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FEMS Microbiology Letters 248 (2005) 133–140

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Lipolytic activity from *Halobacteria*: Screening and hydrolase production

Tej Bhatnagar [a,1], Saad Boutaiba [b], Hocine Hacene [b], Jean-Luc Cayol [c], Marie-Laure Fardeau [c], Bernard Ollivier [c], Jacques C Baratti [a,*]

[a] *Laboratoire de Biocatalyse et Chimie Fine, UMR 6111, Université de la Méditerranée, Faculté des Sciences de Luminy, 163, Avenue de Luminy, Case 901, 13288, Marseille Cedex 9 France*

[b] *Research Laboratory on Arid Zones, Faculté des Sciences Biologiques, USTHB, Alger, Algérie*

[c] *Laboratoire de Microbiologie, IRD, Université de la Méditerranée, Faculté des Sciences de Luminy, Marseille, France*

Received 17 January 2005; received in revised form 18 April 2005; accepted 7 May 2005

First published online 16 June 2005

Edited by S. Mongkolsuk

Abstract

Strains of *Halobacteria* from an Algerian culture collection were screened for their lipolytic activity against *p*-nitrophenyl butyrate (PNPB) and *p*-nitrophenyl palmitate (PNPP). Most strains were active on both esters and 12% hydrolyzed olive oil. A strain identified as *Natronococcus* sp. was further studied. It grew optimally at 3.5 M NaCl, pH 8 and 40 °C. An increase in temperature shifted the optimum salt concentration range for growth from a wider range of 2–4 M, obtained at 25–30 °C, to a narrower range of 3.5–4 M, obtained at 35–40 °C. At 45 °C the optimum salt concentration was 2 M. These results show a clear correlation between salt and temperature requirement. The optimum conditions for the production of hydrolytic activity during growth were: 3.5 M NaCl and pH 8 for PNPB hydrolytic activity and 4 M NaCl and pH 7.5 for PNPP hydrolytic activity; both at 40 °C. The clear supernatant of cells grown at 4 M NaCl showed olive oil hydrolysis activity (in presence of 4 M NaCl) demonstrating the occurrence of a lipase activity in this strain. To our knowledge, this is the first report of a lipase activity at such high salt concentration.

Keywords: *Halobacteria*; Screening; Lipolytic activity; *p*-Nitrophenyl esters; Archaeon; *Natronococcus*

1. Introduction

The *Halobacteria* that grow in highly saline ecosystems, such as hypersaline lakes, marine salterns and salt mines, have optimum salt concentrations for growth that are about ten times that of sea water and are therefore potential producers of extremely halophilic enzymes. They counterbalance high extracellular salt concentrations by accumulating KCl within the cell up to concentrations close to saturation [1]. Their enzymes have evolved particular features that confer to them stability and solubility in high salt concentrations. For example, high surface charges favour the formation of a hydrated ion shell at the protein surface, ensuring solubility [2]. Furthermore, chloride and sodium ions are involved in salt bridges that lock subunits together [3]. Like organic solvents, salts greatly reduce the water activity of the medium. For instance, the water activity (a_w) of saturated NaCl is 0.75, which is equivalent to that of a 60% (v/v) aqueous solution of DMF (dimethyl

* Corresponding author. Tel.: +33 4 9182 9377; fax: +33 4 9182 9362.
E-mail addresses: bhatnaga@luminy.univ-mrs.fr (T. Bhatnagar), jbaratti@luminy.univ-mrs.fr (J.C Baratti).
[1] Tel.: +33 4 9182 9393; fax: +33 4 9182 9362.

0378-1097/$22.00
doi:10.1016/j.femsle.2005.05.044

formamide) [4]. It is quite possible that the distinct features of halophilic enzymes that confer high stability at high salt concentrations will also confer good stability in organic solvents and at high temperatures, thereby making them good candidates as biocatalysts in hostile environments [5,6]. However, in spite of a growing interest in the use of halophilic enzymes for biotechnological applications, there are only relatively few reports in the literature about their production and characterisation. The best studied enzyme of this class is malate dehydrogenase from *Haloarcula marismortui* [3,7–9], while citrate synthase and dihydrolipoamide dehydrogenase from *Haloferax volcanii* [10,11] and 2Fe–2S Ferredoxin [8] have also received some attention. As far as lipolytic enzymes are concerned, in 1970 Gonzalez et al. [12] reported the presence of Tween (20–80) degrading activity on solid media among strains of *Halobacterium*. However, since then, no other work has been published to support this finding.

