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Investigation of Extracellular Highly Thermostable Starch Hydrolyzing Activity from A Novel Thermophilic Bacterium *Anoxybacillus gonensis* A4

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A novel hot spring thermophile, *Anoxybacillus gonensis* A4 was investigated in terms of capability of starch degradation and characterization of its thermostable hydrolytic activity. It was observed that *A. gonensis* A4 has a hydrolytic enzyme responsible for starch hydrolysis with a 0.6 R_f value. The extracellular crude preparation was characterized in terms of pH and temperature optima and stabilities, kinetic parameters and inhibition/activation behaviour toward some chemicals and metal ions. Starch agar assay showed that *A. gonensis* A4 secreted a hydrolytic enzyme and K_m and V_{max} values of its activity were found to be 1.88 mg/mL and 0.54 U/mg protein, respectively. The optimum temperature and pH, for *A. gonensis* A4 hydrolase was 50 ºC and 7.5, respectively. The enzyme activity was not significantly changed by incubating crude extract solution at the temperature range from 20 to 80 ºC for 60 h. pH-stability profile of the crude enzyme for 24 h at 4 ºC of incubation temperature showed that the enzyme had good stability over 65 % at all investigated pH range from 4.5-9.5. The efficiency of metabisulphite on crude amylolytic activity indicated that *A. gonensis* A4 produce a hyrolaze having disulfide bridges were essential for starch hydrolysis.

Key Words: Amylase, *Anoxybacillus*, Thermophile, Thermostability.

INTRODUCTION

α-Amylases (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1)hydrolyze α-1,4-glucosidic linkages in starch and related substrates[1]. Amylases are among the most important enzymes and are of great significance in present-day biotechnology. Although they can be derived from several sources, such as plants, animals and microorganisms[2-4], enzymes from microbial sources generally meet industrial demands. The spectrum of amylase

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application has widened in many other fields, such as clinical, medical and analytical techniques, as well as their wide spread application in starch saccharification and in the textile, food, brewing and distilling industries[5]. Currently, thermostable amylases of *Bacillus stearothermophilus* or *Bacillus licheniformis* are being used in starch processing industries[6]. Thus, the highly thermostable and thermoactive amylolytic enzymes from extreme thermophiles are still being sought for the improvement of the starch hydrolysis process. Except for α-amylases, β-amylases, glucoamylases, pullulanases and isoamylases are known as other industrially significant amylolytic enzymes that hydrolyse α-1,4 or α-1,6 glycosidic bonds. Today, a large number of enzymes are known which hydrolyse starch molecule into different products and a combined action of various enzymes is required to hydrolyze starch completely[7].

Anoxybacillus gonensis A4, a hot spring thermophile, has been isolated from hot springs of Gönen in Turkey and characterization based on its biochemical, chemo-taxonomic and genetic properties in our laboratories[8]. The present study was aimed at screening and partial characterization of thermostable hydrolytic activity responsible for hydrolysis of the starch from *A. gonensis* A4, the strain that formed a large halo around the colony as a amylase producer. The data obtained from this study will gain to focus on the further investigation of immobilization of *A. gonensis* A4 for highly thermostable crude amylolytic activity.

EXPERIMENTAL

All chemicals were of reagent grade and obtained from the following sources: Sigma Chemical Co. (St Louis, MO, USA) and Merck A.G. (Darmstadt, Germany).

Culture conditions and enzyme production: Luria-Bertani (LB) broth medium containing 1 % peptone, 0.5 % yeast extract, 1 % (w/v) soluble starch, 0.5 % NaCl was used to cultivate thermophile. The medium was adjusted to pH 7.5 with 2 M NaOH before sterilization[9]. The bacterium was grown in LB broth medium at 60 ºC for 18 h on a shaker operating at 200 rpm. After incubation cells were pelleted by centrifugation at 5000 rpm for 0.5 h the supernatant was used as crude enzyme extract.

Screening of thermophiles for their amylolytic activity potentials: LBA (Luria-Bertani agar) containing 1 % peptone, 0.5 % yeast extract, 1 % (w/v) soluble starch, 0.5 % NaCl and 1.5 % agar was used for streak plate inoculation. The cells were growth for 2 d at 60 °C. Then, hydrolytic enzyme production was detected with the formation of clear zone arounding the colonies after flooding the plates with iodine solution for 10 min. Iodine solution was prepared by adding 0.3 g iodine, 0.6 g KI and 1 g $NaHCO_3$ in 100 mL distilled water.

