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ORIGINAL ARTICLE

A Mesohaline Thraustochytrid Produces Extremely Halophilic Alpha-Amylases

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Abstract

Halophilic bacteria are well known to produce highly salt-tolerant enzymes that have unusual applications in biotechnology. Production of halophilic proteins is generally not expected in mesohaline microorganisms. *Ulkenia* sp. AH-2, a mesohaline, marine straminipilan thraustochytrid isolated from waters of a mangrove ecosystem, produces halophilic alpha-amylases. Four enzyme fractions, *viz*.., A, B, C, and D, were obtained upon ammonium sulfate fractionation and gel filtration. These had a broad salinity tolerance ranging from 0 to 4 M NaCl, with an optimum at 3 M NaCl. Pools A, C, and D each resolved as a single band on PAGE and zymogram analysis, and the purified proteins were designated *Amy a*, *Amy c*, and *Amy h*. The major activity resided in "pool B," consisting of several amylases which could not be further resolved into pure fractions. Together, these had an optimum at 2 M NaCl. All the enzymes were stable to storage for 2 to 24 h at 4 °C in a range of salt concentrations and even showed enhanced activity following such incubations. True to halophilic enzymes, the complex of "pool B" amylases showed improved activity in the presence of a wide range of organic solvents at 20% concentration. These enzymes are of particular interest by virtue of their constitutive nature as well as production under culture conditions that do not require salinity beyond that of seawater.

Keywords Halophilic · Enzyme · Alpha-amylase · Purification · Thraustochytrid · *Ulkenia* sp.

Introduction

Salts reduce water activity and negatively affect the performance of most enzymes. Halophilic enzymes, however, are stable, highly resistant to denaturation, and active even at salt concentrations as high as 5 M NaCl. Besides, they can function even in non-aqueous media, unlike their non-halophilic counterparts (Marhuenda-Egea and Bonete 2002; van den Burg 2003; Gupta et al. 2003). This enhances their range of applications (Klibanov 2001). As a consequence of their unique properties, halophilic enzymes are of tremendous interest for biotechnological applications in food processing, environmental bioremediation, and biosynthetic processes (Shafiei et al. 2011; Kumar et al. 2016).

Alpha-amylase, an enzyme which cleaves the O-glycosidic bonds in starch, is one of the most commonly studied halophilic enzymes because of its importance in food, fermentation, textile, and paper industries (Kumar et al. 2016). Most studies on halophilic amylases have been reported, understandably, from halophilic bacteria and archaea which are evolutionarily adapted to extreme salt conditions (Amoozegar et al. 2003; Shafiei et al. 2011; Uzyol et al. 2012; Kumar et al. 2016). It might be expected that mesophiles, in general, will not produce halophilic enzymes. We report here the unusual phenomenon of a halophilic amylase from a mesohaline species of marine protists belonging to thraustochytrids.

Thraustochytrids are marine, eukaryotic, osmoheterotrophic, straminipilan fungi that are extremely common in the sea (Raghukumar 2002; Fan and Chen 2006; Bennett et al. 2017; Raghukumar 2017). They are well known for their commercial importance in the production of microbial oil rich in the omega-3 fatty acid, docosahexaenoic acid (Raghukumar 2008). These

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organisms are known to produce a number of enzymes such as cellulases, proteases, and lipases (Bremer and Talbot 1995; Bongiorni et al. 2005; Taoka et al. 2009; Kanchana et al. 2011). We have recently reported the production of alpha-amylases by a number of thraustochytrids (Shirodkar et al. 2017). In this paper, we describe the unusual halophilic property of alpha-amylases from a mesophilic thraustochytrid, indicating the significance and potential usefulness of these enzymes.

Materials and Methods

Organism and Culture Conditions

The thraustochytrid addressed in the present study corresponds to *Ulkenia* sp. AH-2 which had been isolated from mangrove waters and studied earlier for production of various polysaccharide-degrading enzymes including alpha-amylase (Shirodkar et al. 2017). The culture is deposited at the American Type Culture Collection with the accession no. ATCC®PRA-296.

All experiments were carried out using a culture medium that was optimized earlier for α-amylase production using statistical methods (Shirodkar and Muraleedharan 2017). The medium contained (w/v) 0.3% yeast extract, 0.2% corn starch, 0.2% glucose, and 3.4% sea salt. The pH of the medium was 6.8–7.0. A 1% inoculum of a 3-day-old broth culture was added and incubated for 96 h at 25–28 °C in 250-mL Erlenmeyer flasks, on a rotary shaker at 120 rpm. The cells were then separated by centrifugation for 10 min at 4 °C and 10,000 rpm. The clear supernatant served as the crude enzyme preparation for assays and further purification.

