J Ind Microbiol Biotechnol (2009) 36:901–909
DOI 10.1007/s10295-009-0568-1

ORIGINAL PAPER

Production and characterization of esterase and lipase from *Haloarcula marismortui*

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Received: 14 November 2008 / Accepted: 17 March 2009 / Published online: 7 April 2009

Abstract The present study was conducted to investigate the capability of *Haloarcula marismortui* to synthesize esterases and lipases, and the effect of physicochemical conditions on the growth and the production of esterases and lipases. Finally, the effect of NaCl concentration and temperature on esterase and lipase activities was studied using intracellular crude extracts. In order to confirm the genomic prediction about the esterase and lipase synthesis, *H. marismortui* was cultured on a rich medium and the crude extracts (intra- or extracellular) obtained were assayed for both activities using *p*-nitrophenyl esters and triacylglycerides as substrates. Studies on the kinetics of growth and production of esterase and lipase of *H. marismortui* were performed, reaching a maximum growth rate of 0.053 h^{-1} and maximal productions of intracellular esterase and lipase of 2.094 and 0.722 U l^{-1} using *p*-nitrophenyl valerate and *p*-nitrophenyl laurate, respectively. Both enzymes were produced as growth-associated metabolites. The effects of temperature, pH, and NaCl concentration on the growth rate and production of enzymes were studied by using a Box–Behnken response surface design. The three response variables were significantly influenced by the physicochemical factors and an interaction effect between temperature and NaCl concentration was also evidenced. The surface response method estimated the following maximal values for growth rate and productions of esterase and lipase: 0.086 h^{-1} (at 42.5°C, pH 7.4, and 3.6 mol l^{-1} NaCl), 2.3 U l^{-1} (at 50°C, pH 7.5, and 4.3 mol l^{-1} NaCl), and 0.58 U l^{-1} (at 50°C, pH 7.6, and 4.5 mol l^{-1} NaCl), respectively. Esterases were active at different salt concentrations, showing two optimal activities (at 0.5 and 5 mol l^{-1} NaCl), which suggested the presence of two different esterases. Interestingly, in the absence of salt, esterase retained 50% residual activity. Esterases and lipase activities were maximal at 45°C and inactive at 75°C. This study represents the first report evidencing the synthesis of esterase and lipase by *H. marismortui*.

Keywords Halophilic archaea · *Haloarcula marismortui* · Lipase and esterase synthesis

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Introduction

Halophilic archaea are found in natural hypersaline environments such as the Dead Sea, the Great Salt Lake, salterns, and soda lakes, where the concentration of NaCl can reach saturation levels of ~6 mol l^{-1} [7, 26]. In order to proliferate in these hypersaline habitats, these archaea osmoregulate their cytoplasm by the accumulation of K^+ [22]. As a result, intra- and extracellular proteins have to deal successfully with very high salt concentrations [30]. Enzymes from halophilic organisms are stable and active at low water activity, and consequently they are considered as

robust biocatalysts with potential applications in synthesis using nonaqueous media [10, 19, 26].

Lipases and esterases constitute two of the most important biocatalysts for biotechnological applications. These enzymes are essentially distinguished on the basis of their substrate specificity. Lipases preferentially hydrolyze triglycerides composed of long-chain fatty acids and esterases usually hydrolyze water-soluble esters, including short-chain fatty-acid triglycerides [31]. Both enzymes have diverse applications, which include the modification of biologically active molecules, the enhancement of flavor or nutraceutical properties in foods, and the resolution of racemic mixtures [4, 15, 25, 27]. Remarkably, in the presence of some organic solvents, they perform esterification, amidation, and polymerization reactions [4, 25, 27]. Novel lipases and esterases have been isolated from thermophilic and psychrophilic extremophiles, and some of them have been overexpressed in *Escherichia coli* [8, 15]. Recently, screening for lipase activity from halophilic archaea isolated from an aquatic hyperhalobic environment, the Sebkha of El Golea (Algerian Sahara), was carried out [3]. This work reported the discovery of the first true lipase detected in the *Archaea* domain and a preliminary characterization was later performed using a crude enzymatic preparation obtained from *Natronococcus* sp. [5]. Besides, the characterization of extracellular esterase and lipase activities from five halophilic archaeal strains isolated from different hypersaline environments in Turkey was reported [23].

Haloarcula marismortui is a halophilic archaeon whose genome has been recently sequenced [1]. Its genome contains genes encoding for putative esterases and lipase (http://www.genome.ad.jp). However, these genomic predictions have not been experimentally verified. Therefore, in this work the capability of *H. marismortui* to synthesize esterases and lipases was investigated. Furthermore, the effects of pH, temperature, and salt concentration on the growth rate and the production of esterase and lipase were studied. Finally, the effects of NaCl concentration and temperature on enzyme activity were determined.

Materials and methods

Microorganism, medium, and inoculum preparation

Haloarcula marismortui (3752) was obtained from the German Resource Centre for Biological Material (DSMZ) and cultured in ATCC 2185 medium, containing (g l^{-1}): 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^{-1}; $ZnSO_4 \cdot 7H_2O$, 6.6; $MnSO_4 \cdot H_2O$, 1.7; $Fe(NO_4)(SO_4)_2 \cdot 6H_2O$, 3.9; $CuSO_4 \cdot 5H_2O$, 0.7). The medium was adjusted to pH 7.4, prior to sterilization (121°C, 15 min). The reactivated strain was maintained at −20°C in 1.5-ml microtubes. The inoculum was prepared with 9 ml of the above medium and 1 ml of the stored cell culture (at $OD_{600\ nm}$ 3.3), by incubating at 37°C and 200 rpm for 48 h, in 125-ml Erlenmeyer flasks.