Spectrophotometric enzyme assay: Extracellular amylolytic activity was assayed by measurement of the iodine-complexing ability of starch[10]. The reaction mixture contained 500 µL of crude enzyme, 250 µL of 1 % soluble starch and 250 µL 50 mM phosphate buffer at pH 7.5. Reaction was performed for 15 min at 60 ºC. The reaction was stopped by addition of 1 mL of 0.5 N HCl. 100 µL of the mixture was taken in a test tube and 5 mL of iodine solution added on to it. Iodine reagent was prepared by adding 0.5 g iodine and 5 g KI in 100 mL distilled water. Iodine solution was prepared by diluting iodine reagent in the ratio of 1:100 with distilled water. The blue colour developed was analyzed in spectrophometer at 590 nm using working iodine solution as blank. One unit of hydrolytic enzyme activity was defined as the amount of enzyme that hydrolyzes 1 mg soluble starch in 1 min under relevant conditions[10].

Protein determination: Protein quantity in the enzyme extracts was determined according to the Lowry method with bovine serum albumin as standard. The values were obtained by graphic interpolation on a calibration curve at 650 nm[11].

Preperation of growth and activity profile: The isolate was grown in LB broth medium at 60 ºC for 24 h. During this period, 1 mL aliquots from culture medium were harvested every 2 h intervals. Optical densities of the samples were estimated turbidimetrically with a spectrophotometer at 660 nm. Enzyme activity assay was performed as stated in spectrophotometric enzyme assay after preparing crude extract. Growth and activity curves were prepared by plotting optical densities and enzyme activities *vs.* time[12].

Determination of the optimum temperature for enzyme secretion: After determination of optimum time for bacterial growth and enzyme activity, optimum growth temperature for enzyme production was studied. 1.0 mL of seed culture were inoculated to 100 mL Erlenmeyer flasks containing 20 mL of LB broth medium and incubated at 37, 42, 50, 55, 60 and 65 ºC respectively, on a shaker operating at 200 rpm for 18 h. Cells were participated, pellet was discarded and the supernatant was examined for hydrolytic activity[10,13]. Optimum growth temperature of *Anoxybacillus gonensis* A4 for maximum enzyme activity was determined and used for following studies.

Native gel electrophoresis: Native gel electrophoresis was carried out according to the Laemmli method[14], in the absence of SDS. Activity staining was accomplished by the incorporation of starch into the acrylamide matrix of the resolving gel. When preparing the gels, 1 % soluble starch was used in place of distilled water when the gels were cast. To observe the thermophilic amylolytic activity following electrophoresis, the spacers and the gel left between the glass plates were sealed with starch film to prevent desiccation. The gel was then incubated for 3 h at 60 ºC, then stained with

iodine solution. The band containing the hydrolaze responsible for hydrolysis of starch appeared as a clear area in the blue background of the gel[15].

Effect of pH and temperature on enzyme activity: This studies were performed as done by Yildirim *et al.*[16]. The effect of pH on amylolytic activity was determined by measuring the activity at 60 ºC using acetate (50 mM, pH 4.5, 5.5), phosphate (50 mM, pH 6.5, 7.5) and *Tris*-HCl (50 mM, pH 8.5, 9.5) buffers. The temperature optima for activity was determined by assaying activity between 20 and 70 ºC with 10 °C increments in phosphate buffer (50 mM, pH 7.5). Following studies were performed at these optimum pH and temperature values.

Effect of protein concentration on enzyme activity: Hydrolytic activity, as a function of protein concentration, was determined in a protein concentration range of 0-1.5 mg/mL for *A. gonensis* A4 at optimum reaction conditions[17].

Effect of substrate concentration on enzyme activity: Amylolytic activity, as a function of substrate concentration was determined in a substrate concentration range of 0-10 mg/mL for *A. gonensis* A4 at optimum reaction conditions using various volumes of the potato starch solution as substrate. The Michaelis-Menten constant (K_m) and the maximum velocity of the reaction (V_{max}) were calculated from Lineweaver-Burk plot[18].

pH and thermal stability: For the investigation of pH stability, the enzyme solution was 2-times diluted with the buffers used to determine pH optimum and then incubated at 4 ºC for 24 h. The residual activity was assayed[17] at 50 ºC and at pH 7.5.

Thermostability of the enzyme was performed by pre-incubating enzyme samples at temperatures between 20 and 70 ºC with 10 °C increments for 60 h. The residual activity was determined at 50 ºC and pH 7.5. The percentage residual enzyme activity was calculated by comparison with the activity observed in the presence of unincubated enzyme[17,19].

