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Preliminary characterisation of a lipolytic activity from an extremely halophilic archaeon, *Natronococcus* sp.

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Abstract

A crude preparation obtained from an extremely halophilic archaeon, *Natronococcus* sp. strain TC6, hydrolysed olive oil, indicating the presence of a true lipase. This preparation was partially characterised using *p*-nitrophenyl palmitate (pNPP). It hydrolysed pNPP optimally at 4 M NaCl, with no activity in the absence of salt. With 4 M NaCl, activity was maximal at 50 °C and pH 7. The activity was highly thermostable, with more than 90% of original activity being retained when incubated for 60 min at 50 °C. Salt affected the thermostability. The residual pNPP-hydrolysing activity fell to 50% in 76 min when incubated at 80 °C with 4 M NaCl, and in 35 min when incubated without NaCl. The preparation preferentially hydrolysed pNP-esters of long-chain fatty acids (C10–C18), having maximum activity on pNPP (C16). The activity was inhibited by PMSF, indicating the presence of a serine residue in the active site. These results strongly suggest the presence of a true lipase and therefore, this archaeon deserves further attention, since true lipases have not previously been reported in the archaea.

Keywords: Halophile; Lipase; Archaeon; *Natronococcus*; Thermostability

1. Introduction

Lipases find many applications in biotechnology, ranging from the hydrolysis of fats in wastewaters to the synthesis of chiral intermediates of important pharmaceuticals [1,2]. These applications often require aggressive reaction conditions. For example, wastewaters may contain high salt contents, while in biocatalysis high temperatures may be required either to favour stereoselectivity or simply to solubilise high-melting point lipids used as substrates [3,4]. Further, biocatalysis with lipases is often carried out within organic solvents in order to promote synthesis reactions by providing a low water environment. Many lipases lose their activity rapidly under these conditions, and therefore, there is a continuous effort to obtain lipases that are more resistant.

There are two main strategies for obtaining lipases with improved resistance [5], namely the genetic engineering of currently known enzymes and the search for new activities in previously uncharacterised microorganisms. Within the latter approach, the search for lipases in extremophiles seems to be particularly promising since the enzymes of these organisms have particular adaptations to increase stability in adverse environments, which can potentially also increase their stability in the harsh environments in which they are to be applied in biotechnology [6,7].

Archaebacteria are the dominant habitants of extreme environments and include many extreme thermophiles and halophiles. In contrast to the very large number of lipolytic enzymes that have been characterised from the members of the eukaryotic and eubacterial domains, most of which are of mesophilic origin, there has been relatively little effort to search for lipases in the archaea, despite the potential advantages that lipases from extremophilic archaeons might have. The work that has been done with the archaea has focused on the characterisation of esterases from thermophiles. For example, thermostable esterases have been purified and studied from the extremely

Abbreviations: Na-lip, *Natronococcus* sp. lipase; pNP, *p*-nitrophenol; pNPB, *p*-nitrophenyl butyrate; pNPP, *p*-nitrophenyl palmitate

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thermophilic archaeons *Sulfolobus acidocaldarius* [8,9], *S. shibatae* [10], *Pyrococcus juriosus* [11], *Archaeoglobus fulgidus* [12], *S. solfataricus* [13] and *Pyrobaculum calidifontis* [14]. With respect to lipolytic activity in archaea, Gonzalez and Gutierrez [15] showed clearing by isolates of *Halobacterium* on agar–gelatine plates containing Tween 20–80 and $CaCl_2$ but did not characterise the activity any further. The only two reports since then did not demonstrate activity against long-chain triacylglycerides, the substrates for true lipases: an enzyme cloned from *S. acidocaldarius* hydrolysed pNPP and tributyrin but not triolein [8], while an enzyme cloned from *A. fulgidus* was only tested against the shorter chain substrates tributyrin, tricaprylin and *p*-nitrophenyl caproate [16]. Therefore, the recent observation of Hotta et al. [14] that 'as for archaea, a lipase has yet to be identified' still holds true.