Esterase and lipase (lipolytic enzymes) are widely distributed in nature. These enzymes are involved in ester synthesis or hydrolysis and have found numerous biotechnological applications in the pharmaceutical and food industries [13]. Lipases differ from esterases by their capacity to hydrolyse water-insoluble esters of long-chain fatty acids. Up to now, no true lipase activity has been detected in highly saline environments.

The current work describes the screening for lipolytic activity in 35 of 54 *Halobacteria* that had been previously isolated from waters of the El Golea Sebkha, which is located in the middle of Algerian Sahara and contains salt concentrations up to 300 g/l [14]. All these strains required at least 2 M NaCl for growth, with best growth being obtained at 3–5 M. The growth and production of extracellular lipolytic activity under different physiological conditions from a promising strain, strain TC6, were also studied.

2. Materials and methods

2.1. Strains and growth conditions

The 35 bacterial isolates were routinely cultured in modified Gibbons medium [15] which contains (g/l): NaCl (iodine free, Euromedex, Mundolsheim, France), 250; $MgSO_4 \cdot 7H_2O$, 1; KCl, 2; trisodium citrate (Merck, Strasbourg, France), 3; casamino acids (Sigma France, Saint Quentin), 7.5; and yeast extract (Fischer France, Illkirch), 10. When the medium was supplemented with Na_2CO_3 (5 g/l), both NaCl and Na_2CO_3 were sterilised separately before being added to the hot medium. The pH was adjusted to 7.5 with KOH prior to heat sterilisation. Agar plates contained 2% (w/v) agar. Liquid cultures were grown aerobically under reciprocal shaking (400 strokes min^{-1}) at 40 °C in tubes or in Erlenmeyer flasks filled to 15% of the total volume. Growth was recorded either by the increase in optical density at 680 nm or by the amount of cellular protein [16].

2.2. Assay protocols for screening

Two assay methods were used to screen for lipolytic activity: a liquid system (microplates) and a solid system (agar plates). In the liquid system, the broth of 7-day tube cultures was centrifuged at 15000*g* and 4 °C for 10 min, and an aliquot of supernatant was removed to check extracellular activity. A colorimetric method (described below, [17]), adapted for 96-well polystyrene microplates (Greiner), was run at room temperature. The 200 μl of reaction mixture in each well contained 1 mM *p*-nitrophenyl ester, 1% isopropanol, 40 mM Tris–HCl, pH 8, NaCl 2.5 M and supernatant. The absorbance was monitored continuously for 5 min at 405 nm using a BioRad microplate reader 550. The molar extinction coefficient of *p*-nitrophenol under the assay conditions was $8.135 \times 10^3\ M^{-1}\ cm^{-1}$. The solid system was based on the spread plate technique [18]. Sterilised medium (NaCl 25% w/v) was supplemented with 2.5% olive oil (w/v) at 60 °C. The mixture was then homogenised in a blender, Ultra Turrax T25 (IKA-Labortechnik), at 9500 rpm for 5 min and 0.001% rhodamine B (w/v; Sigma) was added at 52 °C before pouring plates. Cultures were grown at 37 °C for 15 days and positive colonies were identified by the presence of an orange–red halo under UV light. The supernatant and pellet from the above-mentioned centrifugations were also examined in wells made in rhodamine B plates. The pellet was disrupted by resuspending it in distilled water without NaCl before being added to the well, the aim of this test being to detect cell-bound activity. Amano lipase PS (Amano pharmaceuticals, Japan) was used as a positive control. The olive oil hydrolysing activity in culture supernatants were also tested for strains TC6 and S250. Enzymatic reaction was initiated at 40 °C by adding culture supernatant to the olive oil emulsion (1:1 v/v). The buffer used was 40 mM Tris–HCl, pH 7.5 and the final concentration of NaCl was adjusted at 4 M. Fatty acids released during hydrolysis were recovered in isooctane phase and absorbency at 715 nm was monitored, after the addition of copper acetate-pyridine reagent (pH 6.1), in a spectrophotometer (Perkin–Elmer Lambda-5). The assay protocol including preparation of emulsion and reagent was the same as described by Kwon et al. [19].