Effect of metal ions and some compounds on enzyme activity: The chloride salts of the some mono-, di- and trivalent metal ions or various compounds were added to reaction mixture seperately that the final concentration of each adjust 10 mM. The activity of enzyme assayed in the absence of metal ion and compound was taken as 100 %. The percentage relative activities were expressed by comparison with standard assay mixture at optimum conditions with no metal ion addition[16]. Na^+, Ca^{2+}, Hg^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, EDTA, $Na_2S_2O_5$ were used to study the effect of metal ions and compounds on amylolytic activity. After addition of each chemical solution at 10 mm final convcentration, the activity was assayed.

Commercial starch hydrolyzing capacity of *A. gonensis* A4: Amylolytic activity was determined at optimum reaction conditions by using some commercial starches, such as potato, corn and wheat as substrates.

Statistical analysis of data: Analysis of variance of the data was performed with SPSS 10.0 for Windows (USA). The LSD multiple range test was employed to determine the statistical analysis. In all figures and tables, data points represent mean of three determinations ($p \leq 0.05$).

RESULTS AND DISCUSSION

A hydrolytic enzyme (responsible for starch degradation) secretion by *Anoxybacillus gonensis* A4 on Luria-Bertani agar plate containing 1 % starch was qualitatively monitored by the formation of clear zone around the colonies by treatment with iodine solution after 2 d incubation at 60 ºC (Fig. 1a). Native gel electrophoresis on crude enzyme from *A. gonensis* A4 indicated the presence of an amylolytic enzyme having *ca.* 0.6 R_f value (Fig. 1b). It can be easily extracted from these results that *A. gonensis* A4 produce an extracellular enzyme responsible for degradation of starch. α-Amylases with the molecular weight in the range of 10-210 kDa from various microorganisms were known. Thermophilic α-amylases from *Bacillus* sp. isolate ANT-6, *Pyrococcus furiosus* and *Cryptococcus* S-2 with the molecular weight as 94.5, 129, 66 kDa, respectively, were reported[3,15,20].

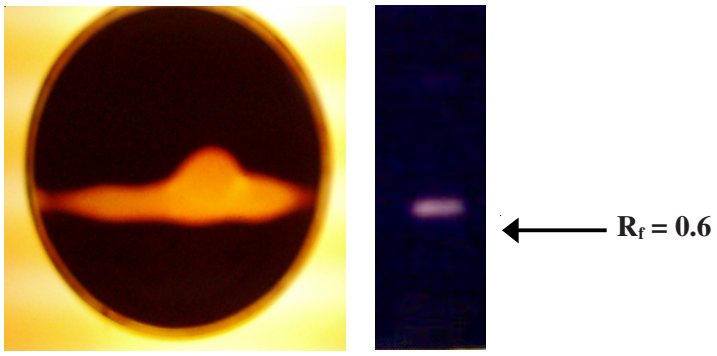

Fig. 1. (A) Screening of an amylolytic enzyme secretion by *Anoxybacillus gonensis* A4 to the culture medium and (B) native gel electrophoresis

After qualitative determination, growth and activity profile of *A. gonensis* A4 *vs.* time (0-24 h) was prepared to observe optimum growth time with the optimum amylolytic activity. The results show that *A. gonensis* A4 secrete a hydrolaze with optimum activity at the end of 18 h growth period (data not shown).

Growth temperature of organism can be related to the hydrolytic enzyme production. *A. gonensis* A4 was grown at different temperatures between 37 and 65 °C for 18 h to screen optimum enzyme secretion to the culture medium. While isolates had been growing, 55 °C was found to be

optimum enzyme secretion temperature for *A. gonensis* A4 (Fig. 2). Lin *et al.*[13] and Busch & Stutzenberger[21] were also reported the same optimum α-amylase secretion temperature for *Bacillus* sp. TS-23 and thermophilic fungus *Thermomonospora fusca*, respectively.