Biomass estimation

Biomass concentration was estimated by measuring optical density at 600 nm using a Spectronic Genesys 2 (Spectronic Instruments, USA) and a standard curve previously determined (Biomass g $l^{-1} = 0.688 \times OD_{600\ nm}$).

Enzymatic assays

Activities of esterase and lipase were assayed spectrophotometrically, according to Beisson et al. [2], by measuring the hydrolysis rate of *p*-nitrophenyl butyrate (pNPB) and *p*-nitrophenyl valerate (pNPV) for esterase and *p*-nitrophenyl laurate (pNPL) for lipase. One milliliter of the substrate solution (10 mmol l^{-1} dissolved in 2-propanol) was mixed with 9 ml solution containing 100 mmol l^{-1} Tris–HCl (pH 7.5), 0.5% (w/v) Triton X-100, and 2 mol l^{-1} NaCl. Then, the mixture was prewarmed at 30°C in a water bath and immediately distributed (0.8 ml) in spectrophotometer cells. The enzymatic assay was started by adding 0.2 ml enzymatic crude extract. The increment in the absorbance with respect to a blank without enzyme was continuously monitored using a Spectronic Genesys 2 (Spectronic Instruments, USA) at 410 nm, for 10 min at 30°C. 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} l mol^{-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 *p*-nitrophenol per minute under assay conditions.

Activities of esterase and lipase were also validated with the pH-stat assay using triacylglycerides (tributyrin and trioctanoin) at 50°C and pH 7.5. One milliliter of sample was added to 10 ml reaction mixture, containing 4 mol l^{-1} NaCl, 0.2% (w/v) polyvinyl alcohol (molecular weight 30,000–70,000), and 10% (v/v) tributyrin or trioctanoin. Free fatty acids released were continuously titrated using 10 mmol l^{-1} NaOH. Spontaneous hydrolysis was subtracted from enzymatic hydrolysis. One lipase unit was defined as the amount of enzyme required to liberate 1 μmol fatty acid per minute under assay conditions. Three enzymatic assays were done for each sample.

Obtaining enzymatic crude extracts

Culture broth (40 ml in 80-ml tubes) was centrifuged at 4°C, 4,500 rpm for 40 min in a Beckman 25R centrifuge

(Beckman Coulter, Inc., Fullerton, CA, USA). To obtain the intracellular crude extract, cell pellets (~0.1 g), were resuspended in 2 ml solution containing 20 mmol l^{-1} Tris–HCl, and 0.15 mol l^{-1} NaCl, and incubated at 5°C for 12 h to promote an osmotic disruption. After, the disrupted cells were sonicated to guarantee complete disruption using an ultrasonic processor Sonics VCX 130 PB (Sonics & Materials, Inc., Newtown, CT, USA) at 4°C, 130 W, 20 kHz, and an amplitude of 80%, during eight periods of 3 min each. Complete breakage was verified by microscope observation and protein quantification, using the Bradford method [6]. Intracellular crude extract and culture broth supernatants were centrifuged at 10,000 rpm for 60 min to remove cell debris and filtered with 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 (Millipore, Billerica, MA, USA) system using 10-kDa polyethersulfone membrane.

Determining the synthesis of lipase and esterase

Cultures of *H. marismortui* were carried out in 0.5-l Erlenmeyer flasks containing 90 ml medium and 10 ml inoculum (at $OD_{600\ nm}$ 3.3), in an Environ shaker (Lab Line Instruments, Melrose Park, IL, USA) at 37°C and 200 rpm, during 50 h. Intra- and extracellular crude extracts were assayed for esterase activity using pNPB and pNPV and for lipase activity using pNPL as substrates [2]. These enzymatic activities were assayed at pH 7.5 and 30°C.

Kinetics of growth and production of esterase and lipase of *H. marismortui* in a bioreactor

Haloarcula marismortui was cultured in a 3-l Bioflo 3000 bioreactor (New Brunswick Scientific, Edison, NJ, USA), using 1,800 ml medium and 200 ml inoculum (at $OD_{600\ nm} = 3.3$) at 300 rpm, 37°C, pH 7.4, and 1 vvm of air. Optical density at 600 nm and intracellular esterase and lipase activities (using pNPV and pNPL as substrates, respectively) were monitored each 6 h during the culture (75 h).

Effect of pH, temperature, and NaCl concentration on the growth rate and the production of enzymes

The effects of pH, temperature, and concentration of NaCl on the growth rate and the intracellular production of esterase and lipase were statistically analyzed by using a Box–Behnken surface response design [13]. Three levels of each factor were studied: pH 6, 7, and 8; temperature 30°C, 40°C, and 50°C; and NaCl concentration 2.5, 3.5 and 4.5 mol l^{-1}. Fifteen cultures were carried out in 500-ml Erlenmeyer flasks containing 90 ml medium and 10 ml inoculum ($OD_{600\ nm}$ 3.3), which were incubated at 200 rpm. pH of the media cultures was buffered by adding 100 mmol l^{-1} of MES, PIPES, and TRIS for pH 6, 7, and 8, respectively. pH was monitored during the cultures, verifying its constant value. Growth and enzyme production at the different experimental conditions were followed until a deceleration phase was detected. Cultures were then stopped by freezing at −20°C. The response variables analyzed were specific growth rate (μ) and intracellular production of esterase and lipase monitored by measuring the hydrolysis of pNPB and pNPL as substrates, respectively.