2.3. Phylogenetic analysis

The 16S rRNA gene was amplified by PCR using total DNA as the template and primers of archaea: 21F 5′-TCCGGTTGATCCTGCCGG-3′ and RD1 5′-AAGGAGGTGATCCAGCC-3′. The amplified DNA was sequenced by Genome Express SA, Grenoble, France.

2.4. Physiological studies

To understand the correlation between growth and the production of extracellular lipolytic activity, strain TC6 was grown in 100 ml shake flasks in the standard medium under the desired combination of salt concentration, pH and temperature. Pre-cultures for inoculation were grown under the same conditions as the main culture. All experiments were run in duplicate. Growth was monitored at 680 nm and expressed as cellular protein using an experimentally determined standard curve.

The hydrolytic activity in supernatants recovered from samples was determined according to Kordel et al. [17], using PNP-esters of butyrate (PNPB for esterase) and palmitate (PNPP for lipase) (Sigma France, Saint Quentin). To 1 ml enzyme solution equilibrated at 40 °C in a 3 ml plastic cuvette was added 0.5 ml of the working substrate solution of PNPB or emulsion of PNPP. Working solution contained: 1 ml of 15 mM substrate in 2-propanol and 9 ml of 66.67 mM Tris–HCl buffer, pH 8. For the PNPP emulsion the Tris–HCl buffer in addition was supplemented with 0.706% (w/w) Triton and 0.176% (w/w) arabic gum. The rate of hydrolysis at 40 °C was monitored continuously at 410 nm in a spectrophotometer (Schimadzu UV-1205) against a blank without enzyme. One enzyme unit was defined as the amount of enzyme that liberates 1 μmol of PNP min^{-1} (extinction coefficient: 12.75×10^3 M^{-1} cm^{-1}). All enzyme assays were run using 3 M NaCl and were done in duplicate using at least three different enzyme concentrations.

3. Results

3.1. Screening of isolates

Thirty-five strains were selected from the Algerian collection [14] for their ability to grow at neutral pH and at 3–5.2 M NaCl and were screened for their potential to produce esterase (PNPB) and lipase (PNPP) activity. No cell-bound activity was detected. On the other hand, a significant lipolytic activity was observed in the culture supernatant for 29 strains using the microplate system (Table 1). All 29 active strains hydrolysed PNPP, but only 22 of these hydrolysed PNPB, suggesting an absolute specificity for long chain fatty acid esters for 7 strains. Two strains (SS1 and S241) hydrolysed PNPB faster than PNPP. Eleven of the 22 strains that hydrolysed PNPB had relatively low activities (15–33% of the highest PNPB hydrolysis activity), whereas 13 out of the 29 strains active on PNPP had relatively high activities (>60% of the highest PNPP hydrolysis activity). Surprisingly, most of the strains that showed activity against PNPP had lag times of 10–100 min before the start of the hydrolysis reaction: the reaction started immediately for only 8 of the strains. To further investigate this lag phenomenon, we selected 6 strains and cultured them in 100 ml Erlenmeyer flasks in order to improve aeration during growth. We observed that the doubling time was halved and enzyme production was increased approximately 2-fold. In addition, no lag phase was observed, suggesting that the lag phase depends on the amount of activity in the culture. It was also noticed that repeated culturing with good aeration improves enzyme production whereas storage of cultures at 4 °C over 15 days decreases the production of PNPP hydrolysis activity by the cells.

