

# Functional expression and characterization of a chitinase from the marine archaeon *Halobacterium salinarum* CECT 395 in *Escherichia coli*

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**Abstract** The *HschiA1* gene of the archaeon *Halobacterium salinarum* CECT 395 was cloned and overexpressed as an active protein of 66.5 kDa in *Escherichia coli*. The protein called *HsChiA1p* has a modular structure consisting of a glycosyl hydrolase family 18 catalytic region, as well as a N-terminal family 5 carbohydrate-binding module and a polycystic kidney domain. The purified recombinant chitinase displayed optimum catalytic activity at pH 7.3 and 40 °C and showed high stability over broad pH (6–8.5) and temperature (25–45 °C) ranges. Protein activity was stimulated by the metal ions Mg<sup>2+</sup>, K<sup>+</sup>, and Ca<sup>2+</sup> and strongly inhibited by Mn<sup>2+</sup>. *HsChiA1p* is salt-dependent with its highest activity in the presence of 1.5 M of NaCl, but retains 20 % of its activity in the absence of salt. The recombinant enzyme hydrolysed *p*-NP-(GlcNAc)<sub>3</sub>, *p*-NP-(GlcNAc), crystalline chitin, and colloidal chitin. From its sequence features and biochemical properties, it can be identified as an exo-acting enzyme with potential interest regarding the biodegradation of chitin waste or its bioconversion into biologically active products.

**Keywords** Chitinase · *Halobacterium salinarum* · Archaea · Extreme halophile · Expression *Escherichia coli*

## Introduction

Chitin, a linear  $\beta$ -1,4-linked homopolymer of *N*-acetylglucosamine (GlcNAc), is one of the most abundant polysaccharides in nature besides cellulose and starch. The main chitin sources in the environment are the shells of crustaceans, insect exoskeletons, and fungal cell walls.

Although widely distributed in nature, it is more common in the marine environment, where 10<sup>11</sup> tonnes of chitin are produced annually. However, there is no accumulation of chitin in marine sediments due to the bioconversion process, which is carried out by very efficient chitinolytic marine microorganisms (Bhattacharya et al. 2007; Dahiya et al. 2006).

Chitinases are glycosyl hydrolases that metabolize chitin, catalyzing the hydrolytic degradation of the  $\beta$ -1,4-glycosidic bonds present in this polymer. Chitin is thereby transformed into its oligo- and monomeric components. These enzymes can be endochitinases, if they act randomly, breaking the chitin in internal sites; or exochitinases, if they cut the polymer from either the reducing or the nonreducing ends, yielding chitobiose and/or *N*-acetylglucosamine. According to the CAZy database (Cantarel et al. 2009), chitinases belong to GH families 18 and 19. GH20 family enzymes that hydrolyse nonreducing terminal residues of oligosaccharides composed of  $\beta$ -*N*-acetylhexosaminides can also act on chitin. Chitinases may work synergistically with chitin-binding proteins (CBPs). CBPs containing a carbohydrate-binding module of family 33 (CBM33) are also able to enzymatically cleave chitin. The name lytic polysaccharide monoxygenases has been suggested for these proteins (Vaaje-Kolstad et al. 2013), and they are classified as auxiliary activity family 10 (AA10). In recent years, chitinases have received more attention because of their broad range of applications. They have been seen to hydrolyse fungal cell walls and insect exoskeletons, and therefore, chitinases could act as a biological pest control providing a very interesting alternative to the use of chemical compounds (Bhattacharya et al. 2007; Dahiya et al. 2006). Moreover, they can transform chitin into its derived products, which are of considerable interest in medicine, pharmacy, and agriculture (Aam et al. 2010; Bhattacharya et al. 2007; Dahiya et al. 2006).

Although several microbial chitinases have been studied and characterized, the diversity of applications and conditions

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in which these enzymes must work also require a large number of different enzymes capable of acting in each condition. Exploration of microbial diversity may well help to find new enzymes with interesting properties (Duo-Chuan 2006).

While many chitinases from different bacteria and fungi have been studied, at present, very little is known about these enzymes in archaea. Although many sequences from the archaea, which probably code for different chitinases, have been annotated on databases, there have been very few studies about them. Indeed, such studies only concern on four chitinases within the Euryarchaeotas and just one within the Crenarchaeotas (Staufenberger et al. 2012). Among these, a chitinase from *Halobacterium salinarum* has been expressed in the extreme halophile *Haloarcula japonica* (Hatori et al. 2006), while the thermophilic chitinases from *Thermococcus kodakaraensis* (Tanaka et al. 1999), *Pyrococcus furiosus* (Oku and Ishikawa 2006), and *Sulfolobus tokodaii* (Staufenberger et al. 2012) have been overexpressed in *Escherichia coli*.

In this present study, we report on the cloning, characterization, and expression in *E. coli* of the *HschiA1* gene encoding a chitinase in *H. salinarum* (strain CECT 395), isolated from codfish. The production, purification, and biochemical characterization of the recombinant enzyme are also described. To our knowledge, this is the first report about the expression in *E. coli* of a chitinase from an extreme halophilic archaea.

## Materials and methods

### Bacterial strains, growth conditions, and vectors

*H. salinarum* CECT 395 was used during this study. The strain for cloning was *E. coli* TOP10 (Invitrogen) and that for protein expression was *E. coli* BL21 (Invitrogen). The vector pET100/D-TOPO® (Invitrogen) was used both for cloning the PCR product and for the expression of the protein (CECT: Spanish Type Culture Collection).

*H. salinarum* was cultured in Halophile II medium (consisting of 250 g/L NaCl, 20 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O, 3 g/L sodium citrate, 10 g/L peptone, 0.2 g/L CaCl<sub>2</sub>, 3.58 mg/L FeCl<sub>2</sub>, and 218 mg/L MnCl<sub>2</sub>) and agar 1.5 %. *E. coli* was cultured in Luria–Bertani (LB) medium. Both microorganisms were grown at 37 °C with shaking, when necessary.

### Colloidal chitin preparation

Colloidal chitin was made following the modified method of Roberts and Selitrennikoff (1988). A total of 90 mL of 37 % HCl and 5 g of chitin from crab shells (Sigma, C7170) were mixed and shaken vigorously at room temperature for 2 h. Subsequently, 0.5 L of frozen 95 % ethanol was added to the mixture, and it was left for shaking for 30 min. The suspension was then centrifuged at 5,000×g for 20 min at 4 °C. The pellet

was washed several times with sterile water until the colloidal chitin achieved a neutral pH. It was frozen until use.