The response function used to study the effect of the factors on growth and enzyme productions was the following second-order polynomial equation:

$$Y = \alpha + \beta \cdot \mathrm{Xe} + \gamma \cdot \mathrm{Xs} + \delta \cdot \mathrm{Xt} + \varepsilon \cdot \mathrm{Xe}^2 + \eta \cdot \mathrm{Xs}^2 + \kappa \cdot \mathrm{Xt}^2 + \lambda \cdot \mathrm{Xe} \cdot \mathrm{Xs} + \rho \cdot \mathrm{Xe} \cdot \mathrm{Xt} + \varphi \cdot \mathrm{Xs} \cdot \mathrm{Xt}, \quad (1)$$

where Y is either growth rate (h^{-1}), esterase activity (U l^{-1}) or lipase activity (U l^{-1}); Xe is pH; Xs is temperature; Xt is NaCl concentration; α is a constant; β, γ, and δ are linear coefficients; ε, η, and κ are quadratic coefficients; and λ, ρ, and ϕ are cross-product coefficients. The coefficients of the response function, their statistical significance, and the calculation of maximal growth rate and productions of esterase and lipase were evaluated using a statistical software program (Statgraphics).

Effect of NaCl concentration and temperature on enzyme activity

The influence of NaCl concentration on pNPV-hydrolyzing activity was tested over the range from 0 to 5 mol l^{-1} at 30°C. The influence of temperature on pNPV and pNPL hydrolyzing activity was tested over the range from 20°C to 80°C at 4 mol l^{-1} NaCl. Both used a crude extract preparation, at pH 7.5.

Results

Detection of esterase and lipase activities in *H. marismortui* cultures

The genome of *H. marismortui* was recently sequenced [1], finding genes which encode for two putative carboxylesterases and for a putative lipase (http://www.genome.ad.jp). In order to verify the ability of this archaeon to produce esterase and lipase, it was cultured in a hypersaline medium using Erlenmeyer flasks, at 37°C and 200 rpm. Crude

extracts (intra- and extracellular) were obtained, and enzyme activities were detected using pNPB and pNPV for esterases and pNPL for lipases. The highest activities for esterase (pNPB hydrolysis) and lipase were reached at 50 h. Intracellular esterase and lipase activities were 0.489 and 0.188 U g^{-1} biomass, respectively; and extracellular esterase and lipase activities were 0.024 and 0.1 U g^{-1} biomass, respectively (Table 1). Since there are some controversies on the use of these substrates to detect esterase or lipase activity, tributyrin and trioctanoin were also used, confirming those enzymatic activities in the intracellular crude extract (3.88 U g^{-1} biomass on tributyrin and 1.34 U g^{-1} biomass on trioctanoin).

Kinetics of growth and production of intracellular esterase and lipase in bioreactor

During the culture of *H. marismortui* using a bioreactor Bioflo 3000 (New Brunswick scientific, Edison, NJ, USA), intracellular productions of esterase and lipase (using pNPV and pNPL, respectively, as substrates) were strongly correlated to microbial growth ($R = 0.948$ and 0.970 for esterase and lipase, respectively) (Fig. 1). The maximal activities of esterase and lipase (2.09 and 0.72 U l^{-1}, respectively) were obtained when a deceleration phase was reached (48 h). Subsequently, the enzyme activities remained constant during the stationary phase. For this culture, specific growth rate and final biomass were 0.052 h^{-1} and 2.35 g l^{-1}, respectively.

Effect of physicochemical conditions on the growth rate and the intracellular esterase and lipase produced by *H. marismortui*

The effect of physicochemical conditions (temperature, pH, and NaCl concentration) on the growth rate and the intracellular esterase and lipase production of *H. marismortui* were studied according to a Box–Behnken central composite design. Coefficients in Eq. 1 were evaluated by multiple regression analysis and tested for significance ($\alpha = 0.1$). No significant coefficients were eliminated and the model was consequently adjusted. The final response

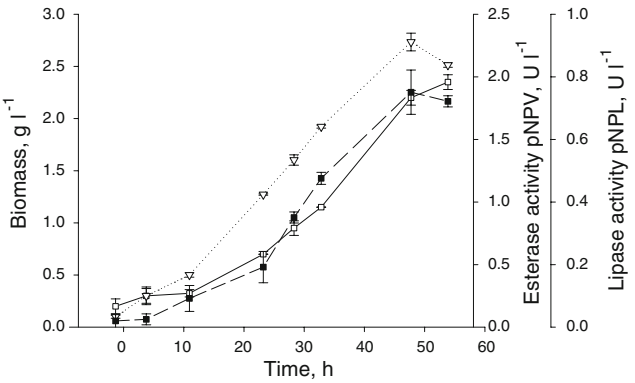

Fig. 1 Kinetic of growth and intracellular esterase and lipase production of *H. marismortui* cultured in a bioreactor. *Open square* denotes biomass (g l^{-1}); *open triangle* denotes esterase activity (U l^{-1}), and *dark filled square* denotes lipase activity (U l^{-1}), using *p*-nitrophenyl valerate (*pNPV*) and *p*-nitrophenyl laurate (*pNPL*), as substrates, respectively. Data represent the mean and the standard deviation of three assays