To confirm that the lipolytic activity detected by PNPP hydrolysis was true lipase activity, the olive oil agar plate assay was run (Table 1). Olive oil was hydrolysed by only 4 of the PNPP-hydrolysing strains, under

Table 1
Lipolytic activity of halobacterial isolates

Strain	Substrates: liquid system			Agar plates
	PNPB (U/l)	PNPP (U/l)	Lag phase (min)	Olive oil
S07	4.2	13.0	72	–
SS9	6.2	10.5	0	–
SS8	6.6	9.4	72	–
SO4	4.0	9.2	10	–
S247	3.8	9.2	0	++
S250	2.0	7.9	0	++
SS1	20.0	7.8	10	–
TC3	0.0	5.5	0	–
S251	1.8	5.3	102	–
SE21	3.0	10.9	72	–
SS6	1.4	5.3	10	–
TC6	4.0	10.8	0	++
SE19	2.6	5.3	72	–
SS4	0.0	4.7	10	–
SO5	3.0	3.9	10	–
SE20	1.2	3.9	72	–
SS2	0.0	2.7	72	–
S241	15.6	9.1	0	–
IS2	0	2.1	10	–
SS11	2.8	8.7	72	–
SE12	3.6	1.2	10	–
SS10	ND	8.3	102	–
SS3	3.8	1.0	10	–
SS7	3.4	8.1	72	–
M21	2.6	1.0	10	–
SS5	0	0		–
SE16	0	0		–
SE17	2.8	6.9	42	+
S248	0	0		–
TC7	0	6.0	10	–
SE22	0	0		–
S252	1.8	6.0	0	–
S256	0	0		–
TC1	0	5.6	0	–
SE18	0	0		–

ND: activity not determined; ++: very clear halo; +: clear halo; –: no halo formed around colony and around microplate wells filled with supernatant. Results are means of two independent assays.

the conditions of the assay. Strain TC6 was selected for further work because it hydrolysed all three substrates, it showed a high activity on PNPP (11 U/l) and it did not show a lag phase prior to PNPP hydrolysis. Cell free extracts from this strain were able to hydrolyze olive oil at 4 M NaCl with activities of 240 and 220 U/l for strain TC6 and S250, respectively. In our opinion, this is the best demonstration that the detected PNPP hydrolysis activity is due to a true lipase activity.

3.2. Strain identification

The phylogenetic position of strain TC6 was determined by 16S rRNA analysis. The complete gene sequence obtained was 1475 nucleotides in length (GenBank, DQ008591), which is comparable to 16S rRNA genes of other halophilic archaeon [20]. It had the 19 nucleotide-long "signature sequence A" of the genus *Natronococcus* [21], positioned 101 nucleotides downstream from the 5′ end (GCCAAACTACCCTCTGGAG), suggesting that strain TC6 belongs to this genus. The G + C mol% of the 16S rRNA gene sequence in strain TC6 was 58.9%. This figure is comparable to the calculated values, 58.8% and 58.2%, obtained from reference sequences of *N. occultus*, strain NCMB 2192, and *N. amylolyticus*, strain Ah-36, respectively. Further, the16S rRNA gene sequence of TC6 was compared by alignment to known sequences (databases: GenBank, EMBL and DDBJ), using BLASTQ2 method [22]. A phylogenetic tree was constructed by neighbour-joining method (Fig. 1). Strain TC6 differed from *N. occultus* (only 96.6% similarity) but it was related closely to *N. amylolyticus* (98.2% similarity) and to *N. xinjiangense* (98.8% similarity, upublished specie).

Morphologically, cells were coccoid, 1–2 μm size, occurred as single motile cells or in pairs during the early growth phase and later formed irregular clusters (Fig. 2). In salt saturated medium they often colonises salt crystals. The colonies were smooth, circular, 1–4 mm in diameter and had bright red colour. The TC6 did hydrolysed starch but failed to degrade gelatine. Morphological and biochemical features have suggested

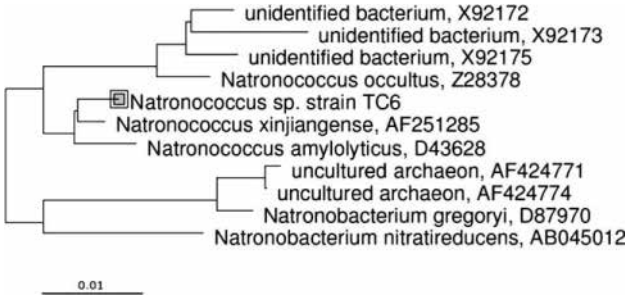

Fig. 1. 16S rRNA tree showing the phylogenetic relationships (neighber-joining method) between strain TC6 and other known sequences of *Natronococcus* sp.