The current work represents the continuation of work that we have recently undertaken into the search for lipase activity in halophiles. We previously isolated 54 strains of *Halobacteria* from an aquatic hyperhalobe environment (296 g/l dissolved salt), the Sebkha of El Golea (a shallow salt lake), located in the middle of Algerian Sahara [17]. Thirty-five of these strains were screened for lipolytic activity, with strain TC6, identified as belonging to the genus *Natronococcus*, being selected for further work on lipase production [18]. The current paper describes a preliminary characterisation of the pNPP-hydrolysing activity of a crude preparation obtained from this strain.

2. Materials and methods

2.1. Strain, growth conditions and production of the enzyme preparation

Strain TC6 was routinely grown in modified Gibbons medium [19] consisting of (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. The pH was adjusted to 7.5 prior to autoclaving. Agar plates contained 2% (w/v) agar. Liquid cultures were grown on a reciprocal shaker (300 strokes min^{-1}) at 40 °C in 250 ml Erlenmeyer flasks filled with 50 ml of medium. Growth was monitored as the increase in the density of cellular proteins. The broth of a culture grown in 4 M NaCl for 5 days was centrifuged (Sorvall RC5C, 10,000 × g) at 4 °C for 15 min. The supernatant was dialysed against 10 mM Tris–HCl buffer, pH 7 at 4 °C to remove NaCl (Spectra/pore 4 membrane tubing, 12–14,000). This dialysed preparation was denominated "crude preparation" and was reactivated by incubation with the desired concentration of NaCl at room temperature for 5 min or on ice for 10 min.

The protein contents in whole cells and in the crude preparation were determined by the method of Bradford [20] with bovine serum albumin as the standard.

2.2. Enzyme assays

The pNPP-hydrolysing activity was determined according to Kordel et al. [21], with the "standard assay" being done at 40 °C with 4 M NaCl and at pH 8. Prior to the preparation of the substrate emulsion in Tris–HCl buffer, sufficient pNPP was dissolved in 2-propanol to give a final concentration in the reaction mixture (1.5 ml total volume) of 0.5 mM. The emulsion was prepared in 40 mM Tris–HCl (Sigma, France). Final quantities of emulsifying agents in the reaction were adjusted to 0.06% arabic gum (w/v, Sigma, France) and 0.36% Triton X-100 (w/v, Fluka–Sigma, France). Emulsifying agents were not added for the soluble substrates with C2–C8 fatty acids. The rate of hydrolysis for fatty acids was monitored at 410 nm using a spectrophotometer (Shimadzu UV-1205) against a blank with dialysed non-inoculated medium. One unit (U) is the amount of enzyme that liberates 1 μmol of *p*-nitrophenol min^{-1}. The molar extinction coefficients of pNP at pH 6, 7, 8, 9 and 10 were 6.52×10^3, 8.28×10^3, 12.75×10^3, 13.41×10^3 and 13.86×10^3 cm^{-1} M^{-1}, respectively.

To test for true lipase activity [22], the dialysed crude preparation was assayed using the previously described olive oil assay [18] with determination of liberated fatty acids with copper salts.

All enzyme assays were done independently in duplicate using at least three different enzyme concentrations. Sigma Plot software (SPSS Science Software, Erkath, Germany) was used for statistical analysis.

3. Results

3.1. Profile for production of pNPP-hydrolysing activity in shake flask culture

Strain TC6, cultured at 40 °C with 4 M NaCl in modified Gibbons medium in shake flasks, grew exponentially for the first 26 h, with a specific growth rate of 0.068 h^{-1} (Fig. 1). Growth decelerated between 26 and 60 h, after which the culture entered into a stationary phase. The pNPP-hydrolysing activity in the culture broth was very low in the early exponential phase, but then increased rapidly from 4 U/l at 14 h to 41.9 U/l at 36 h. The rate of increase in pNPP-hydrolysing activity then slowed, reaching 49.0 U/l at 60 h, after which it remained essentially constant.

