International Journal of Biological Macromolecules 70 (2014) 222–229

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

# Halophilic alkali- and thermostable amylase from a novel polyextremophilic *Amphibacillus* sp. NM-Ra2

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ARTICLE INFO

*Article history:*
Received 7 May 2014
Received in revised form 26 June 2014
Accepted 27 June 2014
Available online 5 July 2014

*Keywords:*
Amylase
Halophilic alkalithermophilic
Wadi An Natrun

ABSTRACT

Extracellular gluco-amylo-pullulanase from *Amphibacillus* sp. NM-Ra2 was purified to homogeneity by ethanol precipitation, anion exchange chromatography and gel filtration chromatography. Molecular mass of the enzyme was 50 kDa (SDS-PAGE). The enzyme showed maximal activity at 1.9 M NaCl, $pH^{50\,°C}$ 8.0 and 54 °C and was active from 0 to 4.3 M NaCl and 37 to 65 °C. The enzyme was inhibited by EDTA and was stable and active in the presence of PMSF, DTT, $H_2O_2$, Triton-X-100, Tween 20 and Tween 80. $Ca^{2+}$ is inessential for activity. The amylase was stimulated with $K^+$ and inhibited with $Cu^{2+}$ and $Mg^{2+}$. $Hg^{2+}$, $Zn^{2+}$ and $Fe^{2+}$ had no effect on activity. Amylase was stable and active in the presence of ethanol, methanol and benzene (25%, v/v). The enzyme hydrolyzed linear and branched polysaccharides including pullulan, glycogen and amylopectin, and hydrolyzed raw wheat starch and raw corn starch (14.6% and 13.5% over 2 h). Amylase activity was inhibited by soluble starch concentrations greater than 0.3%. The major products of soluble starch hydrolysis were maltose and maltotriose. The amylase, being halophilic and alkali-thermostable, in addition to being resistant to surfactants, oxidizing agents and organic solvents, can find applications in the starch processing, pharmaceutical, food and paper/pulp industries.



## 1. Introduction

Amylases (endo-1,4-α-D-glucanohydrolases EC 3.2.1.1) are extracellular enzymes that hydrolyze α-1,4-D glucosidic linkages in polysaccharides to produce different products such as dextrins and smaller polymers of glucose units [1]. The α-amylases have been isolated from animals, plants and microorganisms, but microbial amylases are the mostly used in industrial applications; the pharmaceutical industry uses amylases as digestive aids, the textile industry for sizing of textile fibers [2], and the food industry for the production of glucose and high fructose corn syrups, reduction of viscosity of sugar syrups, reduction of turbidity to produce clarified fruit juice with longer shelf-life and saccharification of starch [2]. The baking industry uses amylases to delay the staling of bread and the paper industry uses amylases for reduction of starch viscosity for the even coating of paper [3].

Many of the commercially available amylases do not withstand harsh industrial reaction conditions. Industries relying on biotransformations in aqueous/organic and non-aqueous media necessitate biocatalysts that are stable and active in low water activity as well as oxidizing conditions and/or presence of surfactants [4]. Amylases used in the food industry need to be independent of divalent cations, particularly calcium, for activity. Many of the amylases currently available require calcium for activity and thermostability [5]. The textile, paper and detergent industries require amylases stable at alkaline pH and high temperature [2]. The present industrial enzyme collection is not sufficient to meet all industrial demands, mainly due to enzyme inhibition and/or instability under industrial applications [6]. Due to this, identifying new amylases that can withstand harsh conditions, such as alkaline pH, high temperature, hypersalinity and/or low water activity, as well as oxidizing conditions and surfactants, is important.

Enzymes isolated from microorganisms inhabiting extreme environments generally show maximal activity and stability under extreme conditions. Halophilic alkaliphilic microorganisms live and multiply in environments with salinities between 0.4 and 3.5 M, alkaline pH values greater than 8 and often temperatures exceeding 40 °C. Extracellular enzymes produced by these

*Abbreviations:* β-ME, β-mercaptoethanol; DNS, dinitrosalicylic acid; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLC, thin layer chromatography.

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http://dx.doi.org/10.1016/j.ijbiomac.2014.06.053
0141-8130/

organisms are expected to show activity in hypersaline conditions, low water activity, alkaline pH values as well as are stable at elevated temperatures [4].

This study describes the purification and characterization of an extracellular amylase with pullulanase activity from a novel *Amphibacillus* sp. NM-Ra2, isolated from the alkaline, hypersaline lakes of the Wadi An Natrun, Egypt. The enzyme is halophilic, thermophilic, alkali-stable and is stable in the presence of different surfactants and organic solvents, and thus has the potential for application in different industries.

## 2. Materials and methods

### 2.1. Organism and culture conditions

*Amphibacillus* sp. NM-Ra2 was isolated from Lake UmRisha, an alkaline, hypersaline lake present within the Wadi An Natrun, Egypt. Mixed water/sediment samples (5 g) were inoculated into enrichment medium consisting of, g $L^{-1}$: soluble starch, 5; yeast extract, 1; NaCl, 100; $Na_2CO_3$, 20; $NH_4Cl$, 1; $KH_2PO_4$, 2; $K_2HPO_4$, 7, $MgCl_2$, 0.1. Enrichment cultures were incubated at $pH^{50\,°C}$ 8.5 and 50 °C for 96 h (The superscript after the pH indicates the temperature at which the pH was measured and pH meter calibrated. Details on accurate measurement of pH at high temperature are given in Wiegel [7]). Pure isolates were obtained by repetitive dilution to extinction in the same enrichment medium supplemented with 1% (w/v) agar. Isolates were screened for amylolytic activity by flooding agar plates with 0.3% $I_2$ (w/v) and 5% (w/v) KI solution. Clear zones of starch hydrolysis against a dark blue background indicated amylolytic activity. Isolate NM-Ra2 produced the largest clearance zone, and was selected for further study.

