evidence of the synthesis of these enzymes by halophilic archaea are scarce. Recently, a screening of lipase activity was carried out from halophilic archaea isolated from an aquatic hyperhalobe environment, the Sebkha of El Golea (Algerian Sahara) (Bhatnagar et al. 2005). In this work, the authors describe the discovery of the first true lipase detected in the *Archaea* domain and make a preliminary characterization of the enzyme activity using an extracellular crude extract from *Natronococcus* sp. (TC6) (Boutaiba et al. 2006). The activities of extracellular esterase and lipase from five halophilic archaeal strains isolated from different hypersaline environments in Turkey were also reported (Ozcan et al. 2009).

Halobacterium sp. NRC-1 is a halophilic archaeon whose genome was sequenced finding that it contains genes coding for putative esterases (Ng et al. 2000) (http://www.genome.ad.jp). However, this genomic prediction has not been experimentally verified. Therefore, in this work, the capability of *Halobacterium* to produce carboxyl ester hydrolases was investigated. Furthermore, the effect of physicochemical conditions (pH, temperature and salt concentration) on the growth rate and the production of carboxyl ester hydrolases was studied. Finally, the effects of NaCl concentration, temperature and pH on enzyme activity were determined.

Materials and methods

Microorganism, medium and inoculum preparation

Halobacterium sp. NRC-1 was obtained from the American Type Culture Collection (ATCC) and cultured in the ATCC 2185 medium, containing (g/l): NaCl, 250; $MgSO_4 \cdot 7H_2O$, 20; trisodium citrate, 3; KCl, 2; tryptone, 5; yeast extract, 3; and 0.1 ml of a trace element solution (containing, mg/ml; $ZnSO_4 \cdot 7H2O$, 6.6; $MnSO_4 \cdot H_2O$, 1.7; $Fe(NH_4)_2SO_4 \cdot 6H_2O$, 3.9; $CuSO_4 \cdot 5H_2O$, 0.7). The pH medium was adjusted to 6.5 prior to sterilization (121°C, 15 min). The reactivated strain was conserved at −20°C in 1.5 ml microtubes. The inoculum was prepared by mixing 1 ml of the stored cell culture ($OD_{600\ nm} = 2.2$) with 9 ml of the above medium and incubated and shaken at 37°C and 200 rpm for 72 h, in 125 ml Erlenmeyer flasks.

Biomass estimation

The biomass concentration was estimated by measuring optical density at 600 nm using a Spectronic Genesys 2 (Spectronic Instruments), and the obtained value was converted to biomass concentration (g/l) using a standard curve previously determined by relating biomass (dry weight) and optical density (Biomass $g/l = 0.66 \times OD_{600\ nm}$). To determine the biomass dry weight, 10 ml of culture broth were centrifuged at 4°C and 4,500 rpm for 30 min in a Beckman 25R centrifuge (Beckman Coulter, Inc.), using pre-weighed tubes. The cell pellet was washed twice, resuspending it in a solution of 2 M NaCl. A third washing step was quickly performed with distilled water. The tubes were dried in an oven at 100°C for 24 h, cooled in a desiccator and then weighed. The biomass dry weight was calculated by the difference between the empty tubes and the tubes with the cell pellets.

Intra and extracellular crude extracts preparation

Samples of culture broth (40 ml in 80 ml tubes) were centrifuged at 4°C and 4,500 rpm for 40 min in a Beckman 25R centrifuge (Beckman Coulter, Inc.). To obtain the intracellular crude extract, a cell pellet ($\sim$0.1 g) was resuspended in 2 ml of 20 mM Tris–HCl pH 7.5 (containing 0.15 M NaCl) and incubated at 5°C for 12 h to promote an osmotic disruption. Intracellular crude extract and culture broth supernatants were centrifuged at 10,000 rpm for 60 min to remove cell debris and then filtered through 0.45 µm cellulose membranes. To obtain the extracellular crude extracts, the culture broth supernatants were concentrated 20 times by ultrafiltration in a MINITAN II system (Millipore), using a 10 kDa polyethersulphone membrane.