Amylase Estimation

The α-amylase activities of crude and purified enzymes were assayed under previously optimized conditions (Shirodkar and Muraleedharan 2017). Thus, 0.5 mL of suitably diluted enzyme was incubated with 1.0 mL of soluble starch (0.3 mg mL^{-1}) prepared in 0.1 M phosphate buffer, pH 7. The reaction mixture was incubated at 50 °C for 10 min and the reaction arrested by adding 0.2 M NaOH. The rate of starch disappearance was measured spectrophotometrically at 578 nm after addition of 1% iodine solution. One unit of alpha amylase activity is defined in terms of milligram starch digested per minute by 1 mL of the enzyme (Fuwa 1954). Protein content was determined according to the Bradford assay (Wilson and Walker 2006). In any experiment, reactions were always carried out in triplicate and the values presented in each dataset are representative of at least three independent experiments.

Effect of NaCl and Organic Solvents

The effect of NaCl on α-amylase activity of the crude enzyme was evaluated at final concentrations of 1–5% NaCl (or 0.17–0.86 M) added in the reaction mixture. For stability studies, the crude enzyme was pre-incubated at 4 °C with NaCl at final concentrations of 0.5 M or 1 M, for 2 h or 24 h, following which the residual activity was assayed. The purified enzymes were tested using concentrations up to 4 M NaCl.

The effects of the organic solvents ethanol, methanol, butanol, acetone, chloroform, benzene, toluene, isopropanol, cyclohexane, and hexane were studied by vortexing the enzyme with 20% (v/v) of the solvent and incubating on a shaker for 1.5 h at room temperature (25 ± 2 °C). The residual amylase activity was estimated and evaluated against the control where solvent was not present.

Enzyme Purification

The crude enzyme preparation was purified by ammonium sulfate fractionation and gel filtration. All steps in the purification procedure were carried out at 2–4 °C, unless specified otherwise.

Fractionation of the crude enzyme was carried out at 0–25%, 25–50%, 50–75%, and 75–90% ammonium sulfate saturations. Another round of fractionation of the crude enzyme was also attempted at 0–20%, 20–40%, 40–60%, 60–80%, and 80–95% saturations. In all these experiments, the pellets obtained in each fraction were re-suspended in a minimum volume of phosphate buffer, pH 7, and dialyzed against the same buffer with three changes after 30 min and 2 h and then continued overnight. A membrane of 10 kDa cutoff was used and the ratio of sample volume to dialysis buffer for each round was typically 1:250. Enzyme activity of fractions was assayed as described above.

A 40–80% ammonium sulfate saturated fraction of the crude enzyme was loaded onto a HiLoad™ 16/600 Superdex™ column (AKTA purifier system, GE Healthcare) equilibrated with 0.1 M phosphate buffer (pH 7.0) containing 0.5 M NaCl. A flow rate of 0.5 mL min^{-1} was maintained and elution was carried out with the same buffer. Two-milliliter fractions were collected and the protein content (A_{280}) as well as the amylase activity were assayed. The fractions obtained in each activity peak were pooled together, and each pool was concentrated overnight at 4 °C by placing in a sealed dialysis bag on a bed of sucrose. These pools were then tested for amylase activity.

Non-denaturing PAGE of each enzyme pool was carried out on 8% polyacrylamide gels and stained for protein as well as for in situ detection of amylase activity as per Dojnov et al. (2008), with slight modifications in incubation temperature and pH, based on the enzyme optima of 50 °C and pH 7. The enzyme activity showed up in the zymogram as clear bands on a dark background. Equal amounts of protein were loaded into each lane to facilitate comparison.

Results

Effect of NaCl

Activity of the crude enzyme was significantly enhanced by sodium chloride at the maximum concentration of 0.86 M that was tested in the assay (Fig. 1). Activity of the enzyme at this salt concentration was 82.58 U mL^{-1}, which was 1.42-fold relative to that in the absence of salt. Salt concentrations lower than 0.86 M did not significantly increase the activity, and activity was lower at 0.17 M compared with the control. The crude enzyme remained stable and active even after storage in 0.5 or 1.0 M NaCl for 2 h or 24 h at 4 °C (Fig. 2). In fact, activity of the enzyme nearly doubled after incubation for 2 h in 1.0 M NaCl. Stability was better when incubated for 2 h rather than 24 h.