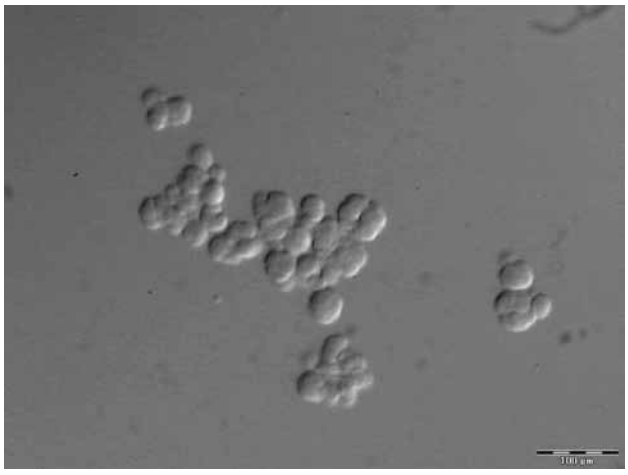

Fig. 2. Phase-contrast micrograph of TC6.

that this strain is related to *N. amylolyticus* [20,21]. However, further work on DNA–DNA homology and biochemical composition of cells, particularly polar lipid pattern are under investigation for the determination of the specie.

3.3. Production of lipolytic activity during growth

To investigate the effect of the growth conditions on the production of extracellular lipolytic activity, strain TC6 was grown at various salt concentrations, pH and temperatures. It was unable to grow at NaCl concentrations below 2 M (Fig. 3). The maximum specific growth rate initially increased with salt concentration, reaching 0.076 h^{-1} at 3.5 M NaCl. At higher salt concentrations the growth rate decreased, but the strain still grew at 5 M NaCl, which represents saturation under the conditions used. The maximum activities for both PNPB and PNPP hydrolysis, obtained at different fermentation times depending on the NaCl concentration used, followed similar patterns, with maxima of 33 U/l at 3.5 M and 49.8 U/l at 4 M, respectively. Interestingly, the ratio of PNPB to PNPP activities decreased slightly with salt concentration, suggesting that the high salt concentration favours the production of lipase activity over the production of esterase activity.

The known species of *Natronococcus* are haloalkalophiles and they require the presence of sodium carbonate in the medium to maintain an alkaline pH during growth. In the present work, it was noticed that the pH of culture medium increased slightly during growth (from pH 7.5–7.8). Hence, sodium carbonate addition was not necessary. Furthermore growth and activity remained unaffected by the presence or absence of sodium carbonate.

The effect of pH on growth rate and production of esterase and lipase activities was investigated using

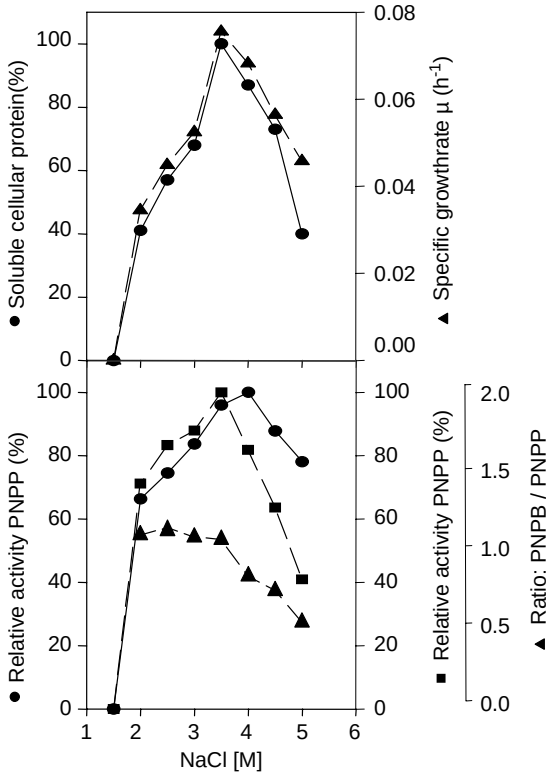

Fig. 3. Effect of NaCl concentration on the growth and lipolytic activity of strain TC6, grown in 100 ml shake flasks at 40 °C and pH 7.5. The percent cellular proteins and the relative activity were defined as the percentage of the maximum value obtained respectively in the assay. Data are the average of two independent experiments (assay conditions: NaCl 3 M, pH 8, 40 °C).