functions to predict the growth rate (Y_1), esterase production (Y_2), and lipase production (Y_3) were as follows:

$$Y_1 = -1.70592 + 0.354458 \cdot \mathrm{Xe} + 0.0101458 \cdot \mathrm{Xs} + 0.136292 \cdot \mathrm{Xt} - 0.0269167 \cdot \mathrm{Xe}^2 - 0.000251667 \cdot \mathrm{Xs}^2 - 0.0229167 \cdot \mathrm{Xt}^2 + 0.001125 \cdot \mathrm{Xe} \cdot \mathrm{Xs} + 0.000775 \cdot \mathrm{Xs} \cdot \mathrm{Xt}, \quad (2)$$

$$Y_2 = -34.7701 + 11.1171 \cdot \mathrm{Xe} - 0.3553 \cdot \mathrm{Xs} + 1.7675 \cdot \mathrm{Xt} - 0.843 \cdot \mathrm{Xe}^2 - 0.42625 \cdot \mathrm{Xt}^2 + 0.0298 \cdot \mathrm{Xe} \cdot \mathrm{Xs} + 0.037175 \cdot \mathrm{Xs} \cdot \mathrm{Xt}, \quad (3)$$

$$Y_3 = -5.3304 + 1.89677 \cdot \mathrm{Xe} - 0.0963625 \cdot \mathrm{Xs} + 0.268885 \cdot \mathrm{Xt} - 0.154269 \cdot \mathrm{Xe}^2 - 0.0897692 \cdot \mathrm{Xt}^2 + 0.008975 \cdot \mathrm{Xe} \cdot \mathrm{Xs} + 0.010675 \cdot \mathrm{Xs} \cdot \mathrm{Xt}. \quad (4)$$

Values of R^2 showed that the equations were highly reliable (0.964, 0.937, and 0.928 for Y_1, Y_2, and Y_3, respectively). Moreover, P values at 95% confidence were <0.05 (0.0008, 0.0010, and 0.0016 for Y_1, Y_2, and Y_3, respectively), indicating that the treatments were significant and the models were adequate to predict Y_1, Y_2, and Y_3 within the range of the employed factors.

Table 1 Intra- or extracellular esterase and lipase produced at 50 h of *H. marismortui* culture

	Enzymatic activity (U g^{-1} biomass)					Hydrolysis ratio
	pNPB	pNPV	pNPL	TC4	TC8	pNPB/pNPL
Intracellular	0.489 ± 0.018	0.451 ± 0.002	0.188 ± 0.011	3.88 ± 0.089	1.34 ± 0.044	2.60
Extracellular	0.024 ± 0.008	0.080 ± 0.022	0.100 ± 0.006			0.24

Esterase activity was assayed using *p*-nitrophenyl butyrate (pNPB), *p*-nitrophenyl valerate (pNPV), and tributyrin (TC4), and lipase activity was assayed using *p*-nitrophenyl laurate (pNPL) and trioctanoin (TC8). Biomass produced at 50 h of culture was 2.60 ± 0.01 g l^{-1}. Data are the mean ± standard deviation of three assays

As shown in Table 2, pH, temperature, and concentration of NaCl influenced significantly the growth rate ($P < 0.10$), while the productions of esterase and lipase, monitored by measuring hydrolysis of pNPB and pNPL as substrates, respectively, were mainly influenced by the concentration of NaCl and pH. As observed in Fig. 2, the growth rate of *H. marismortui* was slow at 30°C (0.048 h^{-1}), increasing to a maximum value at 42.5°C (0.079 h^{-1}), and decreasing slightly at 50°C (0.06 h^{-1}). The slowest growth rate (0.029 h^{-1}) was observed at pH 6, increasing to a maximum value at pH 7.4 (0.083 h^{-1}), and slightly decreasing at pH 8 (0.075 h^{-1}). Growth rate was low at 2.5 mol l^{-1} NaCl (0.049 h^{-1}), reaching a maximum value at 3.6 mol l^{-1} NaCl (0.079 h^{-1}), and slightly decreasing at 4.5 mol l^{-1} NaCl (0.063 h^{-1}).

Since *H. marismortui* did not grow at 2 mol l^{-1} NaCl, an experimental design was carried out using concentrations of NaCl between 2.5 and 4.5 mol l^{-1}. Even this way, very low growth rates were achieved at 2.5 mol l^{-1} NaCl (0.001 h^{-1} at 40°C and pH 6), evidencing the extreme halophilicity of *H. marismortui*. Analysis of response surface calculated a maximum growth rate of 0.086 h^{-1} at 42.5°C, pH 7.4, and 3.6 mol l^{-1} NaCl (Fig. 3).

On the other hand, esterase and lipase production of *H. marismortui* was found to be dependent on NaCl concentration and pH, whose profiles of enzymatic activity were similar, obtaining maximal values at 4.3 mol l^{-1} NaCl and pH 7.5 (2.1 U l^{-1} for esterase and 0.38 U l^{-1} for lipase) (Fig. 2). Esterase and lipase production did not show significant differences ($\alpha = 0.05$), when incubation temperature increased from 30°C to 50°C (from 2.1 to 1.8 U l^{-1} for esterase and from 0.34 to 0.42 U l^{-1} for lipase).

