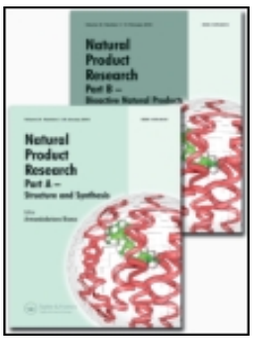

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SHORT COMMUNICATION

Thermostable alkaline halophilic-protease production by *Natronolimnobius innermongolicus* WN18

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This study reports the production and biochemical characterisation of a thermostable alkaline halophilic protease from *Natronolimnobius innermongolicus* WN18 (HQ658997), isolated from soda Lake of Wadi An-Natrun, Egypt. The enzyme was concentrated by spinning through a centriplus, centrifugal ultrafiltration Millipore membrane with a total yield of 25%. The relative molecular mass of this protease determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis ranged from 67 to 43 kDa. The extracellular protease of *N. innermongolicus* WN18 was dependent on high salt concentrations for activity and stability, and it had an optimum temperature of 60°C in the presence of 2.5 M NaCl. This enzyme was stable in a broad pH range (6–12) with an optimum pH of 9–10 for azocasein hydrolysis. This extracellular protease, therefore, could be defined as thermostable and haloalkaliphilic with distinct properties that make the enzyme applicable for different industrial purposes.

Keywords: *Archaea*; haloalkaliphile; *Natronolimnobius innermongolicus* WN18; extremozymes; protease

1. Introduction

Extremely halophilic *Archaea* (haloarchaea) belonging to order Halobacteriales had been isolated from various hypersaline (3–5 M) environments. The haloalkaliphilic *Archaea* form a distinct physiological group which require not only 4–5 M NaCl for growth but also high pH (8.5–11) and low Mg^{2+} concentrations ($\leq$10 mM) (Tindall et al. 1984). The isolation of a haloalkaliphilic *Archaea*, grown optimally at pH 10, from a saline–alkaline soil was reported (Wang et al. 2010).

Many haloarchaea secrete proteolytic enzymes which enable the degradation of proteins and peptides in the natural environment. Several of these extracellular serine proteases have been purified and characterised. These include proteases of 40–66 kDa isolated from neutrophilic haloarchaea including strains of *Halogeometricum borinquense* strain TSS101 (Vidyasagar et al. 2006), *Halobacterium salinarum* (*Halobacterium halobium*) (Ryu et al. 1994), *Natrialba asiatica* 172 P1 (Kamekura et al. 1992) and *Haloferax mediterranei* 1538 (Stepanov et al. 1992) and R4 (Kamekura & Seno 1993). Extracellular proteases have also been isolated from haloalkaliphilic *Archaea* including a 49-kDa protease of unidentified strain A2 (Yu 1991), a 45-kDa protease of *Natrialba magadii* (Gimenez et al. 2000) and an unusual protease of 130-kDa

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from *Natronococcus occultus* (Studdert et al. 1997). In addition, it was reported that the gene encoding the protease Nep secreted by *N. magadii* was cloned and sequenced (De Castro et al. 2008). Most of these proteases have distinct properties since they are dependent on high concentrations of salt for structural stability and display optimum activity in high salt, alkaline pH and temperatures of 50°C or higher. However, despite these studies, proteases from haloalkaliphilic *Archaea* have been less investigated. Therefore, further research efforts are required for the extraction and characterisation of enzymes from haloalkaliphilic *Archaea* due to their biotechnological potential for novel applications (Shameer et al. 2013). In this study, an attempt was made for partial purification and characterisation of thermostable alkaline halophilic protease produced by newly isolated *Natronolimnobius innermongolicus* WN18.

2. Results and discussion

2.1. *Isolation and identification of the strain*

The strain WN18 was isolated from the water sample collected from Hamra Lake (30°23′48.28″N, 30°19′13.39″E) located at Wadi An-Natrun, Egypt. The enrichment and isolation conditions were described by Selim et al. (2012). A set of archaeal specific primers was used to amplify the 16S rRNA according to Ventosa et al. (2004). A volume of 50 ng/μL of each PCR product was used to prepare the samples which were delivered to Macrogen Company in Korea (http://www.macrogen.com) following their specifications. The sequences were analysed using BLAST (http://www.ncbi.nih.gov/BLAST/) to get a preliminary identification of the strains. This revealed that the isolate was *N. innermongolicus* strain; the GenBank accession number was HQ658997. Representatives of maximum homologous (93–98%) sequences of each isolate were obtained and the phylogenetic affiliation was constructed by using MEGA 5 software package. The phylogenetic tree was constructed using only the culturable *N. innermongolicus* (Supplementary Figure S1).