### PCR amplification and cloning of *H. salinarum* *HschiA1* gene

Standard recombinant DNA techniques were performed as described by Sambrook and Russell (2001). Chromosomal DNA from *H. salinarum* was obtained using the DNA Purification Wizard® Genomic kit (Promega). The oligonucleotide primers were designed based on the sequence of the open reading frame (ORF) (AM774415.1) in the genome of *H. salinarum* strain R1. Using a specific program for the archaea (Bagos et al. 2009), a signal peptide of 29 amino acids was located in the N terminus of the protein. Taking this into account, the primers were as follows: HsChiA1: 5'-CACCA TGCCCCACGATAGACGCAG, to amplify the complete gene and HsChiA1-ps: 5'-CACCGACACCCCACCCGAATGGGA to amplify the gene without the signal peptide, as forward primers; and HsChiA1-rev: 5'-CTACTGCCAGGCGTCC CGGACG as a reverse primer. The PCR mixture, to a final volume of 100 µL, was constituted by 3 µL of template DNA (25 ng/µL), 2 µL of forward primer (100 ng/µL), 2 µL of reverse primer (100 ng/µL), 4 µL of dNTP (5 mM), 2 µL of MgCl<sub>2</sub> (50 mM), and 2 µL of Accuzyme DNA polymerase (2.5 U/µL). The thermal conditions for PCR amplification were initial denaturation step for 2 min at 94 °C, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 45 s and extension at 72 °C for 2 min, and finished with a final extension step at 72 °C for 5 min. The PCR products were purified with the GeneClean® Kit (MP Biomedicals), ligated into the pET100-D-TOPO® (Invitrogen) vector, and these TOPO cloning reactions were transformed into *E. coli* One Shot® TOP10 Chemically Competent Cells. The vector had a hexahistidine tag for purification in affinity conditions, and a gene that provided ampicillin resistance. Recombinants were screened on the selective medium LB with ampicillin (100 µg/mL), and the positive clones were identified by PCR amplification, in the same conditions as described above, and by digestion with the restriction enzyme *Hind*III, which does not cut the gene and cuts the vector only once. The PCR products were sequenced and confirmed by Sanger's method (Sambrook and Russell 2001).

### DNA protein sequence analysis

The comparison of DNA and protein sequences, as well as the conceptual translation of the DNA sequence of the *HschiA1* gene, was carried out with the Basic Local Alignment Search Tool (BLAST) program in the NCBI database, using the genetic code Table 11. Protein sequence alignments were performed using the ClustalW 2.0.12 program in EMBL-EBI, and protein analysis was carried out with the ExPASy (Swiss Institute of Bioinformatics) and Pfam (Sanger Institute) tools.

## Protein expression

Protein expression of the *HschiA1* gene without the signal peptide, was made using *E. coli* BL21 (DE3) as the host strain grown in LB medium with ampicillin (100 µg/mL) at 37 °C, with shaking until an OD<sub>600</sub> of 0.8 was reached. Then, 1 mM isopropyl thio-β-D-galactoside (IPTG) was added to the culture. After 4 h of incubation at 37 °C with shaking, the cells were centrifuged at 4,000 rpm for 10 min. A sample of 500 µL was taken after each hour of incubation and centrifuged at 8,000 rpm for 3 min. The final cell pellet was collected and broken for 30 min on ice with a lysis buffer [50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole, 0.05 % (v/v) Tween<sup>®</sup>20, lysozyme (10 mg/mL); pH 8]. Subsequently, a centrifugation of 30 min at 14,000×g was undertaken, and the cell-free supernatant was dialysed overnight with stirring at 4 °C against a dialysis buffer (20 mM Tris–HCl 1.5 M NaCl, pH 7.3). The dialysed product was collected as crude enzyme.

An attempt was made to obtain more soluble protein by adding different IPTG concentrations to a culture of the recombinant strain grown until an optical density (600 nm) of 0.8 was reached. These IPTG concentrations were between 0.5 and 1 mM combined with sorbitol 0.5 M according to Prasad et al. (2011).

## Protein purification and sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis

The cell pellet obtained after protein expression was resuspended in 4 mL of B-PER<sup>®</sup> Reagent (Thermo Scientific) per gram of cell pellet. Subsequently, 2 µL of lysozyme (50 mg/mL) and 2 µL of DNase (2,500 U/mL) per 1 mL of B-PER Reagent were added. The mixture was incubated for 15 min at room temperature and centrifuged at 15,000×g for 5 min. The supernatant and the sediment were used for purification of the protein under native or denaturing conditions, respectively. The supernatant was mixed with equilibration buffer (20 mM sodium phosphate, 300 mM sodium chloride, 10 mM imidazole; pH 7.4), so that the total volume equals two resin bed volumes, and applied to a 0.2-mL resin bed Ni-NTA affinity column (Thermo Scientific). The protocol of Thermo Scientific was followed and modified by washing the column five times with 400 µL of wash buffer (20 mM sodium phosphate, 300 mM sodium chloride, 25 mM imidazole; pH 7.4). The protein elution was carried out four times with 200 µL of elution buffer (20 mM sodium phosphate, 300 mM sodium chloride; pH 7.4, with 100 mM imidazole, 250 mM imidazole, or 200 mM EDTA). For purification of the protein under denaturing conditions, the sediment was resuspended in the abovementioned equilibration buffer supplemented with 6 M guanidine-HCl. The wash and elution buffers were the same as those indicated above, but were also supplemented with 6 M guanidine-HCl. The eluted protein was dialysed as indicated before and stored at –20 °C until use.

The concentration of the protein was determined using Bradford's method (Bradford 1976).

Samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (10 % acrylamide/bisacrylamide) according to Sambrook and Russell (2001). Ten microliters of each sample was mixed with 5 µL of loading buffer (62.5 mM Tris–HCl; pH 6.8, 25 % glycerol, 2 % SDS, 0.01 % bromophenol blue, 5 % β-mercaptoethanol). The gel was stained with EZBlue<sup>™</sup> Gel Staining Reagent (Sigma). Sigma molecular weight markers were used as standard.

## Enzyme activity

### *Enzyme activity using colloidal chitin agar plates as substrate*

Determination of extracellular chitinase activity on the strain of *H. salinarum* CECT 395 was detected by culturing it in Halophile II agar medium supplemented with 1 % chitin. Twenty microliters of a suspension of this strain was placed on the colloidal chitin agar plate. After incubation for 21 days at 37 °C, the chitinase production was checked.

### *Enzyme activity using colloidal chitin as substrate*

The activity was assayed using 1 % colloidal chitin as substrate. The reaction mixture consisted of 125 µL of colloidal chitin and 125 µL of the purified protein diluted in 20 mM Tris–HCl buffer at pH 7.3, with a final NaCl concentration of 0.75 M. After incubation for 18 h at 40 °C, the concentration of reducing sugars in the supernatant was determined by the Somogyi–Nelson method (Nelson 1944), using GlcNAc as the standard. One unit (U) of activity was defined as the amount of enzyme releasing 1 µmol of GlcNAc in the assay conditions. Assays were carried out in triplicate, and the results were expressed as mean ± SD.

### *Enzyme assay using (GlcNAc)<sub>n</sub>-p-NP as substrate*

The reaction mixture contained 125 µL of 0.5 mM (GlcNAc)<sub>3</sub>-p-NP; 0.5 mM (GlcNAc)<sub>2</sub>-p-NP; or 0.5 mM (GlcNAc)-p-NP in distilled water and 125 µL of the purified protein diluted in 20 mM Tris–HCl buffer. A final NaCl concentration of 1.5 M was present in the reaction. After incubation for 2–4 h at 37–40 °C, the reaction was stopped by an addition of 3 M Na<sub>2</sub>CO<sub>3</sub>. *para*-Nitrophenol liberated during the reaction was determined spectrophotometrically at 410 nm. One unit of enzyme activity was defined as the amount of enzyme releasing 1 µmol of *p*-nitrophenol in 2 or 4 h, depending on the assay. Assays were carried out in duplicate, and the results were expressed as mean ± SD.