Enzyme Purification

Prior to further investigations on its halophilic activity, the crude enzyme was subjected to different purification strategies. Protein staining (Fig. 3a) and zymogram analysis (Fig. 3b) after non-denaturing PAGE of the crude as well as the ammonium sulfate fractions of the enzyme revealed several bands. The four ammonium sulfate fractions at 0–25%, 25–50%, 50–75%, and 75–90% saturations presented eight activity bands on the zymogram, revealing the presence of multiple amylases (Fig. 3b). These were labeled "a", "b", "c", "d", "e", "f", "g", and "h" as per the band sequence observed. Protein content as well as amylase activity appeared to be largely concentrated in the 25–50% and 50–75% fractions in the zymogram. Six of the eight bands also showed up as protein bands after PAGE, while the bands "d" and "g" which appeared in the zymogram did not stain for protein, probably on account of the relatively low concentration.

In an attempt to further refine the separation of the desired proteins, yet another range of ammonium sulfate fractionations at 20% intervals was tested. Maximal enzyme activity was localized in the 40–60% and 60–80% fractions (Table 1). Since ammonium sulfate fractions at 40–60% and 60–80% saturations had concentrated most of the multiple α-amylase activities (Table 1), these were pooled together as a "40–80% saturated" fraction and subjected to further purification by column chromatography. Three distinct protein peaks and four amylase activity peaks were detected (Fig. 4). The four active amylase peak fractions were separately pooled as pool A, pool B, pool C, and pool D. These were then individually concentrated overnight at 4 °C using sucrose. The maximum specific activity of 2650 U/mg (average of multiple runs) was concentrated in pool B. The specific activities in the other three pools A, C, and D were much lower, being 27.97, 46.13, and 32.63 U/mg, respectively.

Non-denaturing PAGE of each of the enzyme pools A, B, C, and D obtained upon gel filtration chromatography, was carried out on 8% polyacrylamide gels and stained for protein as well as for in situ detection of amylase activity (Fig. 5). The pool D band (Fig. 5a) was extremely faint due to the comparatively low protein concentration. Identical pools obtained from three independent FPLC runs were put together for pools A, C, and D while fractions from 13 independent gel filtration runs were put together for pool B. The zymogram as well as Coomassie Brilliant Blue–stained gels of these PAGE profiles (Fig. 5a, b) indicated the same broad distribution of amylase activity evidenced in ammonium sulfate fractions (Fig. 3). The relative positions of most of the multiple amylases as seen after native PAGE correlated well with the sequence of their elution during molecular sieving, suggesting very similar charges on the individual amylase proteins.

The zymogram analyses and corresponding protein staining showed that pool A, pool C, and pool D displayed a single band each, indicating purification to homogeneity (Fig. 5b). These corresponded to bands "a," "c," and "h" detected in PAGE runs of the ammonium sulfate fraction (Fig. 3). These purified amylases from pools A, C, and D, respectively (Fig. 3), have been labeled *Amy a*, *Amy c*, and *Amy h*.

Pool B PAGE patterns presented several bands. This pool clearly displayed "a," "b," "c," "e," "f," and "g" bands, with different intensities (Fig. 5a, b). The band "h" was found to be

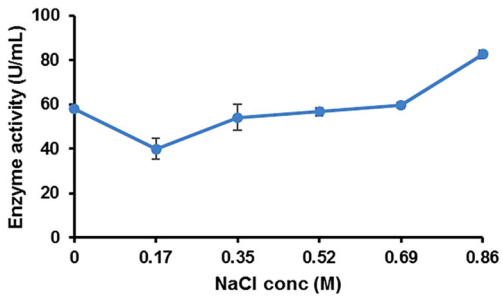

Fig. 1 Effect of NaCl on the crude alpha amylase activity of thraustochytrid isolate AH-2