Carboxyl ester hydrolase activity determination

Carboxyl ester hydrolase activity was assayed by measuring the hydrolysis rate of *p*-nitrophenyl esters (butyrate, valerate and laurate), according to Beisson et al. (2000). 1 ml of the substrate solution (10 mM dissolved in 2-propanol) was mixed with 9 ml of a solution containing 100 mM Tris–HCl (pH 7.5), 0.5% (w/v) Triton X-100 and 2 M NaCl. Then the mixture was pre-warmed at 30°C in a water bath and immediately distributed (0.8 ml) in thermostated cells at 30°C. The enzymatic assay was started by adding 0.2 ml of intra or extracellular crude extract, and the substrate hydrolysis was continuously monitored for 15 min at 410 nm using a Spectronic Genesys 2 (Spectronic Instruments). The background

substrate hydrolysis was accounted for with blanks containing non-fermented culture medium instead of intra or extracellular crude extract for all tested conditions. The reaction rate was calculated from the slope of the absorbance curve versus time by using a molar extinction coefficient of 11,983 cm^{-1} M^{-1} for *p*-nitrophenol, which was calculated at the assay conditions. One enzyme unit (U) was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute under assay conditions.

Alternatively, carboxyl ester hydrolase activity was assayed with the pH–stat technique using triacyl-glycerides (tributyrin and trioctanoin) at 50°C and pH 7.5. One ml of sample was added to 10 ml of the reaction mixture, containing 4 M NaCl, 0.2% (w/v) of polyvinyl alcohol (mw of 30,000 to 70,000) and 10% (v/v) tributyrin or trioctanoin. Free fatty acids released were continuously titrated using 10 mM NaOH. A blank was prepared using non-fermented culture medium and analyzed to measure the spontaneous hydrolysis, which is subtracted from the enzymatic hydrolysis. One lipase unit was defined as the amount of enzyme required to liberate 1 μmol of fatty acid per minute under assay conditions. All enzyme assays were done in triplicate.

Proving the synthesis of carboxyl ester hydrolase from *Halobacterium* sp. NRC-1

Cultures of *Halobacterium* sp. NRC-1 were carried out in 500 ml Erlenmeyer flasks containing 90 ml of medium and 10 ml of inoculum (at $OD_{600\ nm}$ 2.2) in an Environ shaker (Lab Line Instruments) at 37°C and 200 rpm, for 45 h. Intra and extracellular crude extracts were assayed for carboxyl ester hydrolase activity using *p*-nitrophenyl butyrate (pNPB), *p*-nitrophenyl valetare (pNPV) and *p*-nitrophenyl laurate (pNPL) as substrates.

Kinetics of growth and production of carboxyl ester hydrolase from *Halobacterium* sp. NRC-1

The halophilic archaeon *Halobacterium* sp. was cultured in a 3 l Bioflo 3000 bioreactor (New Brunswick scientific) using 1,800 ml of medium and 200 ml of inoculum (at $OD_{600\ nm} = 2.2$) at 300 rpm, 37°C, pH 6.5 and 1 vvm of air. Samples were taken every 5 h during the culture (55 h) and used to measure the optical density at 600 nm (biomass) and the intracellular carboxyl ester hydrolase activity (using pNPV as substrate).

Effects of pH, temperature and NaCl concentration on the growth rate and enzyme production

The effects of pH, temperature and NaCl concentration on the growth rate and intracellular esterase production were statistically analyzed by using a Box Behnken surface response design (Gutiérrez and De la Vara 2004). Three levels for each factor were studied: pH 6, 7 and 8; temperature 30, 40 and 50°C; and NaCl concentration of 2, 3.5 and 5 M. As a result of this experimental design, 15 cultures were carried out in 500 ml Erlenmeyer flasks containing 90 ml of medium and 10 ml of inoculum (at $OD_{600\ nm} = 2.2$), which were incubated and shaken at 200 rpm. The pH of the culture media was buffered by adding 100 mM of MES, PIPES and TRIS for pH 6, 7 and 8, respectively. The archaeal growth and the enzyme production at the different experimental conditions were followed during 50 h, and the cultures were then stopped by freezing them at −20°C. The response variables analyzed were the specific growth rate (μ, h^{-1}) and the intracellular esterase production (U/l, using pNPB as substrate).