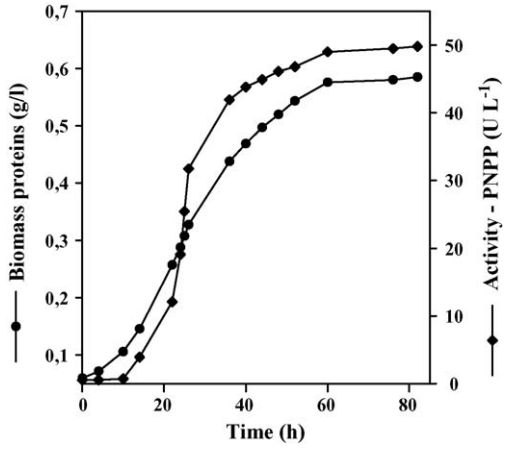

Fig. 1. pNPP-hydrolysing activity in the culture supernatant during growth of *Natronococcus* sp. strain TC6 cultured in the presence of 4 M NaCl at 40 °C, pH 7.5. Enzyme assays were done at 40 °C, pH 8 and NaCl 4 M.

The production of activity was associated to cell growth with a constant ratio of 0.012 ± 0.001 units per gram of biomass for the period 22–82 h.

The crude preparation obtained from a 5 days shake flask culture had a pNPP-hydrolysing activity of 0.371 U/mg and hydrolysed olive oil with an activity of 2.09 U/mg, both in the presence of 4 M NaCl. These results clearly demonstrate the presence of a true lipase activity in the preparation. Attempts to purify further the enzyme responsible for the lipolytic activity were not successful due to the very low enzyme concentration in the culture broth and difficulties encountered in attempts to concentrate the activity. Most of the activity was lost upon concentration by ultrafiltration most probably due to enzyme inactivation by adsorption on the membrane or to enzyme aggregation (usual for lipases). Concentration using an ion exchange resin was also attempted and not successful. The current investigation on the pNPP-hydrolysing activity of the crude preparation was undertaken to ascertain whether future work, to isolate the gene and express it in another organism, would be justified.

3.2. Effect of salt concentration, pH and temperature on pNPP-hydrolysing activity

The influence of NaCl concentration on pNPP-hydrolysing activity was tested over the range of 0–5 M (Fig. 2) using a dialysed enzyme preparation. There was no activity in the absence of added NaCl. The activity increased with salt concentration up to 4 M NaCl, and then decreased again: at 5 M NaCl, the activity was 75% of that at 4 M NaCl. This result clearly indicates that the lipase activity is salt dependent. The effect was not specific for NaCl since the addition of KCl to the crude preparation also restored activity. For example, by addition of 4 M KCl, the activity was 82% of that observed with 4 M NaCl when samples were reactivated in ice bath for 10 min. No activity was detected at KCl concentrations below 1 M. It should be noted that the activity was restored immediately with both salts but for obtaining reproducible results, a short period of incubation was required depending on the temperature (results not shown).

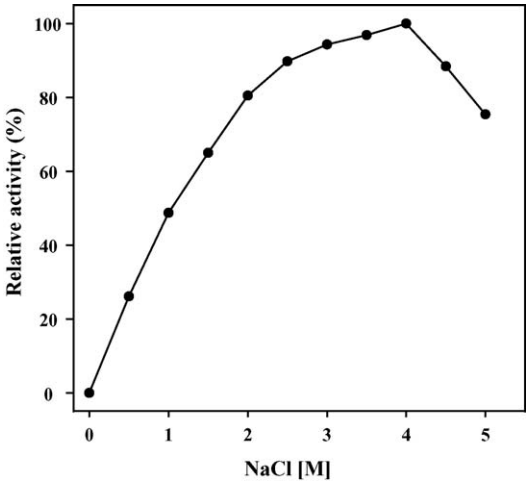

Fig. 2. Effect of NaCl concentrations on pNPP-hydrolysing activity of the dialysed preparation obtained from strain TC6. Assays were done at 40 °C, pH 8.