### Effects of salinity, temperature, pH, and metal ions

Optimum salinity for the chitinase *HsChiA1p* was determined from 0 to 3.5 M of NaCl. Optimum temperature for the purified enzyme was determined to be between 15 and 50 °C. Temperature stability was measured by preincubation of the enzyme at 25, 30, and 45 °C for 10, 20, and 40 min each. Optimum pH was tested from pH 5.5 to pH 9, while pH stability was measured by preincubation of the purified enzyme at pH values of 6, 6.5, 8, and 8.5 for 10, 20, and 40 min each. Enzyme activity was also tested after adding some metal ions, such as K<sup>+</sup> (KCl), Ca<sup>2+</sup> (CaCl<sub>2</sub>), Mg<sup>2+</sup> (MgSO<sub>4</sub>), and Mn<sup>2+</sup> (MnCl<sub>2</sub>), at a final concentration of 5 mM, always in the presence of NaCl at a final concentration of 0.9 M. Assays were carried out in duplicate, and the results were expressed as mean ± SD.

## Results

### Gene isolation and sequence analysis

The strain of *H. salinarum* CECT 395, cultured in Halophile II medium supplemented with 1 % colloidal chitin, showed capacity to hydrolyse chitin. This was demonstrated by a hydrolysis halo observed around the productive colonies after 21 days.

The genomes of two different strains (R1 and NRC-1) of *H. salinarum* were sequenced. The analysis of these genomes made it possible to locate an ORF of 546 amino acids in strains R1 and NRC-1 (accession numbers YP\_001688891.1 and NP\_279794.1, respectively) and an ORF of 572 amino acids also in both strains R1 and NRC-1 (accession numbers YP\_001688892.1 and NP\_279795.1, respectively), which would encode uncharacterized proteins with possible chitinolytic activity. In addition, a third ORF that would encode a protein comprising a family 5 carbohydrate-binding module (CBM5) was identified in the R1 and NRC-1 strains (accession numbers YP\_001688893.1 and NP\_279796.1, respectively).

To isolate these protein-encoding genes, three oligonucleotide pairs were designed based on the sequences mentioned above. It was only possible to amplify a fragment of 1,641 bp from the *H. salinarum* CECT 395 DNA with the primers *HsChiA1* and *HsChiA1-rev*, which flank the ORF corresponding to YP\_001688891.1 protein. This sequence corresponds to the complete gene called *chiA1*. DNA fragments compatible with the other two ORFs could not be successfully amplified from the strain used in this study. Using a specific program for the archaea (Bagos et al. 2009), analysis of the deduced sequence from the *HschiA1* encoded protein (named *HsChiA1p*) showed that the first 29 amino acids from the N-terminal end probably correspond to a signal peptide. A DNA fragment of 1,554 bp was amplified from the same genomic DNA using a pair of primers (*HsChiA1-ps* and *HsChiA1-rev*),

**Fig. 1 a** Comparison of *H. salinarum* CECT395 carbohydrate-binding domain with other CBM5 domains. Conserved aromatic residues responsible for the interaction of binding domain and sugar molecules are in white letters. The consensus sequence AKWWTK is in bold letters. **b** Multiple sequence alignment of *H. salinarum* CECT 395 catalytic domain GH18 (according to Pfam) with other chitinases from family 18. *HsCECT395*, *H. salinarum* CECT 395; *HsR1* (a), *H. salinarum* R1 (YP\_001688892.1); *HsR1* (b), *H. salinarum* R1 (YP\_001688893.1); *NatrinemaspJ7-2*, *Natrinema* sp. J7-2 (YP\_006542007.1); *NaJCM12890*, *N. altunense* JCM 12890 (ZP\_2153664.1); *HnJCM10879*, *Halobioforma nitratireducens* JCM 10879 (ZP\_2170069.1); *HeATCC-BAA-1513*, *H. elongans* ATCC BAA-1513 (ZP\_21652427.1); *HbDSM11551* (a), *H. borinquense* DSM 11551 (YP\_004038214.1); *PfJG1*, *P. flavipulchra* JG1 (ZP\_11231058.1); *EfE1590*, *E. faecium* E1590 (ZP\_19491270.1); *He*, *H. elongans* (WP\_008326747.1); *Ha*, *H. amylolytica* (WP\_008313948.1); *HbDSM11551* (b), *H. borinquense* DSM 11551 (YP\_004038212.1); *SspHarcht-Bsk1*, *Salinarchaeum* sp. Harcht-Bsk1 (YP\_008054875.1); *Ht*, *Haloterrigena turkmenica* (WP\_012943345.1); *Tk*, *T. kodakaraensis* (WP\_011250716.1); *Na*, *N. altunense* (WP\_007110886.1); *Bc*, *B. circulans* (AAF74782.1); *Pb*, *Paenibacillus barengoltzii* (WP\_016312329.1). Identical amino acids are represented by an asterisk, the ones with conserved replacements are marked by a colon, and those with less conserved replacements are indicated by a dot. The consensus sequences of family 18 chitinases SXGG and DXXDXDXE are boxed in gray. The arrow shows the catalytic residue of family 18 chitinases. **c** Phylogenetic tree, constructed using the maximum likelihood method (PhyML software), based on amino acid sequences of the GH18 domains aligned

which exclude the sequence corresponding to the signal peptide.

The BLAST analytical program was used to compare the *HsChiA1p* protein from the strain CECT 395 with those deposited in the database. The *HsChiA1p* protein showed an identity of 100 % with the one deduced from the *chiA1* gene of the strain R1 (YP\_001688891.1) and 47 % with the deduced protein from the *chiA2* gene of *H. salinarum* R1 (YP\_001688892.1).

Analysis of the amino acid sequence from *HsChiA1p*, obtained from the amplified nucleotide sequence, revealed that the enzyme had a multi-domain structure. The first 29 amino acids of the N-terminal end form a signal peptide, which is followed by a 40-amino-acid fragment (E<sup>34</sup>–W<sup>73</sup>) that, according to Pfam, shows homology with CBM5. Figure 1a shows the alignment of this domain with the sequences deposited in the database using the ClustalW 2.0.12 program. This sequence shows high identity values with sequences from different archaea: 78 % with *Natrinema altunense*; 74 % with *Haloferax elongans*; and 71 % with *Halogeometricum borinquense*. It also shows identity values with sequences from different bacteria: 59 % with *Pseudoalteromonas flavipulchra* and 50 % with *Enterococcus faecium*. Moreover, it shows an identity of 78 % with another hypothetical protein from *H. salinarum* R1. The sequence between the T<sup>94</sup> and T<sup>158</sup> amino acids has been identified as a polycystic kidney domain (PKD) present in many sugar-utilizing enzymes. Finally, a characteristic catalytic domain from family 18 chitinases (GH18) has been located in the sequence. The alignment of this region (R<sup>177</sup>–D<sup>532</sup>), containing an alpha + beta insertion between amino acids