The response function *Y,* used to describe the effect of the factors pH, temperature and NaCl on growth and enzyme production, was expressed as a second-degree quadratic polynomial:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3, \quad (1)$$

where Y is either the specific growth rate (μ, h^{-1}) or the esterase activity (U/l); X_1 = pH; X_2 = temperature (°C); X_3 = NaCl concentration (M); b_0 is a constant; b_1, b_2, b_3 are linear coefficients; b_{11}, b_{22}, b_{33} are quadratic coefficients; and b_{12}, b_{13}, b_{23} are cross-product coefficients. The coefficients of the response function, their statistical significance and the maximum values for the growth rate and the production of esterase were evaluated using a statistical software program (Statgraphics).

Effects of NaCl concentration, pH and temperature on esterase activity

The effects of salt concentration, pH and temperature on esterase activity (using pNPB as the substrate) were studied as follows: The effect of salt concentration was investigated using a buffer solution (100 mM Tris–HCl) containing NaCl in the range of 0–5 M and maintaining the temperature and pH at 30°C and 7.5, respectively. The effect of pH was analyzed while fixing the temperature and the salt concentration at 30°C and 2 M, respectively. The pH was buffered from 5 to 6.5 using 100 mM MES and from 7 to 9 using 100 mM Tris–HCl. In addition, the molar extinction coefficient values for *p*-nitrophenol were calculated for each assayed pH value. To study the effect of temperature, the pH and NaCl concentration were fixed at 7.5 and 2 M, respectively, using a 100 mM Tris–HCl buffer, and the reaction temperatures ranged from 30 to 80°C.

Results and discussion

Detection of carboxyl ester hydrolase activity in *Halobacterium* sp. NRC-1 cultures

The genome of *Halobacterium* sp. NRC-1 was sequenced and genes coding for putative carboxyl-esterases were identified (Ng et al. 2000; http://www.genome.ad.jp). To verify whether this halophilic archaeon is able to produce these enzymes, it was cultured in a hypersaline medium at 37°C and 200 rpm for 45 h. Intra and extracellular crude extracts were obtained and used to assay hydrolytic activities toward *p*-nitrophenyl butyrate (pNPB), *p*-nitrophenyl valerate (pNPV) and *p*-nitrophenyl laurate (pNPL).

In agreement with the genomic prediction, the enzyme activity in question was positively detected on the intracellular crude extract, using the above substrates. Nevertheless, no carboxyl ester hydrolase activity was detected on the extracellular crude extract at the assay conditions. It should be mentioned that the lack of activity detection in extracellular extracts could be due to activity losses during the concentration step by ultrafiltration. The highest esterase activity was reached at 45 h (0.87 ± 0.04, 0.77 ± 0.01 and 0.09 ± 0.02 U/g biomass, respectively, for pNPB, pNPV and pNPL). To confirm the expected enzyme activity in the intracellular crude extract, tributyrin and trioctanoin were also used as substrates. The carboxyl ester hydrolase activities were 2.79 and 1.25 U/g biomass, respectively, for both triglycerides; confirming the previous results obtained using *p*-nitrophenyl esters. Furthermore, it was elucidated that *Halobacterium* sp. principally synthesized esterases, hydrolyzing short chain fatty acid esters, since a high pNPB/pNPL hydrolysis ratio of 10.1 was found. In agreement with our results, other researchers have reported that halophilic organisms usually produce higher esterase than lipase activity (Martin et al. 2003; Ozcan et al. 2009; Sanchez-Porro et al. 2003). On the other hand, Boutaiba et al. (2006) tested the enzyme specificity of *Natronococcus* sp. extracellular crude extracts, using several *p*-nitrophenyl esters (containing from 2 to 18 carbons in the fatty acid chain), finding the highest lipolytic activity with *p*-nitrophenyl palmitate.

Kinetics of growth and intracellular esterase production of *Halobacterium* sp. NRC-1

During the culture of *Halobacterium* sp. in a bioreactor, intracellular esterase was produced as a growth associated metabolite (correlation coefficient = 0.997), reaching a maximum value of 2.4 U/l (using pNPV) after the deceleration phase (45 h), and then remaining constant during the stationary phase (Fig. 1). For this culture, a specific growth rate of 0.142 h^{-1} and a maximum biomass concentration of 1.3 g/l were obtained. A similar correlation for growth and enzyme production was observed for *Natronococcus* sp. TC6. Remarkably, this strain produced a maximum value of lipase activity of 52 U/l, using *p*-nitrophenyl palmitate as the substrate (Boutaiba et al. 2006).