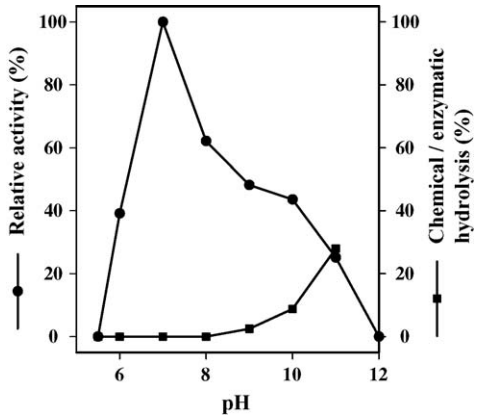

Fig. 3. Influence of pH on the hydrolysis of pNPP in the presence of 4 M NaCl at 40 °C. Plot with circular symbol shows activity of crude preparation of strain TC6 and square symbol shows chemical hydrolysis presented as the percent of the enzymatic hydrolysis.

Before determining the effect of pH on pNPP-hydrolysing activity of the crude preparation at 4 M NaCl, two checks were undertaken. Firstly, it was shown that the addition of NaCl up to 4 M did not affect the molar extinction coefficients for pNP at different pH values. Secondly, the rate of chemical hydrolysis of pNPP was determined at 4 M NaCl and 40 °C, and at various pH values in the range 5.5–11 (Fig. 3). Chemical hydrolysis occurred above pH 8. However, it represented a maximum of 28%, at pH 11, of the enzymatic rate and therefore did not interfere too much in the assay. At pH 5.5, no activity was detected, but as the pH was increased the activity increased rapidly, reaching a maximum at pH 7. Above pH 7 the activity decreased, although at pH 10, it was still over 40% of that at pH 7. Fig. 4 shows the effect of temperature on the pNPP-hydrolysing activity at 4 M NaCl and pH 7, the maximum activity occurred at 50 °C, falling sharply on both sides. However,

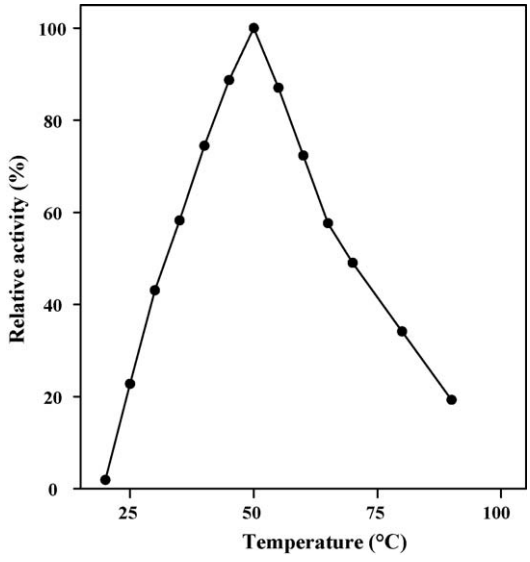

Fig. 4. Temperature dependence of the pNPP-hydrolysing activity of the dialysed preparation obtained from strain TC6. Assays were done with 4 M NaCl at pH 7.

the preparation was still active at 90 °C (21% of the total activity) which probably reflect a good stability of the enzyme.

