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RESEARCH ARTICLE

Production and characterization of thermostable α-amylase from thermophilic *Anoxybacillus flavithermus* sp. nov. SO-19

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This study was concerned with isolation and identification of thermophilic bacteria from hot spring in Afyonkarahisar (Gecek) and optimization of α-amylase production, partial purification of α-amylase, and characterization of extracellular enzyme from isolated thermophilic strain 19. To characterize and identify the thermophilic isolated bacteria, morphological analysis and biochemistry tests were studied. Besides, for classification 16S rRNA gene, G–C content and DNA–DNA hybridization analysis were performed. These results indicated that strain 19 is a novel species, *Anoxybacillus flavithermus* sp. nov. The effects of different fermentation conditions, such as incubation time, temperature, and pH, different carbon and nitrogen sources, and surfactants on α-amylase production were investigated. Various parameters such as temperature and temperature stability, pH and pH stability, detergents and surfactants, different starches, and metal ions on influence of enzyme characterization were assayed. About 93, 87, and 81% of the activities were retained after heating the partially purified enzyme solution at 50, 60, and 70 for 240 min, respectively. Enzyme was excessively inhibited by Hg^{2+} (6%). The enzyme was activated by Co^{2+} (212%) and Mg^{2+} (142%). Enzyme degradated 82% of starch content in apple juice at 70°C in 30 min. The molecular weight of enzyme was estimated as 96 kDa.

Keywords:
Anoxybacillus flavithermus / α-Amylase / Apple juice industry / Detergent industry / Thermostable

1 Introduction

Thermophiles live at high temperatures where most of the other organisms fail to survive. The enzymes from thermophiles are known as thermophilic enzymes which are thermostable and thermoactivity. From this point of view, these enzymes find a number of commercial applications [1, 2]. Among the thermophilic bacteria, thermophilic bacilli have increased as the source of thermostable enzymes such as amylases, cellulases, glucose-isomerases, lipases, proteases, pullulanases, xylanases, and DNA restriction endonucleases [3].

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Starch and its components, amylose, and amylopectin are hydrolyzed by α-amylases which are the most important enzymes related to the hydrolysis of α (1→4) glycosidic bonds [4]. α-Amylases (endo-1,4-α-d-glucan glucanohydrolase EC 3.2.1.1) are extracellular endoenzymes [5]. Thermostable α-amylases are very important for industrial uses [6], which are widely used for glucose and fructose syrups, baking, brewing, food, fruit juices, pharmaceuticals, textiles, papers, and detergent industries [6–10].

Thermostable α-amylases are especially available from the thermophilic bacteria such as *Geobacillus stearothermophilus*, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus cereus*, and *Anoxybacillus* [11]. Among these thermophilic bacteria, the genus of *Anoxybacillus* was first reported by Pikuta [12]. Poli reported 11 new *Anoxbacillus* species which are *A. pushchinoensis*, *A. flavithermus*, *A. gonensis*,

A. contaminans, *A. voinovskiensis*, *A. ayderensis*, *A. kestanbolensis*, *A. kamchatkensis*, *A. amylolyticus*, *A. rupiensis*, and *A. bogrovensis* [13].

Bacterial classification and determination are based on 16S rRNA gene sequencing. 16S rRNA gene analysis is essential of bacterial classification due to almost presence in all bacterial species, unchanged function of the gene, and enough length of DNA (1500–bp) [14]. Universal 16S rRNA gene primers were used for identification and study of bacterial community. Moreover, DNA–DNA hybridization is used in classification. DNA–DNA hybridization is confessedly the "gold standard" for defining new species and for the accurate assignment of a strain with tentative properties to the right taxonomic unit [15].

Less than 97% similarity score of 16S rRNA genes of an individual species to a nearest-neighbor species represents a new species, whereas more than 97% similarity does not clarify whether it is a new species or not [16]. This biostatistical information can represent a new species or represent a previously defined taxon. So, DNA–DNA hybridization is needed to answer those questions [15].

This study was carried out for isolation and identification of thermophilic bacteria, optimization of thermostable α-amylase production, partial purification of α-amylase, and its characterization. In addition, the availability of thermostable α-amylases in some industries such as detergents, starch hydrolysis, and apple juice clarification was experimented.