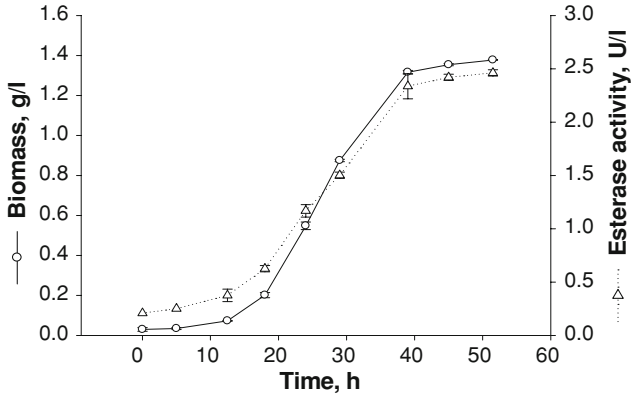

Fig. 1 Kinetics of growth and intracellular esterase production of *Halobacterium* sp. NRC-1 cultured in a bioreactor. Esterase activity was assayed using *p*-nitrophenyl valerate as substrate. Data represent the mean and the standard deviation of three assays

Effects of physicochemical conditions on growth rate and intracellular esterase production of *Halobacterium* sp. NRC-1

The effects of physicochemical conditions (temperature, pH and NaCl concentration) on the growth rate and the intracellular esterase production of *Halobacterium* were studied, according to a Box Behnken surface response design. Coefficients in Eq. 1 were evaluated by multiple regression analysis and tested for their significance ($\alpha = 0.1$). No significant coefficients were eliminated, and the model was consequently adjusted. The final response functions to predict the growth rate (Y_1) and esterase production (Y_2) were as follows:

$$Y_1 = -0.4609 + 0.0083X_1 + 0.0161X_2 + 0.1376X_3 - 0.0003X_2^2 - 0.0205X_3^2 - 0.0090X_1X_3 + 0.0023X_2X_3 \quad (2)$$

$$Y_2 = -5.6149 + 1.9639X_1 - 0.0634X_2 + 0.8616X_3 - 0.1473X_1^2 - 0.1188X_3^2 - 0.0333X_1X_3 + 0.0143X_2X_3 \quad (3)$$

Values of R^2 showed that the equations were reliable (0.912 and 0.873 for Y_1 and Y_2, respectively) and that the correlation between variables was statistically significant (P values <0.05), indicating adequate models to predict Y_1 and Y_2 within the used range of variables.

Table 1 Analysis of variance for the effects of physicochemical conditions on growth rate and esterase production of *Halobacterium* sp. NRC-1

Source	*P* value	
	μ (h^{-1})	Esterase (U/l)
X_1	0.0147*	0.0423*
X_2	0.4265	0.1704
X_3	0.0020*	0.0004*
$(X_2)^2$	0.0277*	0.6609
$(X_3)^2$	0.0029*	0.0739
(X_2) (X_3)	0.0118*	0.1231

Esterase activity was assayed using *p*-nitrophenyl butyrate as substrate

X_1 pH, X_2 temperature, X_3 NaCl concentration

* Significant factors with *P* value <0.05

As Table 1 shows, NaCl concentration, pH and square of temperature, significantly influenced growth rate (*P* value <0.05), while esterase production was mainly influenced by NaCl concentration and pH.

Halobacterium sp. NRC-1 showed a maximum growth rate of 0.136 h^{-1} at 44°C, 4.2 M NaCl and pH 6 (Fig. 2a). This result is in agreement with those values reported from most of haloarchaea (0.057–0.231 h^{-1}) (Dyall-Smith 2006). In the tested interval of temperature, the growth rate was not considerably influenced; nevertheless, the response surface plot shows a maximum for growth rate between 40 and 50°C (Fig. 2a), which is in agreement with the optimum growth temperatures reported for halophilic archaea (43–58°C) (Robinson et al. 2005).