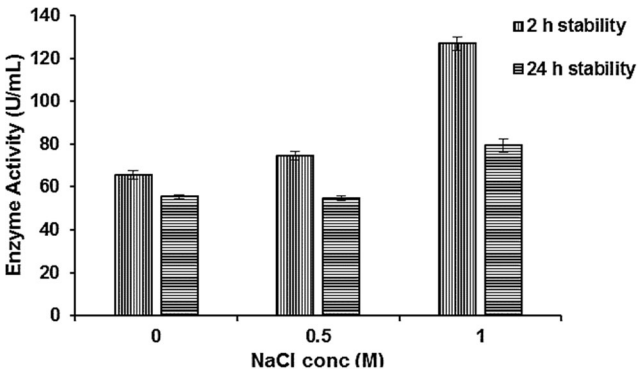

Fig. 2 Effect of NaCl on stability of the crude alpha amylase activity of thraustochytrid isolate AH-2

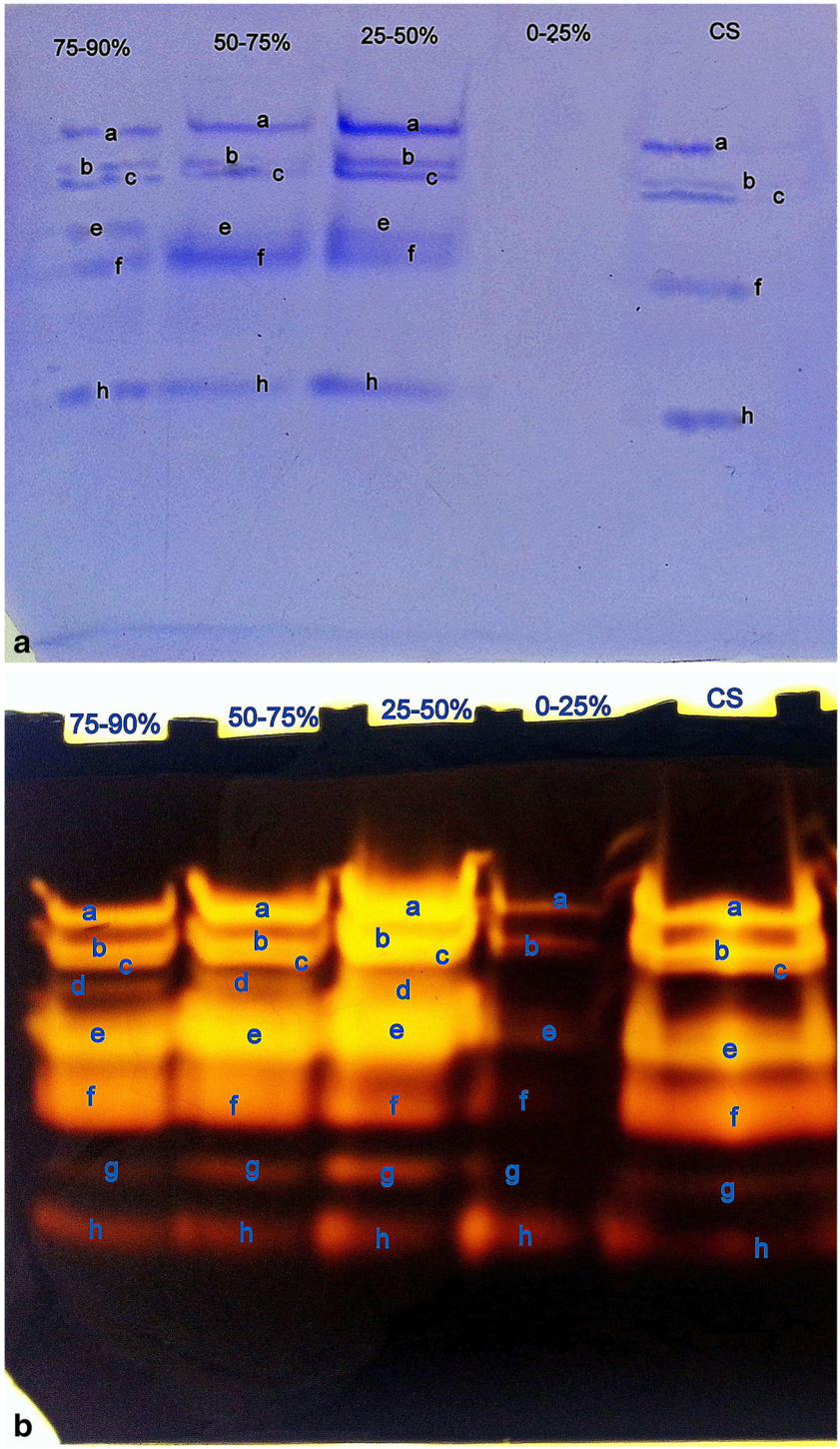

Fig. 3 Protein (**a**) and amylase activity (**b**) profiles of ammonium sulfate fractions on native PAGE

completely separated from among the multiple amylase activity bands. Despite several attempts, we were not able to completely purify and separate out the individual amylase fractions of pool B. The results on purification of these amylases are summarized in Table 2. The amylase activity was purified 4.11-fold, with a recovery of 92.92% after gel filtration on a Superdex™ column.