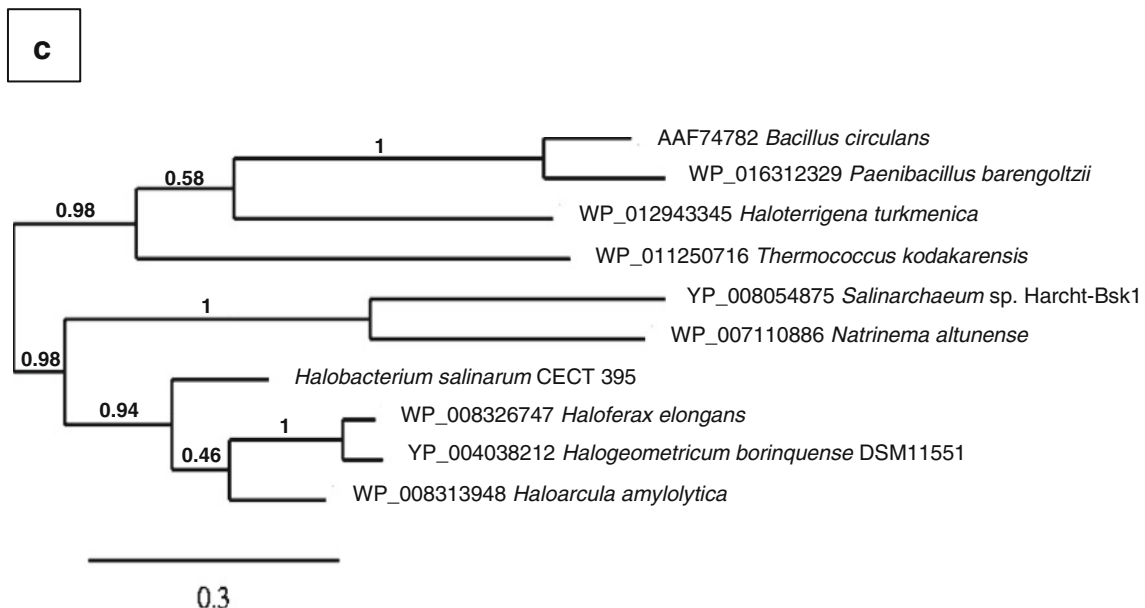
a

		Identity (%)
HsCECT395	EWDPDTVYTDGDQATFDGYVWEAKWTKGDKPG-ADEWGPW	
HsR1 (a)	EWDPDTVYTDGDKATFDGYVWEAKWTKGDKPG-ADEWGPW	98
HsR1 (b)	-WDPDTAYTEGDEVTFDGYVWAAKWTKGDEPG-SDEWGPW	82
NatrinemaspJ7-2	EWDPETVYTSGDRVVYDGYVWEAQWTRGDEPG-ADEWGPW	78
NaJCM12890	EWDPETVYTSGDRVVYDGYVWEAQWTRGDEPG-ADEWGPW	78
HnJCM10879	EWDPETVYTSGDRVIYEGYVWEAQWTRGNEPEGGEWGPW	60
HeATCC-BAA-1513	QWDPDTVYTDGDRVTHDAYIWEAKWTKGDEPS-DD----	74
HbDSM11551 (a)	QWDPDTVYTDGDRVVHDAHIWEAKWTKGDEPT-AD----	71
PfJG1	-WDADKVIYLGDRASYNGVIYEAKWTKGNI PA-ADGSGPW	59
EfeE1590	EWNPEKAVKGDIVTYQGVYQAKWVWNOGEEP-G-SNEWGAW	50
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b

		Identity (%)
HsCECT395	-RVVGYMQWAQWDRDYFPGDIPLDKVTHVNYAFLTNR-----	213
He	-RVVGYMQWAQWREYTPGDIPLDKVTHVNYAFLTN-----	207
Ha	KRVTYGYMQWSQWDRDYFPGDIPLDKVTHVNYAFLTN-----	221
HbDSM11551 (b)	KRVVGYMQWAQWDRDYFPGDIPLDKVTHVNYAFLTVK-----	208
SspHarcht-Bsk1	-RIVGYWQWAQYDRDYVPAIDPKDQVTHLYAFMRPE-----	463
Ht	-RVVGYYSWSIYDRNYQPSDVPDKVTHLNYAFMDVQ-----	315
Tk	-RVIVYYISWGRYARKFYVSDIPWEKVTHTVNYAFLDLK-----	212
Na	-RVIAYYRQWAQYDRDYVPGDIPFDKVTHTVQYAFARPE-----	214
Bc	YKIVGYYPAAAYGRNYVTIDIPTKVTHTVNYAFADICWNGIHGNPDPSPGNPVTVWSCQN	103
Pb	YKIVAYYPSWGAYDRNFVYDIDASKIHTHTVNYAFADIAWDRHGNPDPAGNPVTVWPTQD	102
	: : * : * . : * : : * : : * : * : * : * : *	
HsCECT395	-----EDGAVDYIQENAAAMRVLKPKS-----WHDHTGFDDLVD--PETSFLFISIGG	258
He	-----QDGSVDYINENAAKRVLEPE-----WHDHTGFDELVD--PTTEFLFISIGG	252
Ha	-----QNGAVDYIQQNAAMRVLKPKS-----WHDHTGFDELVD--PTTKFLFISIGG	266
HbDSM11551 (b)	-----QDGSVAYINENAAAMRVLKPKS-----WHDHKGFNELVD--PTTKFLFISIGG	253
SspHarcht-Bsk1	-----SDGTISGLGSSAQYLFPPQ-----WHDVT---SFGDIASN-TDVTCLISIGG	508
Ht	-----PNGTVTYGDNENADR-----QNLAKFQELKQAHPDTPKQLSISIGG	353
Tk	-----EDGTVAFYDVTYADP-----LNLEAMKEYKRYPAVKVLSVSGG	250
Na	-----TDGSINLVGDSYGQMFYAEK--WQGPDRGKTFAGYAAEREDTKFVLSISIGG	264
Bc	EKSQTINVPNGTIVLGDPIWIDTGKQFAGDTWQPYPYAGNINQLNKLKQVNP NLKTIISISIGG	163
Pb	EKGQTINVPNGTIVLGDPIWIDTGKLFPGDTWQPYPYAGNIHLNKLKQANPHLKTIIISVGG	162
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HsCECT395	WNSDYFNSAAEQSQASRERFADTAIEIMRTHNFDGLDIDWEYPGGGGNS-----GNV	310
He	WNSDYFNSAAETEASRQRFAKTAVEIMRAHNF DG LDIDWEYPGGGGKS-----GNI	304
Ha	WNSDYFNSAAEQSQASRERFADTAIEIMRKHNF DG LDIDWEYPGGGGKS-----DNI	318
HbDSM11551 (b)	WNSDYFNSAAETEASRQRFAKTAVEIMREHNF DG LDIDWEYPGGGGKS-----GNI	305
SspHarcht-Bsk1	WSDSENFSDVALEESRQKFADECVRIVREAGVDGVDIDWEYPGGGGHP-----DNT	560
Ht	WLSLTHFSDAATQENRERFASVSKLMRQYDFDGLDIDWEYFDPGGGQA-----GNS	405
Tk	WTL SKYFSVVAADPAKQRFAETAIEILRKYNLDGLDIDWEYPGGGGMA-----GNY	302
Na	WGDSENFSDAALTQENRERFASNCVDLVEKANLDGLDIDWEYFDPGGGGTAADPVCVDVNA	324
Bc	WTVSNRFSDVAATSATREVFANSAVDFLRKYNFDGVDLNWEYPVSGGLD-----GNS	215
Pb	WTVSNRFSDVAASQVTRTFANSAVDFLRKFNFDGLDLDWEYPVAGGLP-----GNS	214
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HsCECT395	VRDGDQRYTELLQTVREKLDVAEDED---GKRYQLTALSADP-----EKNTGLDHA	360
He	VRDGDKKRYTDLLAAVRSELDAAEKED---GQEYLLTALSADP-----QKNEGLDHQ	354
Ha	VRDGDKERFSKLLREVRSQLDEAESE---GQEYLLTAALSADQ-----KKNQGLNHD	368
HbDSM11551 (b)	VRDGDKKRYSDLLAAVRAELDAEED---GQEYLLTALSADP-----KKNGLDHD	355
SspHarcht-Bsk1	VRDGDQERFTLLVQAVRDALDAAGAED---GKDYKLTAAVMSVSG-----GSKA-EGLEHG	610
Ht	ERPDPQNYVLLLQEVREELNEAEQQD---GKEYELSVAAASNP-----QKTAQLDVP	455
Tk	ESPDDGNFVLLKDLREALDKAKEDE---HKDYLLTAA TPADP-----VKAGRIDWV	352
Na	VRDGDQERFTLLCREVREQLDAAEAEDPDRDEPYELSAAVSPNPEIVEGKTAGNDGLEHD	384
Bc	KRPEDKQNYTLLSKIREKLDAAEAVD---GKEYLLTASGASP-----TYAANTELA	265
Pb	YRPEDKQNYTKLLQEI RNKLDASAGVD---GKRYLLTASGASP-----SfVSNTELG	264
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HsCECT395	ANAEALDFLNVMYTYDHGAFNDYTNHQAPLYGTEADPS---PNADEFYVDASMSFWLDTA	417
He	QNSELLDFLNVMYTYDFHGAFDEYTNHQSPLYYKDNDS---PRAQDFNVDAAMTYWANTA	411
Ha	DNAELLDFLNVMYTYDHGAFDENNTHQSPLYGTDDPS---PRADDFYVDASMSYWTDTA	425
HbDSM11551 (b)	RNTGLDFVNVMTYDFHGAFDDYTNHQSPLYYKENDPS---PRAEDFNVAAMTYWANTA	412
SspHarcht-Bsk1	TL SGLDFVSI MTFDMRGGFSDYTGHPSPFQNPNDPS---SKASTYNVSSGVSWSVVDK	667
Ht	GIAEQVDYVSVMYNYDYTGTSWSSQTNHNSKLYSASDD---PSPDHFNGDAGMRGWADAG	510
Tk	EASKYLDSINIMTYDHGAWETTGH LAPLYCDPNAPYTDENVKYHFCVNYTVQWY IQHV	412
Na	KLSDILDFAQVMTFDRGWISETTGHHA PLKANPDDPY---ADSDVWNAQFALEYWADQ	441
Bc	NIASIVDWINIMTYDFNGAWQKISAHNAPLNADPAAASAGVPDSENTFNVAAGAQGHNLNAG	325