The lowest growth rate was achieved at 2 M of NaCl (0.023 h^{-1} at 40°C and pH 8, and no growth was observed at 50°C and pH 7). These results show evidence of the extreme halophilicity of *Halobacterium* sp. NRC-1. Furthermore, a clear interaction effect between NaCl concentration and temperature on growth rate and enzyme production was identified (Fig. 3a, b). At 30°C, no significant differences ($\alpha = 0.05$) on the growth rates (0.022 h^{-1}) were identified, when NaCl concentration increased from 2 to 5 M NaCl. At temperatures higher than 30°C, the growth rate decreased for 2 M NaCl (from 0.022 h^{-1} at 30°C to no detectable growth at 50°C), while it greatly increased for 5 M NaCl (from 0.022 h^{-1} at 30°C to 0.1 h^{-1} at 50°C). Interestingly, the optimum growth rate obtained at 5 M NaCl was observed in a range of temperatures from 44 to 50°C, suggesting an effect of thermal protection of NaCl on *Halobacterium* sp. NRC-1 growth (Fig. 3a). At low salt concentration (2 M NaCl), esterase production from *Halobacterium* sp. NRC-1 decreased from 0.59 U/l at 30°C to no detectable activity at 50°C; however, at a higher salt concentration (5 M NaCl), esterase production increased from 1.25 U/l at 30°C to 1.42 U/l at 50°C (Fig. 3b). Similar effects of thermal protection have been observed for *Natronococcus* sp. (Boutaiba et al. 2006) and *Brevibacterium* sp. (Mimura and Nagata 1998) and

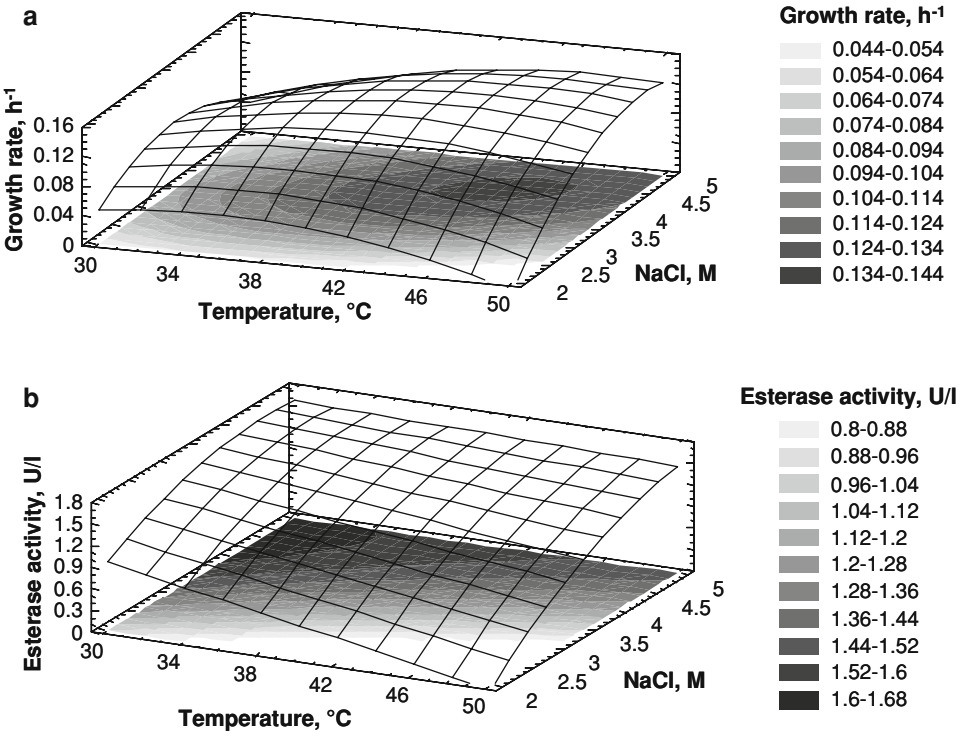

Fig. 2 Estimated response surfaces at pH 6, for growth rate (**a**) and intracellular esterase production (**b**) of *Halobacterium* sp. NRC-1. Esterase was assayed using *p*-nitrophenyl butyrate as substrate