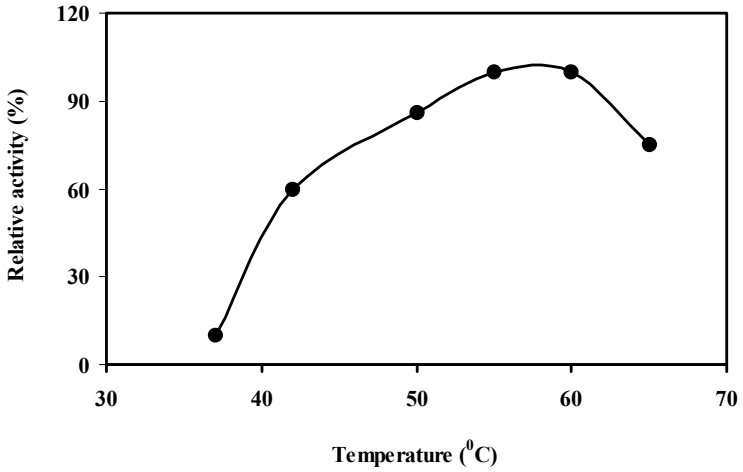

Fig. 2. Amylolytic enzyme secretion by *Anoxybacillus gonensis* A4 at different growth temperatures

Effect of protein concentration on enzyme activity: The amylolytic activity of *A. gonensis* A4 was found to be protein concentration dependent. The enzyme activity increased and reached the maximum value as 0.35 U/mg protein at the 1 mg/mL final protein concentration in the reaction mixture and then remained constant after this amount of protein (figure not presented). This specific activity value was lower than the values obtained for purified *Bacillus* sp. TS-23[13] and *Bacillus flavothermus*[1] extracellular α-amylase, relatively. Optimum protein concentration was almost close to declared concentration levels of purified *Enterobacter cloacae* IIT-BT 08[10].

Effect of substrate concentration on enzyme activity: *A. gonensis* A4 amylolytic enzyme showed a Michaelis-Menten type kinetics when hydrolyzing starch. Substrate saturation curves indicated that *A. gonensis* A4 hydrolaze saturated with 2 mg/mL starch concentration. As calculated from Lineweaver-Burk plots the K_m and V_{max} values at 60 °C were 1.88 mg/mL and 0.54 U/mg protein, respectively (Fig. 3). Thermophilic *E. cloacae* IIT-BT 08 saturated with 8 mg/mL starch concentration[10]. Kinetic parameters for crude extract of *A. gonensis* A4 hydrolaze in good agreement with the earlier reports for other purified thermophilic amylases when used soluble starch as a substrate. The K_m values for *B. flavothermus*[1], *E. cloacae* IIT-BT 08[10] and *Bacillus* sp. TS-23[13] were reported as 2.2, 0.9

and 2.7 mg/mL, respectively. The V_{max} value of *A. gonensis* A4 hydrolase was also well consistent with the purifed *E. cloacae* IIT-BT 08 amylase as 18.18 U/mL[10]. It is naturally expected that maximum activity of purified enzyme is higher than crude one.

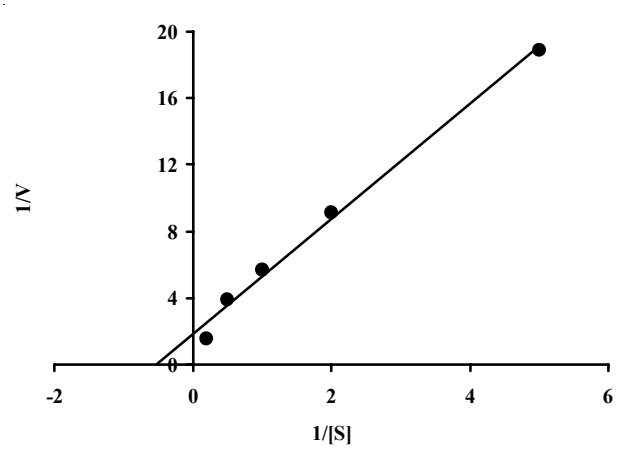

Fig. 3. Dependence of *Anoxybacillus gonensis* A4 amylolytic activity on substrate concentration

Effect of pH on enzyme activity and pH stability: The optimum pH of the enzyme was 7.5. It could be considered from Fig. 4 (inset) that the enzyme has a broad pH range of activity over 75 % between 5.5-9.5. The long-term (24 h) pH stability of the crude enzyme at 4 °C of incubation temperature indicated that the enzyme had good stability over 65 % at all investigated pH, range from 4.5-9.5. However, it was seen that *A. gonensis* A4 hydrolaze responsible for starch degradation displayed minimal pH stability when it was incubated at optimum pH. Optimum pH for known amylases shows[1,10,13,15,22] variety in the range of pH 4-9. Consequently, it can be said that *A. gonensis* A4 hydrolaze is fairly stable for pH changes. The stability of the enzyme in acidic and basic pHs and at high temperatures suggests its usefulness in industrial applications.