3.3. Stability of the pNPP-hydrolysing activity

The pNPP-hydrolysing activity was stable for at least 24 h when the crude preparation was incubated in 4 M NaCl at 4 °C. In fact, the stability was remarkably high at neutral pH since no loss in activity was found after 6 months and only 40% was lost after 9 months. The effect of temperature on the stability of pNPP-hydrolysing activity was studied in greater detail. Crude preparation was incubated with and without 4 M NaCl for 1 h at pH 7, at temperatures from 0 to 98 °C (Fig. 5), the activities prior to and after incubation being determined in the standard assay. The pNPP-hydrolysing activity was quite stable at 50 °C, with residual activities of 90 and 85% for incubation with and without 4 M NaCl, respectively. Even at 70 °C the residual activity after the incubation was above 60%, with only a minor influence of NaCl. However, at higher temperatures NaCl had a significant effect, as demonstrated by the divergence of the two profiles. For example, when the crude preparation was incubated for 1 h at 80 °C in the presence of NaCl, the residual activity was 48%, double the residual activity (23%) obtained without NaCl. No activity was detected in the salt free preparation incubated at 98 °C for 1 h while the preparation incubated with NaCl still had a residual activity of 29%. These results clearly show that the stability of the lipase activity to temperature was high and it was increased by NaCl addition.

The kinetics of decay of pNPP-hydrolysing activity during incubation of the crude preparation at 80 °C were studied in the presence and absence of 4 M NaCl (Fig. 6). Residual activity remained above 95% for the first 15 min in the absence of NaCl and for the first 30 min in the presence of 4 M NaCl. Since decay profiles are not first order, there is no characteristic constant that represents the half-life, although it is useful to compare the times necessary for the residual activity to fall to 50% of the original value, these being 35 min for incubation with 4 M NaCl and 76 min for incubation in the absence of NaCl. These results show the high thermal stability of the lipase activity and the important stabilising effect of NaCl addition.

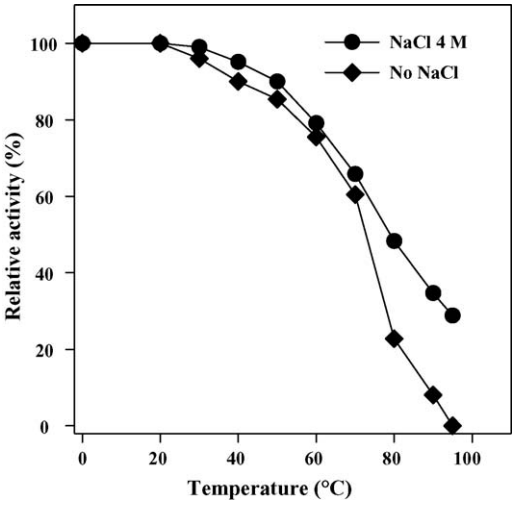

Fig. 5. Thermostability profile of the pNPP-hydrolysing activity of the dialysed preparation obtained from strain TC6. Samples were heated at the designated temperature for 1 h, without or with 4 M NaCl. Assays were done with 4 M NaCl at 40 °C, pH 7.

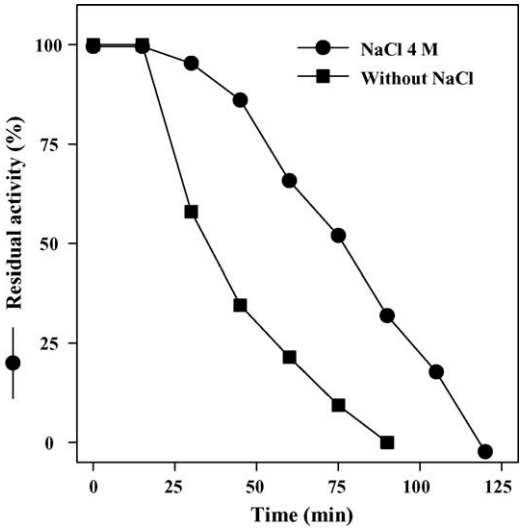

Fig. 6. Kinetics of the loss of pNPP-hydrolysing activity during incubation of the dialysed preparation obtained from strain TC6, at 80 °C, without or with 4 M NaCl. Assays were done with 4 M NaCl at 40 °C, pH 7.