2 Materials and methods

2.1 Morphological and biochemical tests

In this study, a bacterium, which was isolated by Dr. Sadin Özdemir and Dr. Veysi Okumus from hot-spring water of Gecek, Afyonkarahisar in Turkey, was used. The morphological and biochemical identification of the isolated bacteria were tested. To determine the characteristics of the bacterium Gram, spore-staining methods and motility tests were used. The biochemical tests such as starch, gelatin, and casein hydrolysis, and catalase and lipase activities were examined for determination of some characteristics of the isolate.

2.2 DNA isolation and polymerase chain reaction (PCR) and phylogenetic analysis of 16S rRNA

For DNA isolation, first bacterial sample (number 19) which is taken from solid agar plate was overnight incubated in 50 mL liquid broth agar medium that was prepared with tap water at 60°C in rotary shaker at 120 rpm for 16 h. After that, total genomic DNA isolation was done by using Fast DNA Spin Kit For Soil (MP Bio, USA) following manufacturer's protocol. Isolated DNA was quantified using Qubit fluorometer (Invitrogen, USA). Then, total DNA was run in 1% (0.5 g agar/50 mL 1× TAE buffer) agarose gel at 110 V and visualized under Gel Doc (Bio-Rad, USA) imaging system.

To amplify 16S rRNA gene region, PA/PH primer sets (PA: 5′-AGAGTTTGATCCTGGCTCAG-3′, PH: 5′-AGGGAGGTGATCCAGCCGCA-3′) were used in PCR [17]. PCR constructed with 1 μM of each primer set, 0.2 mM dNTP, 10 ng of DNA as template, and 1 U i-StarTaq™ DNA polymerase in the buffer was provided by the manufacturer (INTRON Biotechnology, Inc., USA) in a 40 μL PCR volume totally. A C1000™ Thermal Cycler (Bio-Rad, USA) was used in PCR, and the program was set as 5 min at 95 °C, 30 s at 95°C, 35 cycles of 30 s at 55°C, 45 s at 72°C; and extension for 10 min at 72°C. After PCR, PCR product was purified by QIAquick PCR Purification Kit (Qiagen, Germany) following instruction's protocol and quantified using Qubit fluorometer (Invitrogen, USA). Purified DNA was analyzed with 1% (0.5 g agar/50 mL 1× TAE buffer) agarose gel at 110 V and visualized under Gel Doc (Bio-Rad, USA) imaging system.

Last, sequencing of 16S rRNA gene which belongs to bacterial sample numbered as 19 was performed with PA primer on an AB1373 Automated Sequencer (Invitrogen, Carlsbad, USA) at Iontek Company (Istanbul, Turkey).

2.2.1 Phylogenetic analysis

Sequence data were submitted to GenBank database and its accession number is KJ095001. Homology search of sequence data was performed using Basic Local Alignment Search Tool (BLAST) server of the National Centre for Biotechnology Information using the BLAST algorithm. Nucleotide sequence was queried against sequence database (BLAST), and most closely related 16S rRNA genes of species were determined.

The evolutionary history was inferred using the neighbor-joining method of Saito and Nei [18]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [19]. The evolutionary distances were computed using the *p*-distance method [20] and are in the units of the number of base differences per site. Six nucleotide sequences [sequenced nucleotide (sample 19, accession no. KJ095001) and the closest species nucleotide sequence (accession no. KF952570.1)] and four close species nucleotide sequences (taken from NCBI). All positions containing gaps and missing data were eliminated. There were a total of 1100 positions in the final data set. Evolutionary analyses were conducted in MEGA6 20 [21].

2.3 Determination of GC content of DNA by HPLC and DNA–DNA hybridization

GC content of newly isolated thermophilic bacteria and DNA–DNA hybridization were determined in DSMZ, Germany. For this purpose, cells were cultivated and

disrupted by using a Constant Systems TS 0.75 kW instrument (IUL Instruments, Germany). The DNA was purified on hydroxyapatite according to the procedure of Cashion et al. [22]. The DNA was hydrolyzed with P nuclease and the nucleotides dephosphorylated with bovine alkaline phosphatase [23]. The resulting deoxyribonucleosides were analyzed by HPLC. GC was calculated from the ratio of deoxyguanosine and thymidine according to the method of Mesbah et al. [23]. *Bacillus subtilis* DSM 402 (43.518 mol% G + C), *Xanthomonas campestris* pv. *campestris* DSM 3586^T (65.069 mol% G + C), *Streptomyces violaceruber* DSM 40783 (72.119 mol% G + C), and nonmethylated Lambda DNA (49.858 mol% G + C; Sigma) were used as references for DNA.