Effect of temperature on α-amylase activity and thermal stability: To determine the optimum temperature, the enzyme activity was measured at different temperatures (10-70 °C) and the optimum amylase activity was observed at 50 °C. Fig. 5 (inset) shows the thermal activity profile having a shoulder with a relative starch hydrolyzing activity over 68 % between 20-70 ºC for extracellular crude amylase from *A. gonensis* A4. A higher amylase activity at higher temperatures over 40 ºC is well consistent with the opinion which thermostable enzymes could be extracted from thermophilic microorganisms are active at high temperatures. Similar

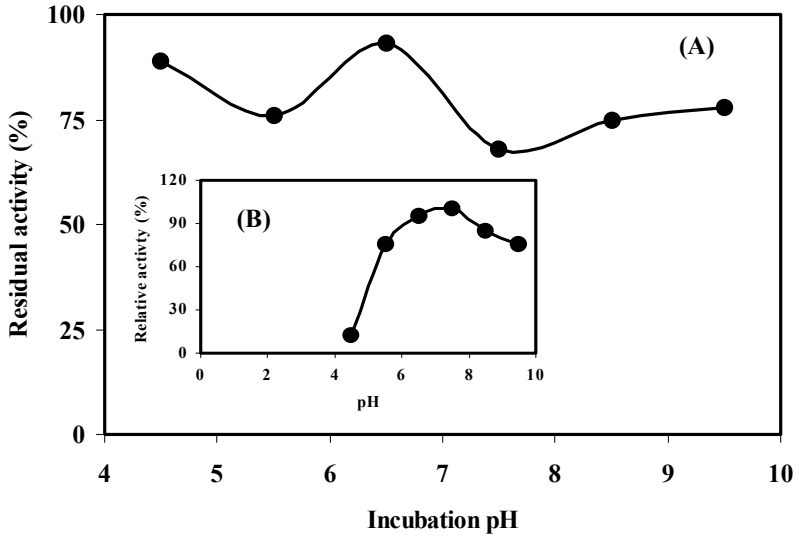

Fig. 4. (A) pH stability profile of *Anoxybacillus gonensis* A4 hydrolase and (B) Effect of pH on Anoxybacillus gonensis A4 amylolytic activity

results were reported for *E. cloacae* IIT-BT 08[10] and *Bacillus* sp.TS-23 amylases[13]. For determination of thermal stability, crude enzyme was pre-incubated at the temperatures between 20 and 80 ºC for 60 h and activity was measured at optimum temperature. Up to 60 ºC, original activity was almost retained for all incubation temperatures. After 30 h incubation period at 70 and 80 ºC, a decline at enzyme activity was observed. However, enzyme maintained its original activity over 80 % both 70 and 80 ºC for 60 h incubation period. It can be said that *A. gonensis* A4 α-amylase was fairly thermostable. From both thermal activity and thermal stability profiles, it can also be speculated that crude enzyme exctract prepared from *A. gonensis* A4 can be used in any temperature from 30 to 70 °C in a biotechnological applications. The thermal stability of amylases can be attributed to some factors such as substrate and presence of stabilizers[23]. The stabilizing effect of starch was reported for α-amylases from *B. licheniformis* CUMC 305 and *Bacillus* sp. WN 11[24,25]. Thermal stability of *A. gonensis* A4 amylase is superior to those of other notable amylases from some *Bacillus* sp.[26,27]. In addition, α-amylase from *Anoxybacillus gonensis* genus is heat stable and may be used starch hydrolysis at high temperature.

Effect of metal ions and some compounds on amylolytic activity: The effect of different metal ions and compounds *e.g.*, Na^+, Ca^{2+}, Hg^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, EDTA and $Na_2S_2O_5$ on *A. gonensis* A4 amylolytic activity was investigated using crude enzyme extract pre-incubated with some chemicals at room temperature for 10 min. The relative activity was