Table 2 Analysis of variance for growth and production of esterase and lipase of *H. marismortui*

Source	*P* value		
	μ	Esterase	Lipase
Temperature	0.0899	0.1107	0.1071
pH	0.0003*	0.0009*	0.0024*
NaCl concentration	0.0599	0.0205*	0.0138*
Temperature^2	0.0012*	NS	NS
pH^2	0.0008*	0.0004*	0.0014*
NaCl concentration^2	0.0019*	0.0151*	0.0213*
Temperature and NaCl	0.1146	0.0232*	0.0082*
Temperature and pH	0.0366*	0.0535	0.0182*

Esterase and lipase activities were assayed using *p*-nitrophenyl butyrate (pNPB) and *p*-nitrophenyl laurate (pNPL), respectively

NS not significant

* Significant factors with $P < 0.05$ ($\alpha = 0.05$)

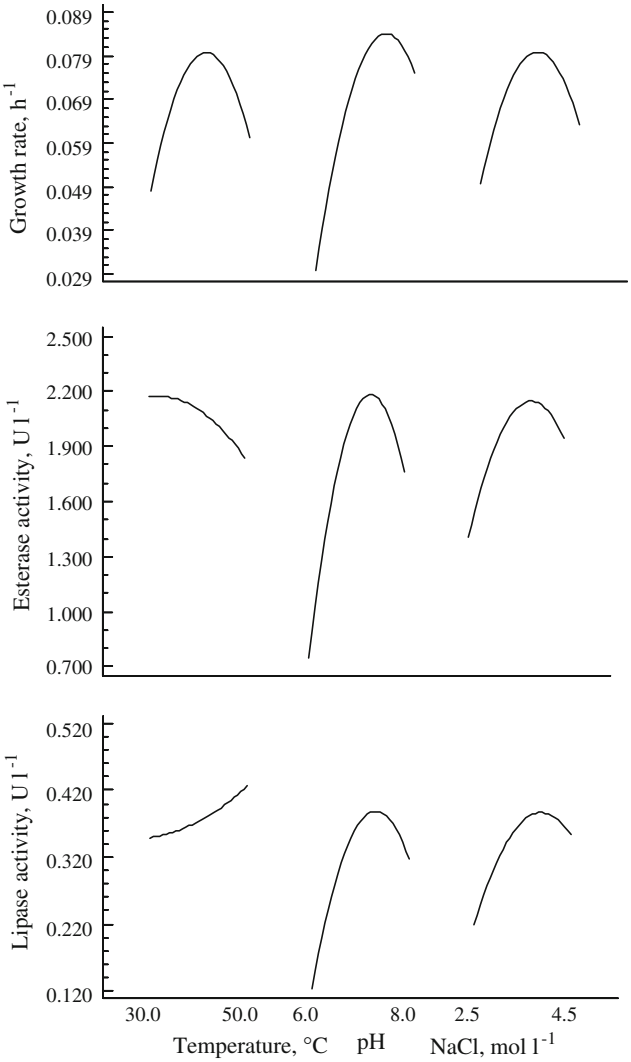

Fig. 2 Main effects: pH, temperature, and NaCl concentration on growth rate and production of esterase and lipase of *H. marismortui* monitored by hydrolysis of *p*-nitrophenyl butyrate (pNPB) and *p*-nitrophenyl laurate (pNPL), respectively

Enzyme production data of *H. marismortui* were also analyzed using the surface response method, estimating the following maximal values: esterase production of 2.3 U l^{-1} at 50°C, pH 7.5, and 4.3 mol l^{-1} NaCl; and lipase production of 0.58 U l^{-1} at 50°C, pH 7.6, and 4.5 mol l^{-1} NaCl (Fig. 3).

A clear interaction between NaCl concentration and temperature on growth rate and production of esterase and lipase was identified (Fig. 4). However, profiles of growth and enzymes production were different. For growth rates, at 30°C no significant differences ($\alpha = 0.05$) were identified as NaCl concentration increased from 2.5 to 4.5 mol l^{-1} (0.025 h^{-1}). Growth rates reached maximal values at 40°C (0.049 and 0.065 h^{-1}, respectively, for 2.5 and 4.5 mol l^{-1} NaCl) and declined for higher temperatures. Interestingly, high incubation temperatures