Effect of NaCl and Organic Solvents on Purified Amylases

All three purified amylases, *Amy a*, *Amy c*, and *Amy h*, were halophilic and showed increasing activity up to a concentration of 3 M NaCl (Fig. 6a). Specific activity of the fraction *Amy a* increased by nearly 100% at 3 M, compared with the

Table 1 Distribution of α-amylase activity upon ammonium sulfate fractionation

Ammonium sulfate fraction	α-Amylase activity (U mL^{-1})
Crude enzyme	39.05
0–20%	53.33
20–40%	87.60
40–60%	1564.88
60–80%	1063.14
80–95%	244.63

control that lacked NaCl. The other two fractions showed an increase of 50 to 80% at 3 M NaCl concentration. Relative activity of all three purified fractions declined at 4 M. However, the activity of *Amy a* was nearly 50% more than the control even at this salt concentration. While the specific activity of the enzyme complex of pool B was much higher, it showed optimal activity at lower salt concentrations of 1–2 M NaCl rather than at 3 M (Fig. 6b).

The purified enzyme samples were tested for their stability at final concentrations of 1 to 4 M NaCl for 2 h at 4 °C (Fig. 7a). All three enzyme fractions not only were stable at concentrations of 1 to 3 M NaCl but also showed increasingly enhanced activity. The activities of *Amy a*, *Amy c*, and *Amy h* improved by about 47, 12, and 32%, respectively, after incubation in 3 M NaCl. While the relative activity of *Amy a* and *Amy h* decreased when pre-incubated at 4 M, *Amy a* continued to show comparatively higher activity in relation to the control. The alpha-amylase activity of the enzyme complex of pool B also increased upon incubation under saline conditions up to 2 M NaCl concentration (Fig. 7b).

The enzyme complex of pool B was active in the presence of all ten organic solvents tested (Fig. 8). Activity was in fact stimulated in the presence of acetone, toluene, chloroform, and hexane compared with the control. Benzene had a slightly negative effect.

Discussion

Salt-tolerant alpha-amylase has been reported almost exclusively from halophiles such as the bacteria *Acinetobacter* sp., *Bacillus dipsosauri*, *Halobacillus* sp., *Halobacterium halobium*, *H. sodomense*, *Halomonas meridiana*, *Halothermothrix orenii*, *Haloferax mediterranei*, *Marinobacter* sp., *Micrococcus halobius*, *M. varians*, *Natronococcus amylolyticus*, and *Nesterenkonia* sp., besides the fungus *Aspergillus penicillioides* (Amoozegar et al. 2003; Ali et al. 2015; Kumar et al. 2016). The present study demonstrates the unusual presence of highly salt-tolerant alpha-amylase enzymes in the mesohaline thraustochytrid protist, *Ulkenia* sp. AH-2. This species, originally isolated from mangrove region waters of approximately 31 ppt (or 3.1%) salinity, was earlier found to be able to grow over a wide range of salt concentrations of 1–10% (w/v), with 3.4% salt yielding maximum production of alkaline proteases and lipases (Kanchana 2007). By virtue of their predominantly saprobic role in the marine ecosystem, thraustochytrids are known to produce a variety of degradative enzymes. They are also commercial sources of omega-3 PUFA-rich oils (Bongiorni 2012; Raghukumar 2008; Bennett et al. 2017).

Purification of halophilic amylases has generally yielded a single protein (Shafiei et al. 2012; Kumar et al. 2016). Prakash et al. (2009) purified two different amylases from *Chromohalobacter* sp. The presence of at least three distinct halophilic alpha amylase proteins in *Ulkenia* sp. AH-2 appears to be unique. Halophily was observed in the crude enzyme and also in every single purified fraction of amylase, viz., *Amy a*, *Amy c*, and *Amy h*. Halophily was also noticed in pool B, the rich pool of mixed amylases. The complex of enzyme bands designated a, b, c, d, e, f, and g found therein could not be separated out, probably because these proteins had very similar pI values.