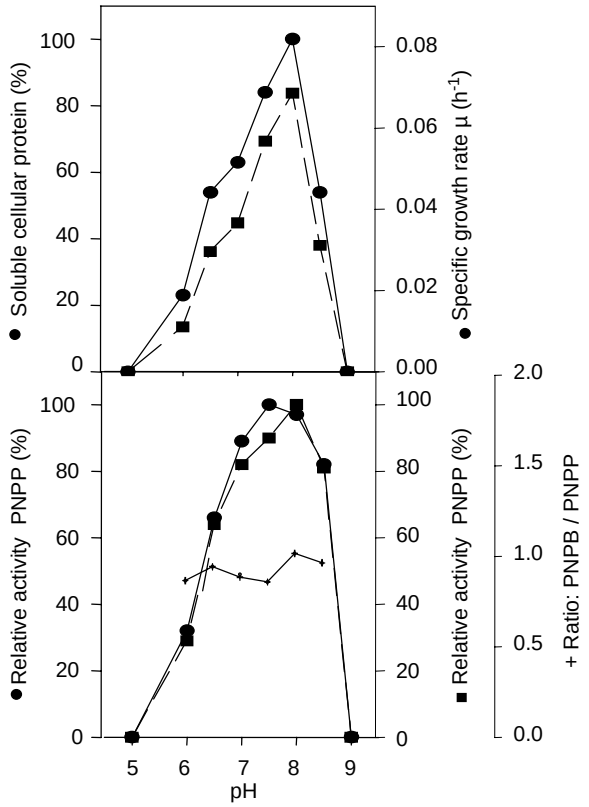

Fig. 4. Influence of pH on the growth and lipolytic activity of TC6 at 40 °C. The medium contained 3.5 M NaCl and was buffered with 100 mM Tris–HCl. For other explanations, see Fig. 1.

standard medium buffered with 100 mM Tris–HCl within a pH range of 5–11 (Fig. 4). Strain TC6 was able to grow well from pH 6 to 8.5. Growth at acidic pH was extremely slow (doubling time 62 h) whereas cell lysis occurred at and above pH 9. The specific growth rate initially increased with pH, forming a sharp peak at pH 8 before falling at higher pH values. Lipolytic activity was produced over the full pH range where growth occurred. A relatively high level of activity (85–100%) was measured within the pH range of 7–8.5. Esterase activity was highest at pH 8 and lipase activity at pH 7.5.

In highly saline medium, the solubility of oxygen, water availability and viscosity are highly affected by temperature. The combined effect of temperature (range 20–50 °C) and NaCl (range 2–5 M) on growth rate and production of lipolytic activity were studied. Results are shown in Figs. 5 and 6. Strain TC6 grew at a temperature range of 25–50 °C, but there was no growth in the presence of 5 M NaCl at the two extreme temperatures tested (25 and 50 °C). The optimum temperature for growth was 40 °C, with the temperature peak being sharper for cultures containing 3–5 M NaCl and broader for cultures containing 2 M NaCl (Fig. 5). The doubling time calculated at 35 °C with 3–5 M NaCl was 2-fold higher than the one obtained at 40 °C. The dependence of PNPB and PNPP hydrolytic activities on

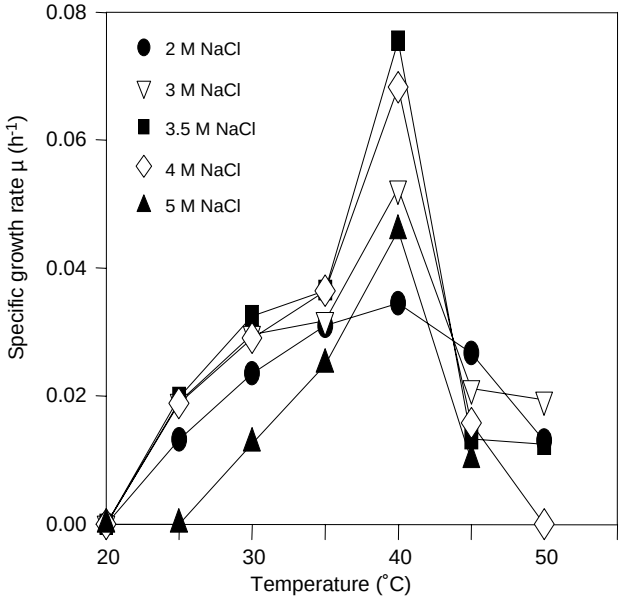

Fig. 5. Temperature dependence of the specific growth rate of strain TC6 at different NaCl concentrations at pH 7.5. Results are averages of two independent assays with standard deviations of less then 8%.