Isolate NM-Ra2 was identified by 16S rRNA amplification and sequencing. The 16S rRNA gene was amplified using universal primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′). The amplified products were sequenced using a 3730xl capillary DNA analyzer (Applied Biosystems) operated at the Georgia Genomics Facility (University of Georgia, Athens, GA USA). The complete 16S rRNA gene sequence for strain WN-NM-Ra2 was compared with GenBank entries by BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For phylogenetic analysis, the complete gene sequence was aligned with closely related sequences using the ClustalX program (http://bips.u-strasbg.fr/fr/Documentation/ClustalX/) and phylogenetic trees were constructed using the PHYLIP software package (http://evolution.genetics.washington.edu/phylip.html).

The culture medium used for enzyme production was the same as the enrichment medium described above. One hundred milliliters of culture were grown in 250 mL Erlenmeyer flasks at 50 °C with constant shaking. After 96 h of incubation, the culture was centrifuged at 3500 × *g* for 30 min at 4 °C and the supernatant was used for amylase purification.

### 2.2. Purification of the amylase

Pre-chilled ethanol (−15 °C) was added slowly to the supernatant to 80% (v/v) saturation and the mixture was incubated at −15 °C for 4 h. The precipitate was collected by centrifugation for 5 min at 3500 × *g*, and dissolved in a minimum volume (~50 mL) of 50 mM Tris–Cl, pH 8.0. Suspended protein was dialyzed against 2 L of the same buffer overnight, with one buffer change. The sample was applied to a Q-sepharose FF column (10 × 1.6 cm, GE Healthcare) which had been equilibrated with the same buffer. Bound proteins were eluted with a linear gradient of 0–1.5 M NaCl in the same buffer at a flow rate of 1.5 mL/min. Fractions showing amylase activity were pooled (final sample volume was ~10 mL), desalted by dialyzing overnight against 2 L of 50 mM Tris–Cl, pH 8.0, and concentrated by ultrafiltration (Vivaspin, Sartorius AG). The concentrated protein was loaded on a Superdex™ 75 gel filtration column (1.5 × 20 cm) pre-equilibrated with 50 mM Tris–Cl, pH 8.0 containing 150 mM of NaCl, and eluted with the same buffer at a flow rate of 0.1 mL/min. Fractions with amylase activity were pooled, dialyzed against Tris–Cl, pH 8.0 to remove residual salt, and concentrated by ultrafiltration as described above. The purified enzyme was used for further biochemical characterization.

### 2.3. Amylase assay

Amylase activity was determined by measuring the amount of reducing sugars released during starch hydrolysis using the dinitrosalicylic acid method [8]. The reaction mixture, 200 μL final volume, consisted of 50 mM Tris–Cl pH 8.0, 1.7 M NaCl and 20–30 μg of purified protein. Reaction mixtures were incubated at 50 °C for 20 min. The reaction was stopped by adding 100 μL of DNS solution and the mixture was boiled for 15 min. After cooling to room temperature, the reaction mixture was diluted with 700 μL of distilled water and absorbance at 540 nm was measured. For all enzyme assays described, linearity of the assays was assessed over time (30 min), and a no-enzyme control was run to correct for non-specific hydrolysis of the substrate. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol of maltose per minute at $pH^{50\,°C}$ 8.0, 1.7 M NaCl and 50 °C. Protein concentration was measured by the Bradford method [9] using bovine serum albumin as standard.

### 2.4. Polyacrylamide gel electrophoresis

The molecular weight and purity of the enzyme was estimated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [10]. Samples were boiled for 5 min in Laemmli sample buffer before loading onto 8% polyacrylamide gels. After electrophoresis, the gels were stained with the SERVA silver staining kit (Serva Gmbh, Heidelberg, Germany). The molecular weight of the enzyme was estimated using an EZ-Run ™ *Rec* protein ladder (Fisher Bioreagents). Molecular weight of the protein was confirmed by native PAGE.

To demonstrate amylase activity of the purified protein, samples that had not been boiled were run on an 8% non-denaturing polyacrylamide gel. After electrophoresis, the gel was incubated in 50 mM Tris–Cl, pH 8.0 buffer containing 0.2% (w/v) soluble starch and 1.7 M NaCl at 50 °C for 30 min, then stained with iodine reagent (0.3% $I_2$/0.7% KI, w/v). Amylase activity was demonstrated by a white area against a dark blue background.

### 2.5. Biochemical properties of purified amylase

#### 2.5.1. Effect of salt

To demonstrate the effect of NaCl on enzyme activity, amylolytic activity was measured using the standard assay in the presence of increasing concentrations of NaCl (0–4.3 M), at $pH^{50\,°C}$ 8.0 and 50 °C.

#### 2.5.2. Effect of pH and temperature

To determine the effect of $pH^{50\,°C}$ on amylase activity, amylase activity was tested over a pH range 7.0–11.5 (measured at 50°C), at 50 °C in the presence of 1.7 M NaCl. The buffer system used was 50 mM Tris ($pH^{50\,°C}$ 7.0–9.0) and 50 mM glycine-NaOH ($pH^{50\,°C}$ 9.0–12.0).

To evaluate the optimal temperature for enzyme activity, enzyme assays were conducted at different temperatures ranging from 30 to 80 °C, at $pH^{50\,°C}$ 8.0 in the presence of 1.7 M NaCl. Thermal stability of the enzyme was determined by pre-incubating the