2.3.1 HPLC apparatus

The HPLC system (Shimadzu Corp., Japan) consisted of the following modules: LC-20AD solvent delivery module, DGU-20A3 online degasser, CTO-10AC column oven, SIL-20 automatic sample injector, and SPD-20A UV spectrophotometric detector. Chromatograms were analyzed by using the CLARITY (Version 2.4.1.93) software package (DTA Apex Ltd., Czech Republic). The analytical column was a VYDC 201SP54, C_{18}, 5 μm (250 × 4.6 mm) equipped with a C_{18} guard column.

2.3.2 Chromatography conditions

Temperature 45°C, 10 μL sample, solvent 0.3 M (NH_4) H_2PO_4/acetonitrile, 40:1(v/v), pH 4.4, and 1.3 mL/min. These conditions were adapted from Tamaoka and Komagata [24].

2.3.3 DNA–DNA hybridization

Cells were disrupted by using a Constant Systems TS 0.75 kW (IUL Instruments, Germany), and the DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion et al. [22]. DNA–DNA hybridization was carried out as described by De Ley et al. [25] under consideration of the modifications described by Huss et al. [26] using a model Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with in situ temperature probe (Varian).

2.4 Submerged fermentation

All experiments were studied in duplicates using 250 mL Erlenmeyer flasks containing 50 mL fermentation media, as per experimental design is adjusted to optimum pH with 0.1 M HCl or NaOH. These flasks were autoclaved at 121°C for 15 min. Then each flask was inoculated with overnight culture of 1 mL cell suspension prepared in nutrient broth. Inoculated flasks were incubated on a rotary shaker incubator at 55°C and 120 rpm for 24 h.

2.5 Enzyme assay

α-Amylase activity was experimented according to the Bernfeld method [27]. According to this method, 100 μL enzyme solution and 200 μL starch solution (0.5%) were incubated for 30 min at 70°C. After 30 min of incubation, the reaction was stopped by adding 400 μL 3,5-dinitrosalicylic acid, the reaction mixture was then heated in the boiling water bath for 5 min. The reducing sugars released were measured by UV/VIS spectrophotometer at 489 nm. One unit of enzyme activity was defined as the amount of enzyme that produced 1 mmol of reducing sugars maltose per minute under the assay conditions.

2.6 Effect of incubation time on α-amylase production

The effect of different incubation time (0–96 h) on α-amylase production by thermophilic bacteria was studied at 55°C in nutrient broth (NB) liquid media at 120 rpm in shaker at pH 7.0. A 2 mL culture sample was taken for 0, 4, 8, 12, 16, 24, 36, 48, 72, and 96 h and then was centrifuged at 7000 rpm for 10 min. Supernatant was used for enzyme activity based on the standard assay procedure described in Section 2.5.

2.7 Effect of incubation temperature and pH on α-amylase production

In order to determine the best incubation temperature on α-amylase production, bacteria were cultured at various incubation temperatures (30–80°C) on optimum incubation time. The optimum pH for amylase production by thermophilic bacteria was experimented at different pH values (5.0–10.0) of NB culture medium on optimum incubation conditions. The pH was adjusted with 0.1 M HCI and NaOH. After incubation, the culture medium centrifuged and the supernatant was used for α-amylase activity as given procedure above.

2.8 Effect of carbon and nitrogen sources on α-amylase production

Anoxybacillus flavithermus sp. nov. was cultured in the NB liquid medium containing different carbon sources such as glucose, galactose, maltose, lactose, fructose, sucrose, wheat, and maize starch at a concentration of 1% (w/v), in order to study their effect on enzyme production. After incubating the organism under the optimum culture conditions, the crude enzyme was harvested and tested to determine the enzyme activity. To determine the best nitrogen sources (1%, w/v) for maximum enzyme production was tested using various nitrogen sources such as glycine, tryptone, urea,

ammonium sulfate, ammonium nitrate, natrium nitrate, yeast extract, and bacto liver under the best medium conditions. At the end of incubation, the crude enzyme was harvested and used to determine the enzyme activity.