Pb	NIASIVDFWINIMTYDFNGGWQQITAHNAPLYYDPAAGNAGVPSASVFNADAQVGHNLNAG	324
	: . * . : * . : * * : . : * : :	:
HsCECT395	FDPAQLSLGLPFYGRSFGNVASSD-----NNGLYQPFDFG-----TPDGTWG-----	458
He	FDSSQLSMGLPFYGRSFGNVASSD-----NGGLYQSFDG-----SPDGTWG-----	452
Ha	FDSKQLSIGLFPFYGRSFGNVASKD-----NNGLYQPFDFG-----SPDGTWG-----	466
HbDSM11551 (b)	FDSSQLSMGLPFYGRSFGNVASSD-----NNGLYQSFDG-----SPDGTWG-----	453
SspHarcht-Bsk1	WDPGQLNIATPFYGRSFSVEAPSGDLGNGTDDGLFQNFSG-----TGGGSFP---P--D	717
Ht	MPKEKLVYGAFFFGYGFEGVPDR-----NNGLYQSFTG-----PADVGW-----	549
Tk	PDKTKITVGLPFYGRSFGNVASSD-----NNGLYQPFDFG-----TPAGTWGP-----A	454
Na	WDPGQLNMVAPFYGRSFTDVPPEGDFGTGTDGGLFQKYEGRDASGEGSYPSWDPMSG	501
Bc	VPAAKLVGLVFPFYGRGWDGCAQA-----NNGQYQTCSG-----GSSIG-----	363
Pb	VPANKLVGIFPYGRGWDGVNNA-----GNGQYQSASG-----GSSVG-----	362
	: : . . * : . . : .	
HsCECT395	-QDNGIKEYWDI-----TQNLAPESSDYETFWDDTAKVPWLYSPSKNVLVSYSRPS	508
He	-QDNGIMEFWDI-----NQNLPSSEYEHWDDTAKVPWIYSSSDVLSYDNPKS	502
Ha	-QDNGIMEFWDI-----KNNLAPSCKYAESWDDTAKVPWIYSESEDILVSYDNARS	516
HbDSM11551 (b)	-QDNGIMEFWDI-----NQNLPSSEYEHWDDTAKVPWIYSSSDVLSYDNPKS	503
SspHarcht-Bsk1	TESSGIYDYWDLSTVGTARSTSQVDLSSSDWTTSYDDTAKATWSYNQTDLSLMI SHPTPDS	777
Ht	TVADGATDYRSV-----QELIDNDSSYVRYWDEAKVPYVYSARDNVFITTYEDPES	600
Tk	YETYGVMDYWDV-----AEKN-QSSEYEHWDPIAQVAWLYSPSKRIFITFDDPRA	504
Na	TSYAGVWEWFDLGS DGRGGSN-PVDLESGAWETYFDEDAVTAWSWNPEERLMI SHETEQS	560
Bc	TWEAGSFDYDL-----EANYINKNGYTRYWNDTAKVPFLYNASNKRFI SYDDAES	414
Pb	TWEAGSFDYDL-----EANYINKNGYTRYWNDTAKVPFLYNASNKRFI SYDDAES	413
	* : : : .	
HsCECT395	VEAKTAYAAQHDIGMMFWTFSGD	532
He	VGIKTDYALENNLGLMFWAFSGD	526
Ha	VGLKTEYAIENDFGMMFWTFSGD	540
HbDSM11551 (b)	IGLKTDYAVDNDIGMMFWAFSGD	527
SspHarcht-Bsk1	VQQRASWLANS DYGGMIMIALSHD	801
Ht	MAIKAEYVKENGYGGMMFWFYG	624
Tk	IGIKVDYMLKNGLGGVMIWEITAD	528
Na	MEAKMDWLRDSPYGGTMLWALS	584
Bc	IGHKTAYIKSKGLGGAMFWELSGD	438
Pb	IGFKTDYIKSRGLAGAMFWELSGD	437
	: : : .	