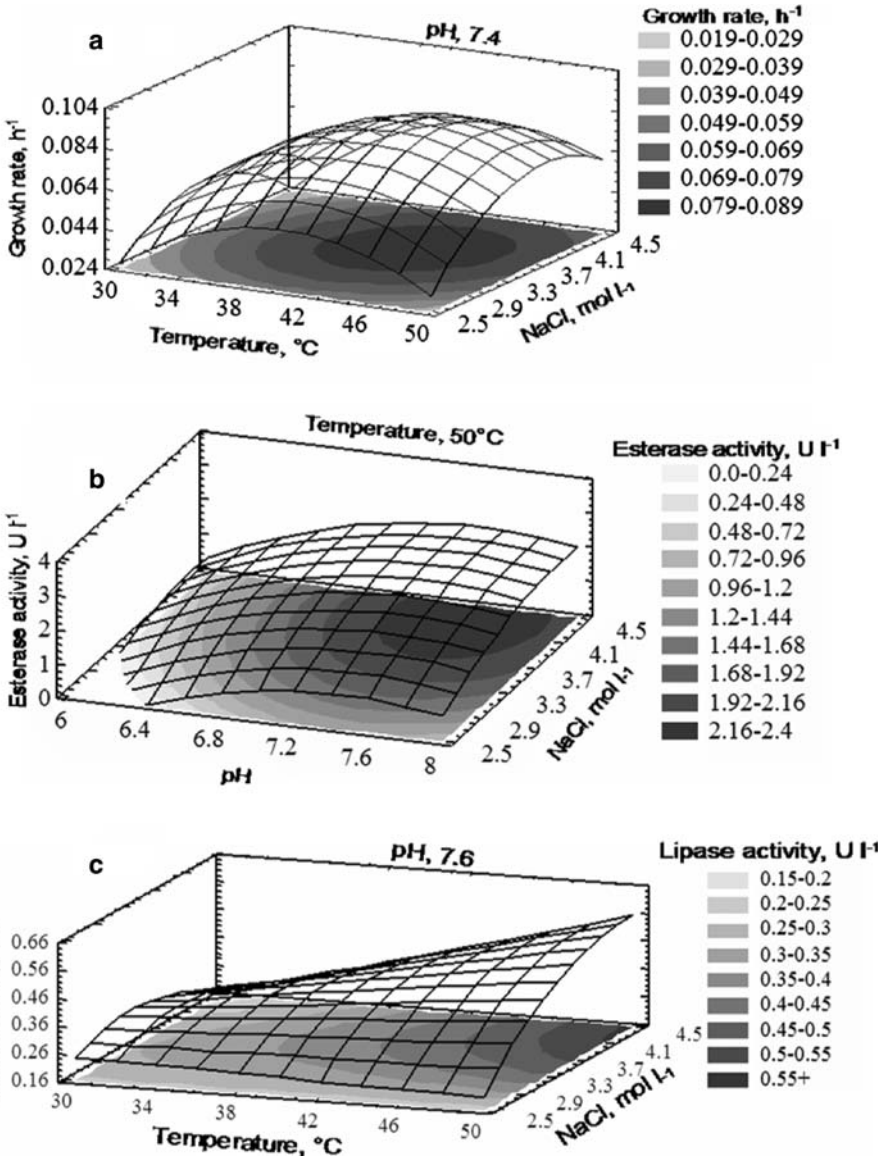

Fig. 3 Estimated response surface for growth rate (**a**) and production of esterase (**b**) and lipase (**c**) [monitored by hydrolysis of *p*-nitrophenyl butyrate (pNPB) and *p*-nitrophenyl laurate (pNPL), respectively] of *H. marismortui*

stimulated esterase and lipase production at 4.5 mol l^{-1} NaCl and had the opposite effect at 2.5 mol l^{-1} NaCl, revealing an effect of thermal protection by salt. At 50°C, esterase and lipase productions were 2 and 0.51 U l^{-1}, respectively, for 4.5 mol l^{-1} NaCl, and 0.77 and 0.16 U l^{-1}, respectively, for 2.5 mol l^{-1} NaCl.

Furthermore, a clear interaction between pH and temperature on growth rate and production of esterase and lipase was identified (Fig. 5). For the lowest temperature tested (30°C), no significant differences ($\alpha = 0.05$) in growth rate and enzymes production were identified as pH increased from 6 to 8. Interestingly, high values of pH (8) and temperature (50°C) stimulated growth and enzymes production. At pH 8 and 50°C, growth rate, esterase, and lipase productions were 0.067 h^{-1}, 1.86, and 0.45 U l^{-1}, respectively, while no growth or enzymes production was observed at pH 6 and 50°C.

Effect of NaCl concentration and temperature on enzyme activity

Figure 6 shows intracellular esterase activity (using pNPV as the 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, whose optimum activity was situated at 0.5 mol l^{-1}, and the second one more active at high salt concentrations, whose optimum activity was located in a range of salt concentrations from 3 to 5 mol l^{-1}. On the other hand, the profiles of enzymatic activity as a function of temperature were

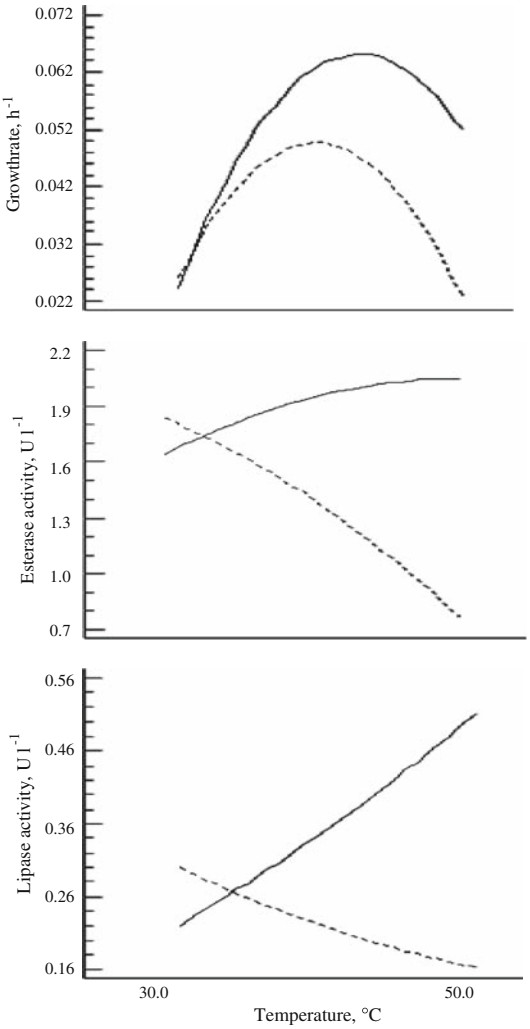

Fig. 4 Effect of temperature and NaCl interaction on growth rate and production of esterase and lipase [monitored by hydrolysis of *p*-nitrophenyl butyrate (pNPB) and *p*-nitrophenyl laurate (pNPL), respectively] of *H. marismortui*: *dashed line* 2.5 mol l^{-1} NaCl, *solid line* 4.5 mol l^{-1} NaCl