The purified *Amy a*, *Amy c*, and *Amy h* alpha-amylases from *Ulkenia* sp. AH-2 were active from 0 to 4 M NaCl. The alpha-amylases from this isolate were thus versatile and active even in the absence of salt. However, true to their

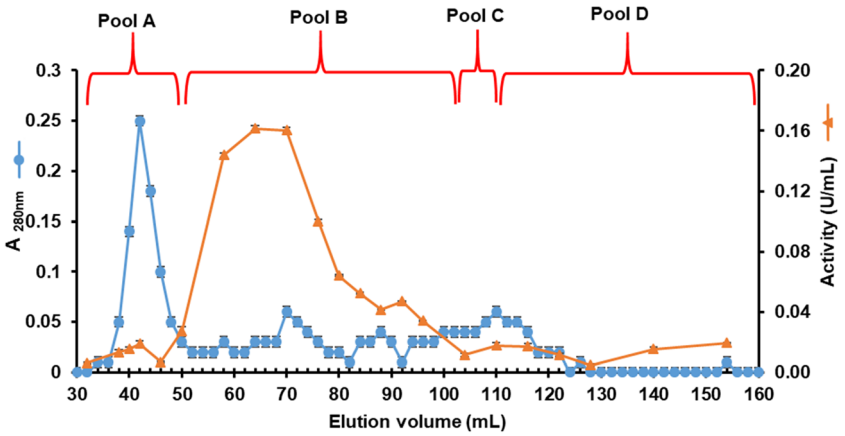

Fig. 4 Elution pattern of protein and α-amylase activity using HiLoad™ 16/600 Superdex™ column

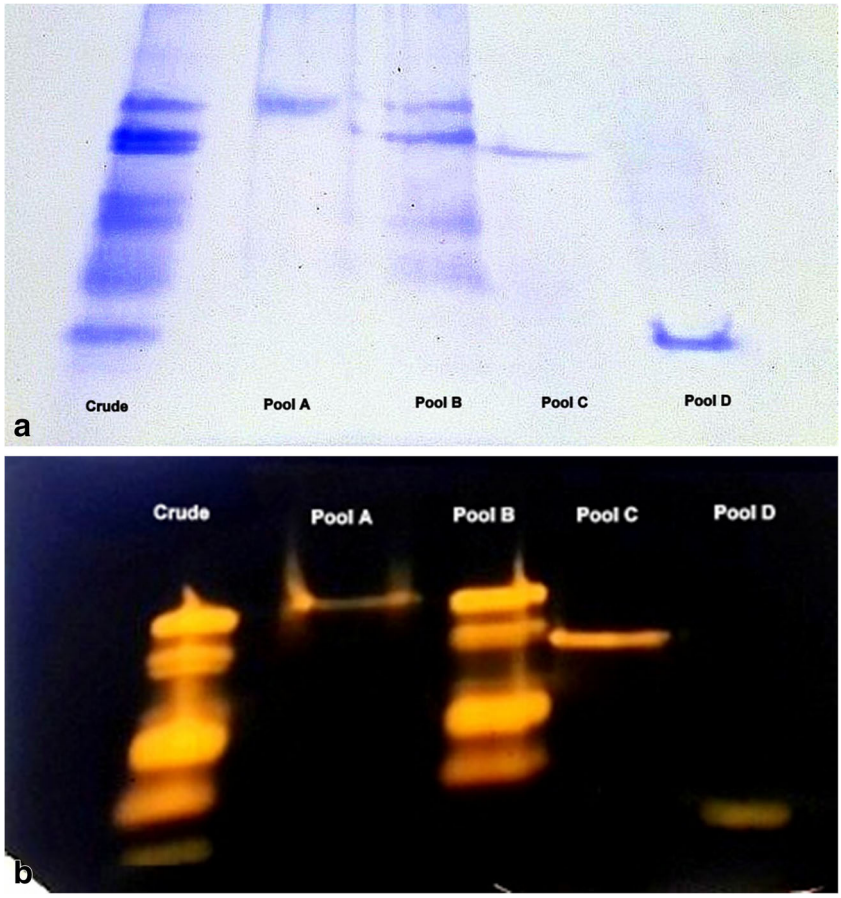

Fig. 5 Protein (**a**) and amylase activity (**b**) profiles of the various pools from chromatography fractionation of amylases, as separated by native PAGE