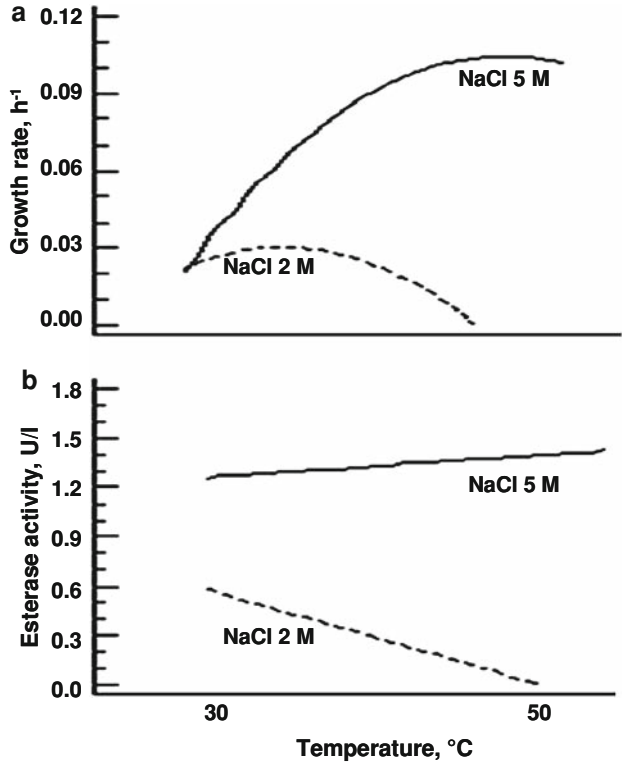

Fig. 3 Effects of temperature and NaCl interaction on growth rate and intracellular esterase production of *Halobacterium* sp. NRC-1. Esterase activity was assayed using *p*-nitrophenyl butyrate

also for halophilic enzymes: malate dehydrogenase from *H. marismortui* (Tehei et al. 2002) and nitrate reductase from *Haloferax mediterranei* (Martinez-Espinosa et al. 2001).

On the other hand, a maximum esterase production of 1.8 U/l was experimentally reached at 30°C, pH 6 and 3.5 M NaCl. This value was in agreement with that estimated value using the surface response method (1.6 U/l at 30°C, pH 6, NaCl 4.6 M, Fig. 2b).

Esterase synthesis as a function of NaCl concentration and pH responded similarly to the archaeal growth. A similar correlation was observed for *Natronoccocus* sp. (Bhatnagar et al. 2005). Conversely, temperature requirements were different for optimal esterase production (30°C) and growth (44°C).

Effects of NaCl concentration, pH and temperature on enzyme activity

Figure 4a shows the intracellular esterase activity (using pNPB as substrate) as a function of NaCl concentration. This figure suggests the presence of two esterase activities, one of them more active at low salt concentrations, and the other one more active at high salt concentrations, with optimal activities located at 1 and 5 M, respectively. Scarce studies on the characterization of carboxyl ester hydrolases by halophilic archaea have been reported. Boutaiba et al. (2006) reported that extracellular lipase activity (from *Natronococcus* sp.) increased as the concentration of NaCl increased, reaching a maximum value at 4 M of NaCl and showing no activity in the absence of salt. Ozcan et al. (2009) have reported for five halophilic archaeal strains a range from 3 to 4.5 M NaCl for optimal extracellular esterase activities.

It is worth noting that for the few purified enzymes from halophilic archaea, a general pattern for enzyme activity and its salt dependence has not been established (Madern et al. 2000). Most of the studied enzymes are inactive in the absence of NaCl (Giménez et al. 2000; Liu et al. 2002; Studdert et al. 2001). Nevertheless, some halophilic enzymes retain their activity at low salt concentrations or even in its absence (Ferrer et al. 1996; Hutcheon et al. 2005; Johnsen and Schonheit 2004). On the other hand, some halophilic enzymes showed their maximum activity from 4 to 5 M NaCl (Hutcheon et al. 2005; Liu et al. 2002).