3.4. Substrate specificity and sensitivity toward inhibitors

Fig. 7 shows the relative rates of hydrolysis of esters of pNP with acyl chain lengths from C2 to C18 in the presence of 4 M NaCl. The highest activity was obtained against pNPP (C16, 52 U/l). The activity on *p*-nitrophenyl myristate (pNPM, C14) was 94% of this value. Relative activities of approximately 50% or more were observed for other long-chain substrates (C10, C12, C18) and for one short-chain substrate C4 (pNPB). On C2 (acetate), the hydrolytic activity was 10 times less than on pNPP. These results confirm the lipase nature of the enzyme, with it being more active on long-chain fatty acid esters.

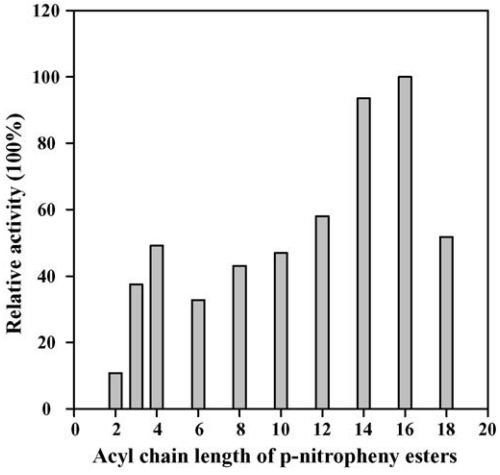

Fig. 7. Relative rates of hydrolysis of pNP-esters by the dialysed preparation obtained from *Natronococcus* sp. strain TC6. Assays were done with 4 M NaCl at 40 °C and pH 7.

Table 1
Effect of inhibitors on pNPP activity

Inhibitor	Concentration (mM)	Residual activity (%)		
		Room temperature		40 °C, 90 min
		60 min	90 min	
PMSF	1.0	52	43	9
Eserine	1.0	97	87	–
EDTA	0.5	90	87	–
DTT	1.0	105	–	–
$HgCl_2$	1.0	106	–	–
SDS	0.5	100	100	–
	1.0	100	–	–

Conditions: Assay and control were run independently in duplicates using dialysed crude preparation (Tris–HCl buffer, pH 7.2) with added NaCl 4 M.

The effect of lipase inhibitors on the pNPP-hydrolysing activity was tested in the presence of 4 M NaCl using the standard assay (Table 1). One millilitre of PMSF inhibited nearly all initial activity, after incubation for 90 min at 40 °C, suggesting the presence of a serine group in the active site. The other inhibitors like inhibitors of metaloenzymes, thiol-reducing agents, and anionic detergent like SDS had no significant effect on the pNPP-hydrolysing activity produced by strain TC6.

4. Discussion

In the current work, we have shown that a crude preparation from the newly isolated strain, *Natronococcus* sp. (TC6), hydrolyses olive oil in addition to pNPP in the presence of high NaCl concentrations, indicating the presence of a true lipase in the preparation used. To our knowledge this is the first time that hydrolysis of olive oil by extracts from extremely halophilic archaeons is reported. This result broadens the field of lipase producing microorganims. Given the potential of lipases as tools in biocatalysis, our work suggests that it is interesting to search for lipases in other extremely halophilic archaeons. Certainly there is current interest in the production of other enzymes from halophiles, both archaeal and eubacterial, given the possibility that enzymes adapted to high salt concentrations will have high activities and stabilities when used for biocatalysis in organic solvents [23] as has been shown for the alkaline phosphatase of *Halobacterium salinarium* [24].