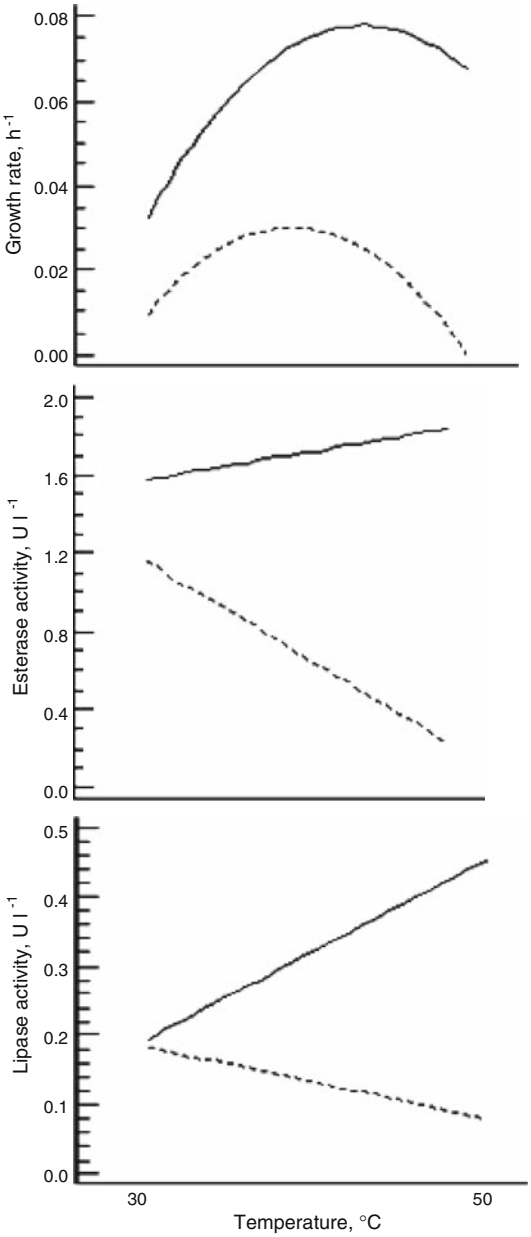

Fig. 5 Effect of temperature and pH interaction on growth rate and production of esterase and lipase [monitored by hydrolysis of *p*-nitrophenyl butyrate (pNPB) and *p*-nitrophenyl laurate (pNPL), respectively] of *H. marismortui*: *dashed line* pH 6, *solid line* pH 8

slightly different for esterase and lipase (Fig. 7). At low reaction temperatures, esterase was more active than lipase. Indeed, at 25°C, esterase showed 55.9% relative activity, while lipase showed only 9.1% relative activity. Both maximal enzymatic activities were reached at 45°C, retaining 23.3% and 19.8% residual activity for esterase and lipase, respectively, at 70°C.

Discussion

In agreement with the genomic prediction, this work showed that *H. marismortui* synthesizes esterases and lipases intracellularly (1.27 and 0.49 U l^{-1}, respectively) and extracellularly (0.06 and 0.26 U l^{-1}, respectively) (Table 1). Interestingly, the hydrolysis ratios of pNPB/pNPL for intra- and extracellular crude extracts were 2.6 and 0.24, respectively, showing that *H. marismortui* secreted mainly lipases, while esterases were mainly retained intracellularly (Table 1).

Esterases and lipases were mostly produced intracellularly (95% and 65%, respectively), with total enzymatic activities of 1.33 and 0.75 U l^{-1}, respectively, lower values than the lipase activity reported for *Natronococcus* sp. TC6 (52 U l^{-1}), where *p*-nitrophenyl palmitate was used as the substrate [5]. Intracellular productions of esterase and

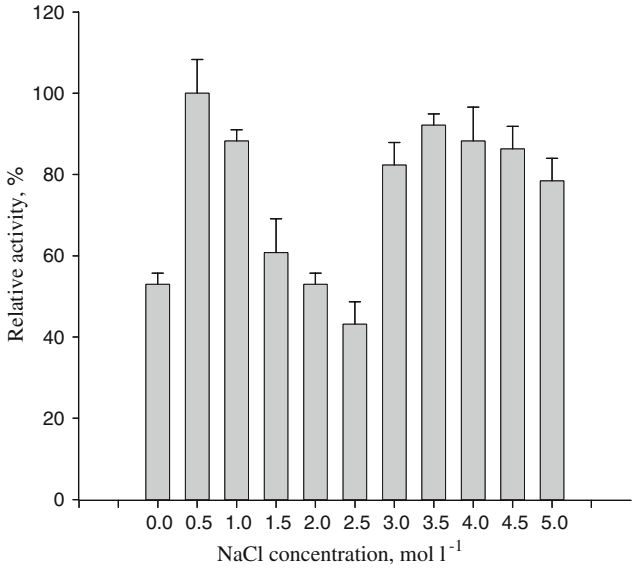

Fig. 6 Intracellular esterase activity as a function of NaCl concentration. Assays were performed using *p*-nitrophenyl valerate; error bars represent standard deviation ($n = 3$)