The effect of temperature (from 30 to 80°C) on the intracellular esterase activity was investigated at 2 M NaCl and pH 7.5 (Fig. 4b). Surprisingly, the maximum esterase activity occurred at 80°C, and it is notably higher compared to other halophilic enzymes. Lipase from *Natronococcus* sp. and esterases from five halophilic archaeal strains exhibited maximal activities, respectively, at 50°C and between 60 and 65°C (Boutaiba et al. 2006, Ozcan et al. 2009). In addition, other halophilic enzymes have shown maximum activities from 50 to 60°C (Giménez et al. 2000; Hutcheon et al. 2005; Johnsen and Schonheit 2004; Studdert et al. 2001).

The influence of pH on the esterase activity of the intracellular crude extract is depicted in Fig. 4c. Interestingly, the results suggest the presence of two esterase activities, one of them more active at moderately acidic pH, and the other one, more active at slightly alkaline pH, with optimal activities located at a pH of 6 and 7.5, respectively. Boutaiba et al. (2006) reported that lipase of *Natronococcus* sp. exhibited an optimum activity at pH 7. Furthermore, Ozcan et al. (2009) reported a range of pH from 8 to 8.5 for optimal esterase activities of five halophilic archaeal strains. Other halophilic enzymes have shown maximum activities in a pH range from 7 to 10 (Giménez et al. 2000; Hutcheon et al. 2005; Johnsen and Schonheit 2004; Studdert et al. 2001).

Remarkably, esterase detected in this work showed the highest activity in extremely high saline conditions (5 M NaCl) and high temperature (80°C). This finding makes it an interesting enzyme for future investigations in biocatalysis using non-aqueous solvents, since it could be considered as a robust biocatalyst, active at low water activity

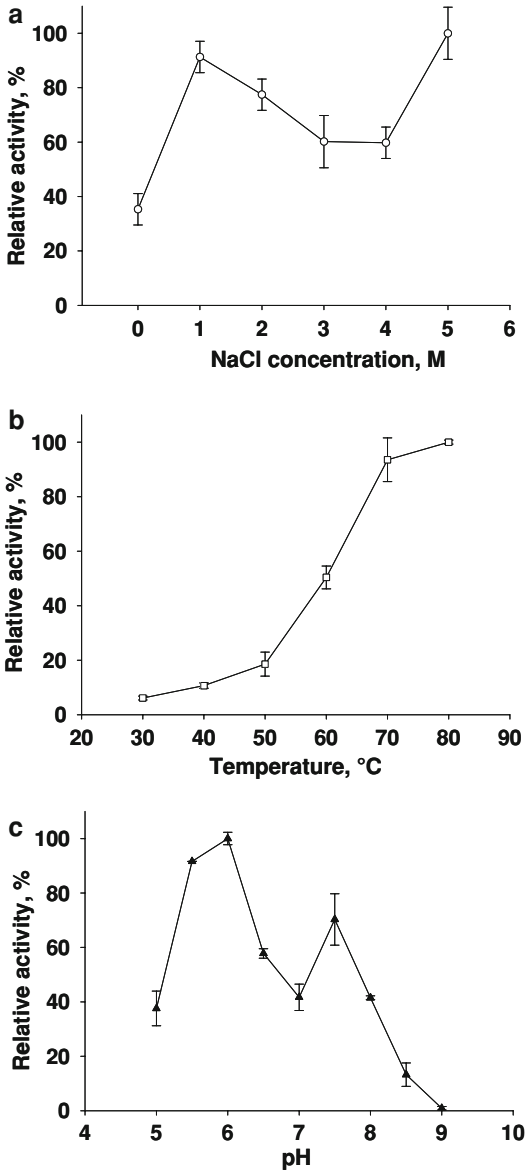

Fig. 4 Intracellular esterase activity of *Halobacterium* sp. NRC-1 as a function of: **a** NaCl concentration (temperature and pH were fixed at 30°C and 7.5, respectively), **b** temperature (NaCl concentration and pH were fixed at 2 M and 7.5, respectively) and **c** pH (NaCl concentration and temperature were fixed at 2 M and 30°C, respectively). Relative activities of 100% were of 3.07, 31.39 and 3.45 U/l, respectively for the NaCl, temperature and pH experiments. Assays were performed using *p*-nitrophenyl butyrate. Data represent the mean and the standard deviation of three assays

and high temperature. Work is currently underway to explore this hypothesis.

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