halophilic nature, their optimal activity was elicited at 17.5% (or 3 M) NaCl concentration. In comparison, halophilic bacteria display varying salt concentration optima for enzyme activity, ranging from 5% as in *Halomonas meridian* to 25% as in *Halobacterium halobium* (Kumar et al. 2016). A number of halophilic fungi, particularly belonging to black yeasts such as *Hortaea werneckii* and *Wallemia* sp., capable of growth at up to 32% salinity, have been studied intensely in recent years (Raghukumar 2017). Production of polyextremophilic alpha-amylase has been described in a fungus *Aspergillus penicillioides* (Ali et al. 2015).

An additional heat treatment step in the purification scheme can at times simplify the purification of thermotolerant proteins, and preliminary experiments on the crude enzyme had indicated some extent of its thermal stability. Accordingly, the crude enzyme preparation was subjected to a 10-min heat treatment at 50 °C prior to fractionation with ammonium sulfate. Likewise, in another set of experiments, the crude enzyme was first fractionated with ammonium sulfate and the individual fractions then subjected to heat treatment. Whether carried out before or after the ammonium sulfate fractionation, heat treatment did not significantly contribute to an increase in specific activity (data not shown) and hence, this was not integrated as a step in the purification procedure.

Ulkenia sp. AH-2 produced a maximum of about 60 U mL^{-1} amylase at 0% NaCl concentration. The highest activity of 5831 U mL^{-1} was measured in the 40–80% ammonium sulfate fraction of the enzyme preparation. It is difficult to compare our results with those of others since different assay methods have been followed by individual researchers

Table 2 Purification of multiple amylases

Purification step	Volume (mL)	Total activity (U)	Protein (mg/mL)	Specific activity (U/mg protein)	Yield (%)	Fold purification
Crude enzyme	1500	91,860	0.095	644.63	100	1
40–80% ammonium sulfate fraction	15.0	87,465	4.667	1249.41	95.22	1.94
Superdex chromatography pool B	54.5	85,355	0.591	2650.00	92.92	4.11

who have reported activities ranging from 0.7 U mL^{-1} in *Halorubrum xinjiangense* to 90 U mL^{-1} in *Micrococcus* sp. (Kumar et al. 2016).

Non-halophilic proteins are generally distorted in the presence of organic solvents. On the contrary, halophilic proteins are resistant to low-water-activity environments, such as in the presence of organic solvents (Marhuenda-Egea and Bonete 2002; Kumar et al. 2016). In conformation with this unusual property of halophilic enzymes, alpha-amylases of *Ulkenia* sp. AH-2 were active even in the presence of organic solvents at a concentration of 20%. This is similar to the results of Shafiei et al. (2012) who found that amylolytic activity of alpha-amylase from *Nesterenkonia* sp. was enhanced not only by 20% of water-immiscible organic solvents but also by acetone, ethanol, and chloroform. Such enzymes that do not require special stabilization such as immobilization, chemical or physical modification, and molecular engineering could be very useful for biotechnological applications that employ organic solvents as reaction media (Ogino et al. 2000; Ogino and Ishikawa 2001).

Halophilic enzymes have adapted to high-salt environments by acquiring a relatively large number of negatively charged amino acid residues on their surfaces to prevent precipitation. Consequently, their solubility is often very poor in surroundings with lower salt concentrations (Shafiei et al. 2011, 2012). Further work on amino acid composition of the alpha-amylase of *Ulkenia* sp. AH-2 will be interesting.