The lipase activity of preparations obtained from strain TC6 was highest at 4 M NaCl and totally absent in the absence of salt. This result is quite interesting since it demonstrates that the protein responsible for pNPP hydrolysis is completely adapted to high salt concentrations and probably optimised, at the molecular level, to be fully efficient only when salt is present in high amount. In fact, many halophilic enzymes require the presence of NaCl or KCl concentrations in the range of 1–4 M for optimum activity and stability [25]. They can be divided into two groups on the basis of whether or not their activity is irreversibly lost upon exposure to low ionic strength. Most of the enzymes studied so far are inactivated irreversibly. For example, the β-galactosidase from *Haloferax alikantei* irreversibly loses its activity within minutes at NaCl concentrations below 0.5 M [26], as does the extracellular serine protease from *N. occulus* [27]. On the other hand, several halophilic enzymes regain their activity upon reestablishment of the high salt concentration. Such is the case for malate dehydrogenase [28], citrate synthase [29] and β-xylanase and β-xylosidase [30] of various extreme halophiles. The lipase activity of strain TC6 clearly falls into the latter group since the initial activity level was re-established by adding 4 M NaCl. This property is quite useful since, for instance, the enzyme purification can be run without salt using classical chromatographic methods such as ion exchange chromatography, and the activity assayed on line in the presence of salt after re-activation. On the molecular level, one may expect a high flexibility of the lipase protein allowing a fast change from the fully active form (in the presence of salt) to an inactive form (without salt).

The thermal stability of the pNPP-hydrolysing activity of strain TC6 was increased by the presence of NaCl. The effect of high salt concentrations on the stability of enzymes is somewhat variable in non-halophilic archaea. For example, NaCl decreased the thermostability of a carboxypeptidase from the thermoacidophilic archaeon *Sulfolobus solfataricus* [31] but increased the thermostability of the *N*5,*N*10-methylenetetrahydromethanopterin reductase of the hyperthermophilic archaeon *Methanopyrus kandleri* [32] and of the shikimate 5-dehydrogenase and the NADP(+)-specific glutamate dehydrogenase from the hyperthermophilic archaeon *A. fulgidus* [33,34]. Surprisingly, the thermostability of enzymes from halophilic archaeons has received relatively little attention. The present work represents the first report of improvement of the thermostability of an extracellular enzyme of a halophilic archaeon by the addition of NaCl. Similar results have already been shown for intracellular enzymes of halophilic archaeons: NaCl improved the thermostability of the glucose dehydrogenase of *Haloferax mediterranei* [35] and of the glutamine synthetase of *H. salinarium* [36]. Typically, the rate of an enzyme-catalysed reaction increases over the temperature range within which denaturation is slow, with the "temperature optimum of the enzyme" deriving from a combination of (1) the increase of the rate of the catalysed reaction with temperature, (2) the increase of the rate of enzyme denaturation with temperature and (3) the manner in which the experiment designed to test the effect of temperature on activity is carried out [37]. Previously characterised thermophilic enzymes perform as expected, with reported temperatures where activity is maximal being within 10 °C of the temperature at which the denaturation rate becomes significant. Such is the case for enzymes from several thermophilic archaea: esterases from *Pyrococcus furiosus* and *A. fulgidus* [11,12], malic enzyme of *Thermococcus kodakaraensis* [38] and a carboxylesterase of *P. calidifontis* [14].

In contrast, in the present study, the pNPP-hydrolysing activity had a clear temperature "optimum" at 50 °C even though the enzyme was fully stable up to 80 °C for at least 15 min, which is much longer than the duration of the activity assay. This phenomenon deserves more attention. The denaturation process can involve multiple steps, such as an initial reversible folding followed by irreversible denaturation [39]. Reversible refolding after high temperature treatment has been demonstrated for the

β-lactamase of the moderately halophilic eubacterium *Chromohalobacter* by Tokunaga et al. [40], who claimed that irreversible aggregation was prevented by the abundance of acidic amino acids, which in turn is a characteristic feature of halophilic enzymes [6]. There is an additional possibility that complicates interpretation of the data that must be considered for enzymes that act at interfaces within emulsions, namely the fact that the increase in temperature may have significant effects on the physicochemical properties of the emulsion and the interaction of the enzyme with it.

5. Conclusion

A dialysed preparation obtained from *Natronococcus* sp. strain TC6 hydrolysed olive oil, indicating the presence of a true lipase. The pNPP-hydrolysing activity of the preparation had optimum activity at 4 M NaCl and this activity was quite thermostable suggesting that is would be interesting to investigate potential applications in biocatalysis.

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