2.2. *Production of extracellular proteolytic activity during growth*

To investigate the occurrence of extracellular protease during growth, samples were taken at different times, and the azocaseinolytic activity of the cell-free medium was measured. Maximum protease production was obtained after one week of incubation (25 U mg^{-1}) during the stationary phase (Supplementary Figure S2). Also, maximum activity of protease was obtained during stationary phase in *N. magadii* (Studdert et al. 1997) and late exponential growth phase in *N. occultus* (Studdert et al. 1997). The enzyme containing supernatant was concentrated by spinning through a centriplus, centrifugal ultrafiltration Millipore membrane (Amicon, Merck Millipore Ltd., Tullagreen, Carrigtwohill, Co. Cork, Ireland) (30,000 molecular weight cut-off) and dialysed against 10 mM Tris–HCl (pH 8.5) containing 3 M NaCl. The relative molecular mass of this protease was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on vertical slab gels containing 10% acrylamide in the presence of 4 M betaine (Supplementary Figure S3), since it partially prevents autolysis of halophilic protease (Studdert et al. 1997). The purified sample of protease revealed two bands, ranging from 67 to 43 kDa, but it is not known that whether these two bands represent different size classes of the same protein. The apparent molecular mass of the major protein bands in *N. occultus* and *N. Magadii* proteases were 90 and 36 kDa, respectively (Gimenez et al. 2000).

2.3. *Effect of salt concentration, pH and temperature on activity and stability*

The extracellular protease of *N. innermongolicus* was found to be active at 60°C (Supplementary Figure S4A); however, no proteolytic activity was detected at 70°C. The activity was still

retained up to 100% and 65% of the initial activity after incubation at 55 and 60°C for 2 h, respectively. This revealed that the enzyme was stable up to 60°C (Supplementary Figure S5). The maximum activity of the enzyme was detected at a concentration of 2–2.5 M NaCl (at 40°C and pH 9), the protease was stable at all NaCl concentrations (100% of residual activity in 1–3 M NaCl), However, the enzyme activity declined at <0.5 M NaCl (Supplementary Figure S4B). The purified enzyme was found to be active and stable in a broad pH range (6–12), and exhibited the highest azocaseinolytic activity between pH 9 and 10 in the presence of 2.5 M NaCl at 40°C (Supplementary Figure S4C). These properties were slightly similar for halolysin 172 P1 (Kamekura & Seno 1990) and for the extracellular serine protease of *N. occultus* (Studdert et al. 1997) and also for *N. magadii* (Gimenez et al. 2000); however, the stability was favoured at 60°C for 2 h.

2.4. *Effect of some metal ions on the protease activity*

The effect of some divalent cations on protease was assessed by measuring the azocasein hydrolysis activity of the purified enzyme in the presence of various ions at a concentration of 2 mM (Supplementary Figure S6). The results indicated that Ca^{2+} was particularly effective in activating the enzyme, while Mn^{2+} and Mg^{2+} displayed less positive effects. Zn^{2+}, Fe^{2+} and Co^{2+} possessed some inhibitory effects, 85%, 75% and 65% decrease in activity, respectively. The observation of the significant effect of Ca^{2+} on protease activity is in agreement with Ward (1983) who reported the role of Ca^{2+} in maintaining the activity of serine protease; also, Towatana et al. (1999) reported that Ca^{2+} has the ability to retard enzyme denaturation. Subtilisin-like protease from hyperthermophilic archaeon exhibited 70% and 80% of the maximal activity in the presence of 1–100 mM $CaCl_2$ (Kannan et al. 2001). Vidyasagar et al. (2006) observed that the thermostability of a haloalkaliphilic extracellular serine protease produced by an extreme halophilic archaeon *H. borinquense* strain TSS101 increased in the presence of Ca^{2+}.

3. Conclusion

Strain *N. innermongolicus* WN18 (HQ658997) isolated from Soda Lake of Wadi An-Natrun, Egypt, possessed the ability to produce thermostable, alkaline halophilic protease. The protease of the strain *N. innermongolicus* exhibited optimum activity for azocasein hydrolysis in the presence of 2.5 M NaCl, pH 9–10, at a temperature of 40°C. Therefore, the possibility of using this extremozyme in industrial process has an advantage of optimal activity at alkaline conditions and high salt concentrations.

Supplementary materials

Experimental details relating to this article are available online, alongside Figures S1–S6.

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