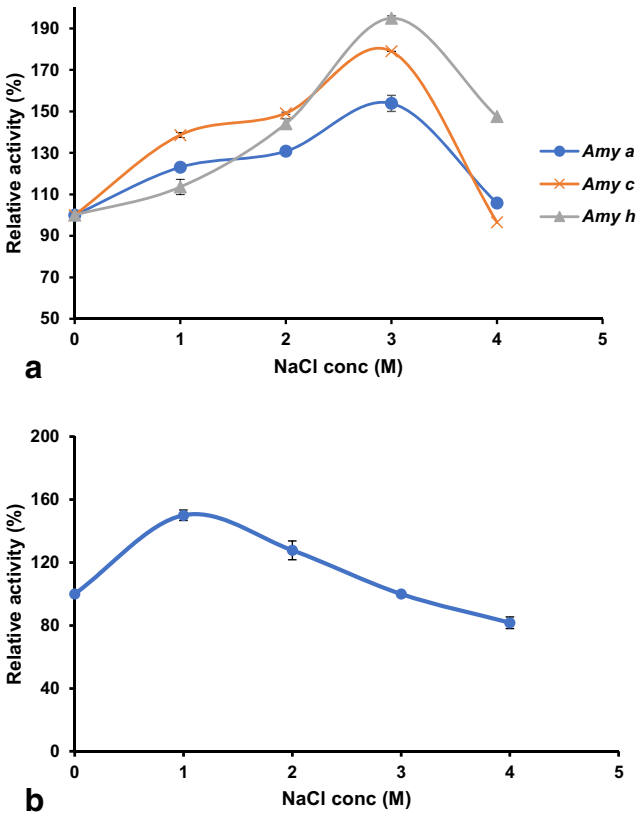

Fig. 6 **a** Effect of NaCl on the activity of purified amylases *Amy a*, *Amy c*, and *Amy h*. **b** Effect of NaCl on activity of the multiple enzymes in pool B

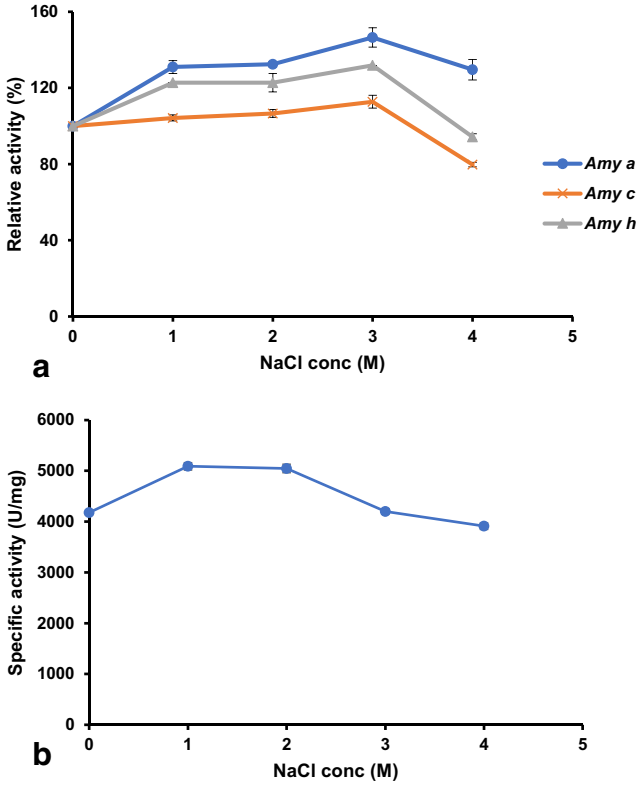

Fig. 7 **a** Effect of NaCl on the stability of purified amylases *Amy a*, *Amy c*, and *Amy h*. **b** Effect of NaCl on stability of the multiple enzymes in pool B

Halophilic alpha-amylases from *Ulkenia* sp. AH-2 are of interest for two further reasons. Their activity being constitutive (Shirodkar et al. 2017), substrate addition in the growth medium was not necessary, thereby diminishing production costs and complexity. These amylases can also be generated in a simple seawater medium, without the addition of high amounts of salts as is generally required for halophilic bacteria. Such production of halophilic enzymes even in the absence of high amounts of salts would particularly increase the feasibility of large-scale production.

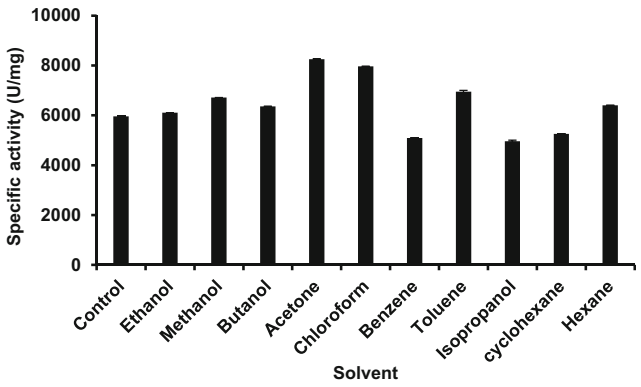

Fig. 8 Effect of solvents on pool B amylase activity

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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