**Fig. 1** (continued)

Y<sup>430</sup> and Y<sup>503</sup>, is presented in Fig. 1b, together with a phylogenetic tree showing the relationship between the sequences (Fig. 1c). The GH18 catalytic domain of *HsChiA1p* shows an identity of 77 % with *H. elongans* and *Haloarcula amylolytica*; 76 % with *H. borinquense*; 44 % with *T. kodakaraensis*; 43 % with *Salinarchaeum* sp.; and 37 % with *Bacillus circulans*.

#### Protein expression and purification

A fragment of 1,554 bp, containing the *HschiA1* gene from *H. salinarum* without the corresponding signal peptide sequence, was cloned into the pET100/D-TOPO<sup>®</sup> vector. The *E. coli* BL21 (DE3) strain was transformed with this construction to

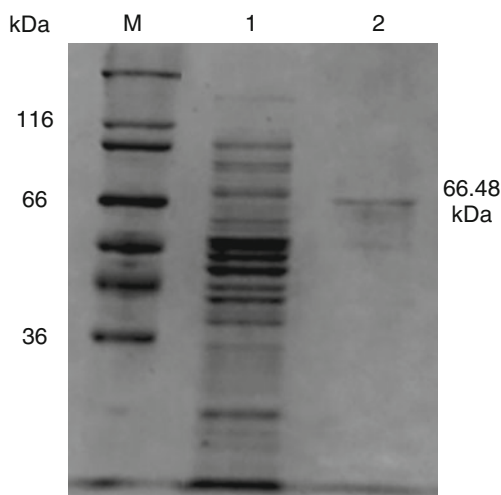
obtain the intracellular expression of the gene, which was under the control of T7/tac promoter and was expressed using the IPTG inducer at a concentration of 1 mM. The soluble protein was purified by taking advantage of the hexahistidine tag, and an apparently homogeneous band of approximately 66.5 kDa was seen on an SDS-PAGE gel (Fig. 2).

The enzymatic activity of the recombinant protein was assayed against different substrates (Table 1). It was observed that *HsChiA1p* can hydrolyse chitin, as colloidal chitin or crystalline chitin, and was more active when the colloidal chitin was used as substrate. Using different *p*-NP-glucosides as substrate, the enzyme showed higher activity against *p*-NP *N*-acetyl- $\beta$ -D-glucosaminide than against *p*-NP  $\beta$ -D-*N,N,N'*-triacetylchitotriose, and no activity (evaluated as *p*-NP released) was detected with the *p*-NP *N,N'*-diacetyl- $\beta$ -D-chitobioside substrate. The *p*-NP *N*-acetyl- $\beta$ -D-glucosaminide substrate was used for enzyme characterization.

The different protein purification steps are detailed in Table 2. Normally, 0.14 mg of the purified enzyme is obtained from 100 mL of culture. In this study, it was seen that the specific activity of the purified enzyme (513.6 mU/mg) was eight times higher than that of the crude enzyme (64.7 mU/mg).

Purification of the enzyme under denaturing conditions showed that some protein was located in inclusion bodies. However, the attempt to obtain a greater amount of soluble protein using 0.5 M sorbitol following the method of Prasad et al. (2011) did not lead to an increase in enzymatic activity.

The enzyme was eluted using imidazole and EDTA. The specific activity for the protein eluted with 200 mM EDTA was about two and four times higher than that detected with 100 and 250 mM imidazole, respectively.



**Fig. 2** SDS-PAGE analysis of purified recombinant protein *HsChiA1p* from *H. salinarum* CECT 395. *M*, high range (Sigma) molecular weight marker. *1*, crude extract from induced cells. *2*, purified enzyme

**Table 1** Substrate specificities of the purified recombinant protein

Substrates	Enzymatic activity (U/mL)	Specific activity (U/mg)
<i>p</i> -NP <i>N</i> -acetyl- $\beta$ -D-glucosaminide	0.49 $\pm$ 0.005	4.27 $\pm$ 0.04
<i>p</i> -NP <i>N,N'</i> -diacetyl- $\beta$ -D-chitobioside	0	0
<i>p</i> -NP $\beta$ -D- <i>N,N,N'</i> -triacetylchitotriose	0.01 $\pm$ 0.004	0.10 $\pm$ 0.03
Colloidal chitin	1.91 $\pm$ 0.25	16.48 $\pm$ 2.17
Crystalline chitin	0.64 $\pm$ 0.35	5.49 $\pm$ 3.05

Reactions were carried out for 18 h at 40 °C at pH 7.3 Tris–HCl buffer. Substrate concentrations were 0.5 mM for *p*-NPs and 1 % for chitins. Results are presented as means  $\pm$  standard deviation with  $n=2$  for *p*-NPs and as means  $\pm$  standard deviation with  $n=3$  for chitins

#### Effect of salinity, temperature, pH, and metal ions on chitinase activity

The effect of salt on the enzyme activity was measured in 50 mM Tris–HCl buffer at pH 8 with different concentrations of NaCl (Fig. 3). The maximum activity was found at a final concentration of 1.5 M NaCl. Without any salt, more than 15 % of the chitinase activity is still conserved. The effect of different metal ions on the protein activity was also assayed (Table 3), showing that Mg<sup>2+</sup>, K<sup>+</sup>, and Ca<sup>2+</sup> increase the chitinase activity, whereas Mn<sup>2+</sup> strongly inhibits it.

The optimum temperature for the recombinant enzyme (Fig. 4a) was determined in 50 mM Tris–HCl buffer at pH 8 with 1.5 M NaCl. The protein was active between 15 and 45 °C, with 40 °C as the optimum temperature, while 30 and 50 % of activity were maintained at 25 and 30 °C, respectively. At 50 °C, the enzyme dramatically lost its activity. Regarding stability, the protein maintained 90 % of its activity after 40 min at 25 and 30 °C. After 40 min at 45 °C, the enzyme conserved more than 65 % of its activity (Fig. 4b).

The optimum pH for the recombinant chitinase (Fig. 4c) was measured at 40 °C in 50 mM Tris–HCl buffer at different pH values with 1.5 M NaCl. The enzyme was active between pH 6 and 9, with 7.3 as the optimum pH, and completely lost its activity at pH 5.5. Regarding stability, the enzyme maintained over 90 % of its activity after 40 min at pH values 6.5, 8, and 8.5. Even at pH 6 for 40 min, the chitinase conserved more than 65 % of its activity (Fig. 4d).

## Discussion

Chitinases are enzymes with various applications. One of the most important is the bioconversion of chitin residues into products with added value (*N*-acetylglucosamine and chitooligosaccharides), which are of considerable interest for the food and pharmaceutical industries. Enzymatic production of these derivatives of chitin is more efficient and less harmful

**Table 2** Purification of recombinant chitinase from *H. salinarum* CECT 395 expressed in *E. coli*

Enzyme	Total activity (mU)	Total protein (mg)	Specific activity (mU/mg)	Yield (%)	Purification fold
Crude enzyme	322.4±22.4	5.0±0.2	64.5±4.5	100	1
Purified enzyme	71.0±3.6	0.14±0.0	513.6±25.7	2.8	8

Reactions were carried out at 40 °C at pH 7.3 Tris–HCl buffer using 0.5 mM *p*-NP *N*-acetyl- $\beta$ -D-glucosaminide as substrate. Results are presented as means  $\pm$  standard deviation,  $n=2$

to the environment than chemical reactions and, therefore, is considered to be an excellent alternative (Aam et al. 2010; Bhattacharya et al. 2007; Duo-Chuan 2006).