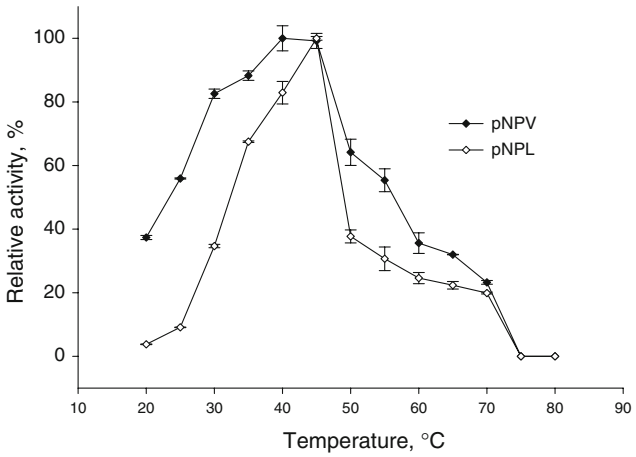

Fig. 7 Intracellular esterase and lipase activity as a function of the temperature. Assays were performed using *p*-nitrophenyl valerate (esterase) and *p*-nitrophenyl laurate (lipase). NaCl concentration and pH were fixed at 4 mol l^{-1} and 7.5, respectively. *Error bars* represent standard deviation ($n = 3$)

lipase were strongly correlated to *H. marismortui* growth, with *R* values of 0.948 for esterase and 0.970 for lipase, reaching maximal values at early stationary phase (Fig. 1). Similar behavior was observed for *Natronococcus* sp. [5]. Intracellular enzyme activities on triacylglycerides were also assayed, finding activities of 3.5 U l^{-1} on trioctanoin (TC8) and 10.1 U l^{-1} on tributyrin (TC4). These results confirmed the experimental results obtained using *p*-nitrophenyl esters, although it is worth noting that the haloarchaeal enzymes showed a marked selectivity toward triacylglycerides compared with *p*-nitrophenyl esters. Additionally, hydrolysis ratio of TC4/TC8 was also similar (2.9) to that obtained using *p*-nitrophenyl esters (pNPB/pNPL = 2.6), confirming the dependability of these assays.

The effects of physicochemical conditions (temperature, pH, and NaCl concentration) on the growth rate and the production of intracellular esterase and lipase of *H. marismortui* were also studied. Analysis of response surface calculated a maximum growth rate of 0.086 h^{-1} at 42.5°C, pH 7.4, and 3.6 mol l^{-1} NaCl (Fig. 3). This value was close to the maximum experimental value obtained (0.081 h^{-1} at 40°C, pH 7, and 3.5 mol l^{-1} NaCl). Additionally, this μ value and the incubation temperature for optimum growth (42.5°C) are in good agreement with values reported from most haloarchaea: 0.057–0.231 h^{-1} [9] and 43–58°C [24].

Growth rate was mainly influenced by NaCl concentration, pH, and temperature in the tested ranges, while esterase and lipase productions were only influenced by NaCl and pH. Besides, growth rate and enzymes production were greatly influenced by the combined effect of temperature and NaCl concentration, reveling an effect of thermal protection by NaCl. A similar effect was observed for *Natronococcus* sp. [5] and *Brevibacterium* sp. [21], and also for halophilic enzymes: malate dehydrogenase from *H. marismortui* [29] and nitrate reductase from *Haloferax mediterranei* [20].

By comparing the profile of esterase activity as a function of NaCl concentration, the presence of two esterase activities was suggested. For one of them, esterase activity decreased as salt concentration increased, whereas for the second one, activity increased as salt concentration increased (Fig. 6). This observation agreed with the genomic knowledge of this haloarchaea, concerning the synthesis of two putative esterases (http://www.genome.ad.jp). 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 [18]. Most of the halophilic enzymes studied are inactive in absence of NaCl, for example, lipases from *Natronococcus* sp. [5], proteases from *Natronococcus occultus* [28] and *Natrialba magadii* [12], and a thiolase from *Halobacterium* ZP-6 [17]. On the other hand, some halophilic enzymes retain their activity at low salt concentrations and even in its absence, such as the xylose dehydrogenase from *H. marismortui* [16], glutamate dehydrogenase from *Haloferax mediterranei* [11], and the α-amylase from *Haloarcula hispanica* [14].

Esterase and lipase detected in this work showed their maximal activities at a reaction temperature of 45°C (Fig. 7), which was slightly lower compared with other studies using halophilic enzymes (with maximal activities between 50°C and 60°C) [12, 14, 16, 28]. Similarly, the lipase of *Natronococcus* sp. exhibited its maximum activity at 50°C [5].

It is worth noting that esterase and lipase detected in this work showed high activities in extremely saline conditions (as high as 5 mol l^{-1} NaCl) (Fig. 6). This finding makes them interesting enzymes for future investigations as biocatalysis using nonaqueous solvents, since they could be considered as active and robust biocatalysts at low water activity. Work is ongoing to explore this hypothesis.

Acknowledgments This work was supported by CONACYT (J1-61207). Rosa Maria Camacho Ruiz acknowledges a Ph.D. grant received from CONACYT.

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