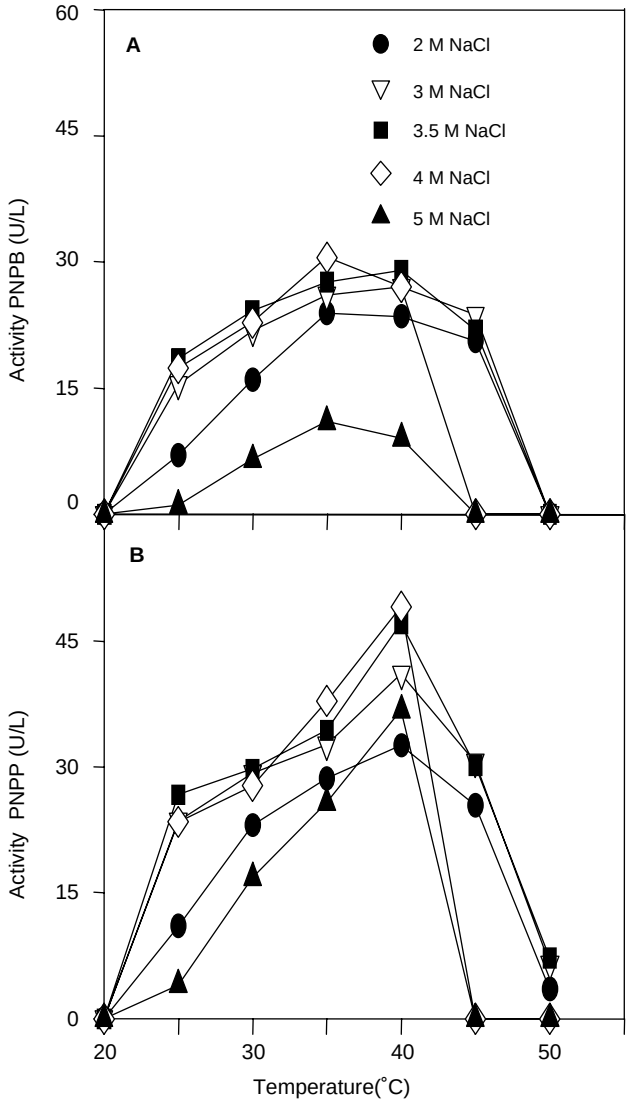

Fig. 6. Combined effect of temperature and salt concentrations on the profile of lipolytic activity of strain TC6 (A: PNPB and B: PNPP). The pH of the flask culture was 7.5. Results are averages of two independent assays.

temperature for different salt concentrations followed patterns similar to those of the specific growth rate, with production peaks at 35–40 °C (Fig. 6). The peaks for PNPB hydrolytic activity were fairly symmetrical around the optimum (Fig. 6A) while those for PNPP hydrolytic activity were skewed (Fig. 6B). At 50 °C both PNPB and PNPP hydrolytic activities were absent at salt concentrations of 4 and 5 M and very low at lower salt concentrations. At 45 °C both activities were absent with 4 and 5 M NaCl.