Although in the sequenced genomes of *H. salinarum* strains R1 and NRC-1 two sequences could be identified that probably encode chitinases, only the ORF of 1,641 bp (YP\_001688891.1) could be amplified from the strain CECT 395 in the assayed conditions, which suggests a different chitinolytic pattern in this strain. The identification of a possible signal peptide at the N-terminal end indicates that it is an extracellular protein, which is consistent with the ability, shown by strain CECT 395, to hydrolyse colloidal chitin on plates.

The carbohydrate-binding module CBM5 of *HsChiA1p* shows a high and moderate identity, respectively, with different sequences of archaea and bacteria deposited in the databases. These sequences bind the enzyme to the substrate through hydrophobic interactions between certain aromatic residues and the sugar molecules, allowing the catalytic domain to hydrolyse the substrate in an effective way. The consensus sequence A<sup>56</sup>KWWTK<sup>61</sup>, well conserved in the CBM5 modules in bacteria, also appears to be conserved in *H. salinarum*, as are the three aromatic amino acids (Y<sup>41</sup>W<sup>58</sup>W<sup>59</sup>) probably responsible for the interaction between the enzyme and the substrate (Brun et al. 1997; Itoh et al. 2006).

Other binding domains, such as the fibronectin type III domain or the PKD, have been found in different chitinases and bacteria. A study has reported on the binding of the PKD to the substrate, which induces the hydrolysis of chitin (Orikoshi et al. 2005). The characteristic conserved sequence of the PKD (Orikoshi et al. 2005) also appears in the chitinase

of *H. salinarum* (W<sup>122</sup>AFGDG<sup>127</sup>) and in numerous sequences of possible haloarchaea chitinases entered in the databases.

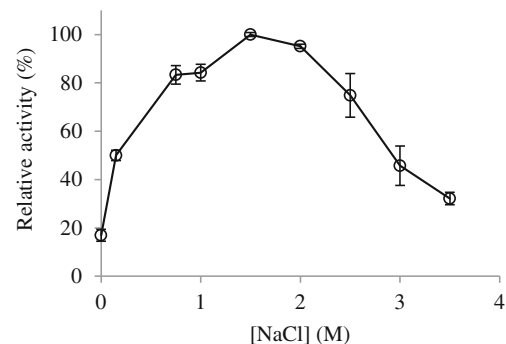
The conserved signature sequence in the catalytic domain of the glycosyl hydrolases of family 18 (DXXDXDXE) present in bacteria, fungi, and archaea (Tsuji et al. 2010) has been localized in the sequence of *HsChiA1p* (D<sup>292</sup>GLDIDWE<sup>299</sup>), which can, therefore, be classified within this group. In addition, from the alignments, it has been deduced that the Asp and Glu amino acids, considered to be essential for chitinolytic activity (Synstad et al. 2004) and that are localized in very conserved positions in the chitinases of bacteria and fungi, are also conserved in the chitinases of different archaea (Gao et al. 2003), including in *HsChiA1p* of *H. salinarum*. In agreement with this, the Glu<sup>299</sup> of *HsChiA1p* would correspond to the residue probably involved in the donation of protons in the catalytic mechanism (Tsuji et al. 2010). The presence of an alpha + beta insertion between amino acids Y<sup>430</sup> and Y<sup>503</sup> allows the classification of the enzyme into the subfamily A of GH18 chitinases (Suzuki et al. 1999).

The enzymes of the halophilic microorganisms, and especially those of the halophilic archaea, are considered to have a high applied potential. They are, therefore, receiving increasing attention because of the following properties: their ability to catalyze reactions in the presence of high concentrations of NaCl; their stability at a broad range of pH values and temperatures outside the optimum; and their capability of developing their activity in media with a low water activity and even in the presence of organic solvents (Delgado-García et al.

**Table 3** Effect of metal ions on the chitinase activity of the purified enzyme

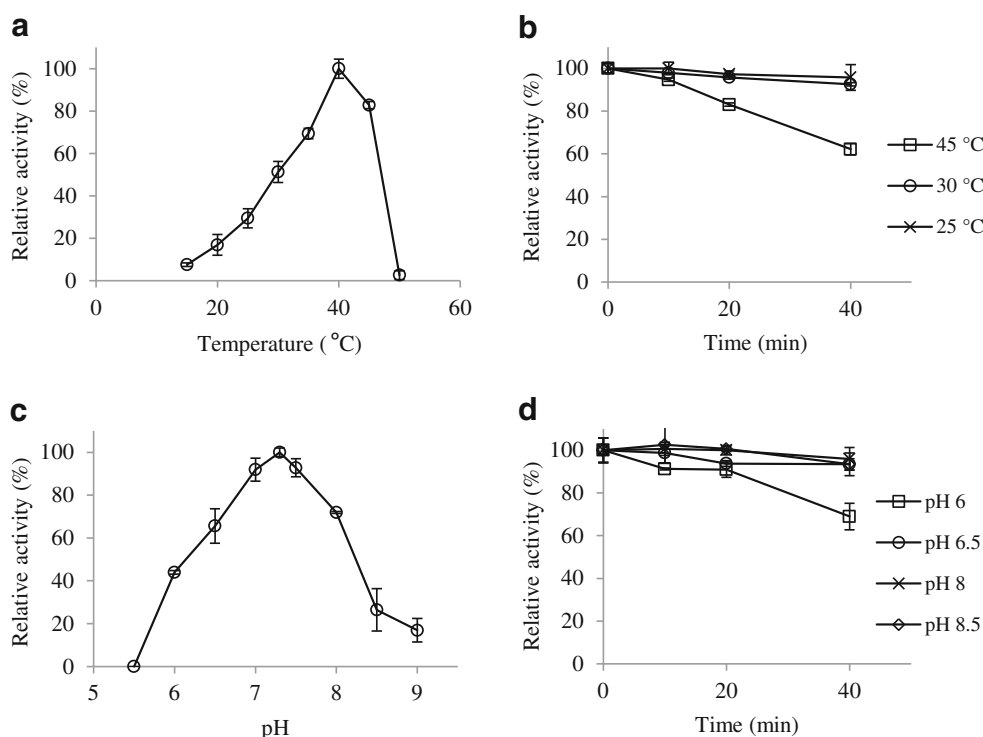
Metal ion	Relative activity (%)
Control	100±7.2
K <sup>+</sup>	121.0±2.1
Ca <sup>2+</sup>	118.0±4.2
Mn <sup>2+</sup>	19.1±3.7
Mg <sup>2+</sup>	132.9±3.3

Reactions were carried out at 40 °C at pH 7.3 Tris–HCl buffer. Metal ions tested were added at a final concentration of 5 mM. Substrate used was 0.5 mM *p*-NP *N*-acetyl- $\beta$ -D-glucosaminide. Results are presented as means  $\pm$  standard deviation,  $n=2$

**Fig. 3** Effect of sodium chloride concentration on the chitinase activity of the purified enzyme. Reactions were carried out at 37 °C at pH 8 Tris–HCl buffer using 0.5 mM *p*-NP *N*-acetyl- $\beta$ -D-glucosaminide as substrate. Results are presented as means  $\pm$  standard deviation,  $n=2$



**Fig. 4** Effect of temperature (a) and pH (c) on the chitinase activity of the purified enzyme. Effects of different suboptimal temperatures (b) and pH values (d) on the stability of the enzyme. a and b Assays were carried out at various temperatures at pH 8 Tris–HCl buffer. c and d Assays were carried out at 40 °C in Tris–HCl buffers with different pH values. Substrate used was 0.5 mM *p*-NP *N*-acetyl- $\beta$ -D-glucosaminide. Results are presented as means  $\pm$  standard deviation,  $n=2$



2012; Litchfield 2011). However, regulation of enzyme synthesis in wild-type strains, the low and delayed production, as well as the need to cultivate halophilic microorganisms in complex media with a high concentration of NaCl, especially for cultivating extreme halophiles, all make it difficult to produce these enzymes for use in biotechnological applications (Litchfield 2011).