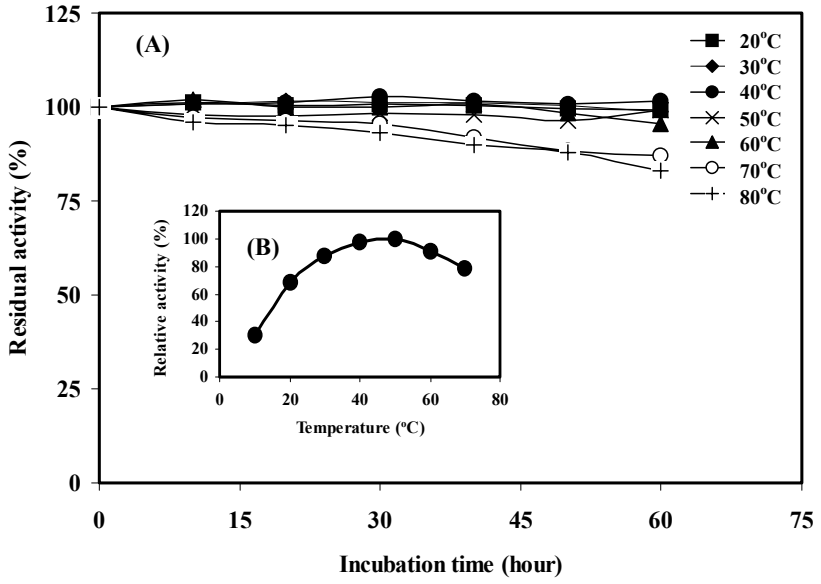

Fig. 5. (A) Thermal stability profile of *Anoxybacillus gonensis* A4 hydrolase and (B) Effect of temperature on *Anoxybacillus gonensis* A4 amylolytic activity

measured at optimum pH and temperature. Among the tested ions, Cu^{2+}, Cd^{2+}, Hg^{2+} and Zn^{2+} at 10 mM final concentration, decreased the activity at the different levels (Table-1). It was reported that many metal ions especially heavy metals totally or partially inhibited α-amylase activity[7,28]. Similar results were found for *Bacillus* sp. TS-23 and *Lactobacillus manihotivorans* LMG 18010T amylases. *Bacillus* sp. ANT-6 amylase was highly thermostable related to inhibition with Zn^{2+} like present study[3]. On the other hand, a stimulated activity was observed in the presence of Na^{+} and Ca^{2+}. *L. manihotivorans* LMG 18010^{T} amylase was also slightly stimulated in the presence of these cations at the same final concentration[28]. It is known that Ca^{2+} generally increase[7], but it is also reported that it had no or slightly enhancement on amylase activity[9,28]. EDTA almost did not affect, but metabisulphite as reducing agent of disulfide bridges strongly inhibited *A. gonensis* A4 amylolytic enzyme activity at 10 mM final concentration in the reaction mixture. It can be concluded from this result that disulfide bridges were essential for starch hydrolysis. It was declared that EDTA and sulphydryl group reagents inhibited amylase activity[7]. Various effects of EDTA at different final concentartion on amylase activity have been reported. Complete inhibition[13], partial inhibition[3] and no inhibition[22] of amylase activity was observed (Table-1).

TABLE-1
EFFECT OF SOME COMPOUNDS ON *A. gonensis* A4 AMYLOLYTIC ACTIVITY

Compounds (10 mM)	Relative amylolytic activity (%)
Control (no compound added)	100 ± 2
NaCl	113 ± 2
$CaCl_2$	107 ± 1
$HgCl_2$	75 ± 1
$CdCl_2$	69 ± 4
$CuCl_2$	37 ± 1
$ZnCl_2$	94 ± 2
EDTA	103 ± 1
Sodium metabisulphite	10 ± 1

Commercial starch hydrolyzing capacity of *A. gonensis* A4 hydrolaze: Some commercial starch substrates such as corn, potato and wheat starches were tested for substrate specificity of *A. gonensis* A4 amylolytic enzyme. The enzymatic hydrolysis of raw starches by the action of *A. gonensis* A4 hydrolase were nearly same with small difference. The relative activites (%) in the presence of potato, corn and wheat starches as substrate were 100, 86 and 85 %, respectively. It is easily seen that the enzyme is the most efficient when used with potato starch and decomposes these substrates with similar mechanisms. Potato starch used as substrate was reported by Lin *et al.*[13] and Bose and Das[29].

In conclusion, a thermophilic bacterium *A. gonensis* A4 isolated from hot springs in Turkey, secretes an extracellular hydrolaze responsible for the degradation of starch. This extracellular crude preparation was investigated in terms of pH and temperature optima, stability, kinetic parameters and commercial starch hydrolyzing capacity. It was found that *A. gonensis* A4 produced a good thermostable extracellular amylolytic enzyme and enzyme was highly stable at all investigated pH values. These characteristics of *A. gonensis* A4 hydrolaze suggest its usefulness in industrial applications.

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