4. Discussion

Lipase and esterase are important industrial biocatalysts [23–26]. The search for new hydrolases active in extreme conditions is very attractive. They can provide distinct advantages over their classical counterparts in the development of new bioconversion processes, potentially providing resistance to high salt conditions or high temperatures and enabling the use of organic solvents at low water activity [27,28]. Therefore, it is very promising to screen microorganisms living in hostile environments for catalysts having new biochemical properties. We have screened new isolates of *Halobacteria* from an Algerian collection. Interestingly, extracellular lipolytic activity was detected in strains collected from a hypersaline lake flooded regularly with agriculture drainage water (palm grower fields with vegetables, legumes and some cash crops) and domestic wastes. At least 83% of these strains hydrolysed both short and long acyl-chain PNP-esters. This is in agreement with the sparse data in the literature, though the substrates and methods used were of different sensitivity. Gonzalez et al. [12] found that 66% of their 53 natural isolates of *Halobacterium* had lipolytic activity on Tween 20–80 at 4 M NaCl when analysed in a solid phase assay. In a recent study, Sanchez-Porro et al. [29] isolated hydrolase-producing moderately halophilic and halotolerant eubacteria from Spanish slatterns. Only 23% of the 892 strains they isolated produced extracellular lipolytic activity when screened on Tween 80 (1.7 M NaCl). In the present work, 24% of the positive strains were selective for PNPP and 76% were active on both PNPP and PNPB. Similarly, at least 80% of the positive strains obtained by Gonzalez et al. [12] hydrolysed 3 or 4 substrates (Tween 20–40), whereas 3% hydrolysed either only Tween 20 or only Tween 40. This indicates low biochemical diversity among extremely halophilic archaeon [30]. On the contrary, for mesophilic soil streptomycetes, Gandolfi et al. [31], using three substrates (Tributyrin, Tween 60 and Triolein), observed a certain diversity among the esterase and lipase activities produced by their screened isolates: 83% out of 185 positive strains were selective for one substrate, 65% preferentially attacked the short carbon chain, 16% were active on two substrates, and only 2.7% hydrolysed the three substrates. In our case, the lipolytic activity was assayed on *p*-nitrophenyl esters, i.e. butyrate for esterase activity and palmitate for lipase activity. Since there are some controversies on the use of these substrates to detect "true" lipase activity, we have confirmed the presence of a lipase activity by hydrolysis of olive oil on both agar and emulsified oil. To our knowledge, it is the first report of a true lipase activity at high salt concentration.

In the present study the conditions for growth and production of lipolytic activity by strain TC6 were investigated. Morphological and phylogenetic observations revealed clearly that strain TC6 belongs to the genus *Natronococcus*. The 16S rRNA gene sequence analysis related it to *N. amylolyticus* strain Ah-36^T with 98.2% similarity. However, physiologically this strain is different

from other reported isolates of the genus *Natronococcus* [20,21]: contrary to these isolates, strain TC6 grows well at high Mg^{2+} concentration ($\geqslant 10$ mM, results not shown) and its cells disintegrated at alkaline pH $\geqslant 9$ (the specific death rate at pH 9 was 0.023 h^{-1}, results not shown). It was extremely halophilic, growing at NaCl concentrations ranging from 2 to 5 M. The doubling time under standard growth conditions (NaCl 3.5 M, 40 °C) was 9 h, corresponding to the values observed for most halophilic archaeon: 8–12 h [32]. Like most halophilic archaeon, strain TC6 did not grow at 20 °C. The salt requirement of halophilic bacteria is believed to be under temperature control [32,33]. In agreement with this, we observed that an increase in temperature shifted the salt range giving near optimum growth from a wider range (2–4 M) at 25–30 °C to a narrower range (3.5–4 M) at 35–40 °C. At 45 °C the optimum salt concentration was 2 M. In liquid culture esterase and lipase activity production followed growth, reaching maximum values during early stationary phase and then remaining stable. The salt, temperature and pH requirement for the enzyme synthesis was correlated with optimum growth conditions, although the optimum conditions for esterase production (pH 8, NaCl 3.5 M) were slightly different from those for lipase production (pH 7.5, NaCl 4 M), although the optimum temperature for both was 40 °C.

Acknowledgements

This work was partly supported with the grant from French Ministry of Foreign Affairs under agreement on scientific cooperation with Algerian Ministry of Education, University of Sciences and Technology Houari Boumedienne, Algiers – Project No. CMEP: 00 MDU 470. The authors wish to thank Dr. David Mitchell of the Federal University of Paraná, Brazil, for help with the English expression in this manuscript and Dr Mohamed Barakat, DEVM, Commissariat à l'Energie Atomique, Cadarache, France for his help in the preparations of illustrations.

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