The enzymes of extreme halophilic microorganisms are not only adapted to tolerate high concentrations of salt (Gomes and Steiner 2004; Litchfield 2011), but also need a variable NaCl concentration (1–4 M) for their correct folding, activity, and stability (Gomes and Steiner 2004). In *H. salinarum*, we noted this dependency, previously described by Holmes et al. (1965), when observing that a crude extract from a culture of strain CECT 395, dialysed against a buffer without salt, completely lost its chitinolytic activity. As *E. coli* does not accumulate salt at the intracellular level, the heterologous expression system based on this bacterium may present problems when expressing extreme halophilic proteins. Despite this, in our study, when the cells were lysed in a lysis buffer with NaCl 300 mM and the extract was dialysed against a buffer containing 1.5 M of NaCl, it was possible to express at the intracellular level in *E. coli* the enzyme *HsChiA1p* of *H. salinarum* as a soluble and active protein. This probably allowed the correct folding of the protein, as has also been described for other halophilic enzymes (Gomes and Steiner 2004). Although chitinases of moderate halophilic bacteria have been expressed in *E. coli* (Aunpad et al. 2007; Essghaier et al. 2012), to our knowledge, this present study

is the first report about the expression in *E. coli* of a chitinase of an extreme halophilic archaeon.

The chitinase activity of the recombinant protein produced in *E. coli* is well demonstrated by its ability to hydrolyse crystalline chitin and, more effectively (three times), colloidal chitin. The recombinant enzyme is also able to hydrolyse (GlcNAc)-*p*-NP in a more effective way (40 times) than (GlcNAc)<sub>3</sub>-*p*-NP. According to the analysis of the protein sequence, the enzyme would carry out its action by an exo-acting mechanism. The presence of an alpha + beta insertion in the GH18 catalytic domain may enhance the exo-type activity by forming a deep substrate-binding cleft on the top of the TIM barrel (Horn et al. 2006; Vaaje-Kolstad et al. 2013).

Although, as would be expected, the recombinant enzyme is dependent on the NaCl concentration, with maximum activity at a concentration of 1.5 M, it is worth pointing out that this recombinant enzyme also conserves 20 % of its activity in the absence of salt, and up to 50 % at a concentration of NaCl of 0.25 M. This does not occur with other halophilic proteins studied, which are strictly dependent on salt (Gomes and Steiner 2004; Shanmughapriya et al. 2009). As has been suggested for other recombinant enzymes of halophilic microorganisms, this could be because the positive charges of the hexahistidine tail may be able to interact with the negative charges of other regions of the protein, aiding its folding and stability in the absence of NaCl (Ishibashi et al. 2001). The broad range of action shown by *HsChiA1p*, regarding NaCl requirements, gives it a greater versatility for various applications.

As with other chitinases, the activity of the recombinant protein is also affected by various metals that were tested in the presence of NaCl. Thus,  $Mg^{2+}$  stimulates its activity (30 %), as occurs with the chitinase C of the moderate halophile *Salinivibrio costicola* (Aunpad et al. 2007), whereas this ion inhibits or does not affect the activity of the chitinases of *Pseudomonas aeruginosa* (Wang et al. 2010). Moreover,  $K^+$  and  $Ca^{2+}$  stimulate the activity of the chitinase of *H. salinarum*, but do not have any effect on the chitinase C of *S. costicola* (Aunpad et al. 2007). It is important to emphasize the dramatic inhibiting effect (80 %) that  $Mn^{2+}$  has on enzymatic activity, similar to that described for other bacterial chitinases, such as those characterized in *P. aeruginosa* (Wang et al. 2010). In contrast, this ion stimulates the enzymatic activity of different chitinases of marine microorganisms, such as those of *Streptomyces* sp. DA11 (Han et al. 2009).

The chitinases of archaea so far expressed in *E. coli* all belong to the thermophiles (Gao et al. 2003; Staufenberger et al. 2012; Tanaka et al. 1999). *HsChiA1p* is the first chitinase of a mesophilic archaeon expressed in *E. coli*. The recombinant enzyme is active between 20 and 45 °C, with an optimal temperature (40 °C) characteristic of a mesophile. The optimal temperature and the dramatic loss of activity at higher temperatures (50 °C) are in accordance with the results found for another glycosyl hydrolase, an amylase, of the halophile *Halobacillus halophilus* (Shanmughapriya et al. 2009). The optimum pH determined for *HsChiA1p* is 7.3, although it is active within the range 6–9, which is slightly lower than that of 8 found for the amylase mentioned above. Regarding the stability of the recombinant enzyme, *HsChiA1p* has been shown to be very stable, conserving between 65 and 90 % of activity after incubation in suboptimal conditions of temperature (25–45 °C) and pH values (6–8.5). These data are consistent with the advantages attributed to extremophile enzymes, in particular those of extreme halophiles, relating to their stability in adverse and suboptimal conditions (Delgado-García et al. 2012; Eichler 2001). The structural stability of halophilic enzymes in these conditions is attributed to the high content of acidic amino acids in the proteins (Delgado-García et al. 2012), which can exceed 20 %. The stability of *HsChiA1p* could, therefore, be due to its high content (17 %) of acidic amino acids (aspartic acid + glutamic acid).

The recombinant chitinase of *H. salinarum* studied in this work displayed a broad spectrum of activity in relation to temperature, pH, and concentration of NaCl, as well as high stability in suboptimal conditions. These capabilities make it an important enzyme of potential interest regarding the processes of biodegradation or bioconversion of chitin residues (rich in NaCl when of marine origin) into products with added value. The enzyme could act alone or in mixtures with other enzymes that have different mechanisms of action for obtaining *N*-acetylglucosamine or oligosaccharides with biological activity. Despite the applied interest that the enzymes

of halophilic archaea may present, due to their interesting properties, their use is still fairly rare, and they are not considered an attractive option, mainly due to the difficulties of producing them from wild-type strains. The most significant of these problems is the need to cultivate halophilic archaea in the presence of high concentrations of salt, which has made it necessary to develop special bioreactors for this purpose (Litchfield 2011). In this context, *HsChiA1p* has the advantage that it can be expressed as a recombinant protein in *E. coli* and produced in larger quantities in simpler culture media, without NaCl, and more rapidly than using the wild-type strain. Ongoing research to improve and optimize the production of this enzyme in *E. coli* is being carried out in our laboratory.

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