2.9 Effect of surfactants on α-amylase production

Tween 40 and sodium dodecyl sulfate (SDS) were tested to determine their effects on α-amylase production at different concentrations under the best incubation conditions in a rotary shaker at 120 rpm.

2.10 Partial purification of α-amylase

Anoxybacillus flavithermus sp. nov. was cultivated in the NB liquid medium for 24 h. After incubation, culture samples were centrifuged at 10 000 rpm for 10 min at 4°C. The partial purification of α-amylase was experimented according to the method described by Özdemir et al. [28]. In further studies, partially purified enzyme was utilized for characterization of enzyme.

2.11 Effect of temperature on α-amylase activity and stability

The optimum temperature of the partially purified enzyme was determined by measuring α-amylase activity at 30, 40, 50, 60, 70, 80, and 90°C in 0.1 M Tris–HCl (pH 7.0). The influence of temperature on partially purified enzyme stability was experimented by measuring the residual activity after 0–720 min of preincubation in 0.1 M Tris–HCl (pH 7.0) at various temperatures (50–70°C).

2.12 Effect of pH on α-amylase activity and stability

The effect of different pH values ranging from 4.0 to 11.0 on the partially purified enzyme was tested by utilizing the following buffers: citrate (0.1 M, pH 4.0, 5.0, and 6.0), Tris–HCl (0.1 M, pH 7.0, 8.0, and 9.0), and carbonate = bicarbonate (0.1 M, pH 10.0, and 11.0). The pH stability of partially purified α-amylase was evaluated. For this purpose, the enzyme was preincubated in various buffers (pH 5.0–9.0) for 0–720 min. The residual α-amylase activity was measured as described earlier.

2.13 Effect of detergents and surfactants on α-amylase activity

To determine the effect of different detergents (Omo, Ariel, Alo, SDS, and Tween 40) on partially purified α-amylase activity were tested. In this experiment, 200 μL of 0.5% detergent solution, 100 μL partially purified enzyme solution, and 200 μL starch (0.5%) were incubated. The residual α-amylase activity was evaluated according to the Bernfeld method [27].

2.14 Effect of some metal ions on α-amylase activity

The influence of various metal ions such as $MnCI_2$, $ZnCI_2$, $MgCI_2$, $CoCI_2$, $CuCI_2$, $CaCI_2$, $FeCI_2$, $CdCI_2$, and $HgCI_2$ on partially purified enzyme activity was experimented by preincubating the enzyme in the presence of metal ions with a final concentration of 1.5 mM for 30 min at optimum temperature and pH. All metals used were in the chloride form. The activity in the absence of any additives was taken to be 100%. The relative enzyme activity was determined under standard assay conditions.

2.15 Effect of different starches on α-amylase activity

To test the degradation of various starches (wheat, maize, potato, and rice) as a substrate by partially purified α-amylase was assayed as given method above.

2.16 Determination of soluble and insoluble starch in apple juice and its degradation by partially purified α-amylase

Unripe apples (green), which were obtained from Afyonkarahisar, Turkey, were collected before usual harvest date. To determine the soluble and insoluble starch in apple juice and its degradation by partially purified enzyme, they were experimented according to processes described by Carrín et al. [29].

2.17 Electrophoresis

Sodium dodecylsulfhate–polyacrylamide gel electrophoresis (SDS–PAGE) was evaluated as described by the method of Laemmli [30]. The molecular weights of proteins were determined by using standard molecular weight markers (20 000–200 000 kDa).

The native PAGE was experimented in acrylamide gel (10%) with Bio-Rad minigel electrophoresis. All electrophoresis buffers were prepared without SDS. After electrophoresis, the gel was incubated at 70°C in 3% soluble starch solution. Subsequently, the gel was stained with KI-I_2 solution and clear bands indicate the presence of α-amylase activity.

3 Results and discussion

3.1 Morphological and biochemical tests, 16S rRNA gene sequence analysis, GC content, and DNA–DNA hybridization

It was determined that the obtained isolate is Gram-positive, motile rod and has with terminal, ellipsoidal spores form. The isolated strain was catalase and lipase positive. It was

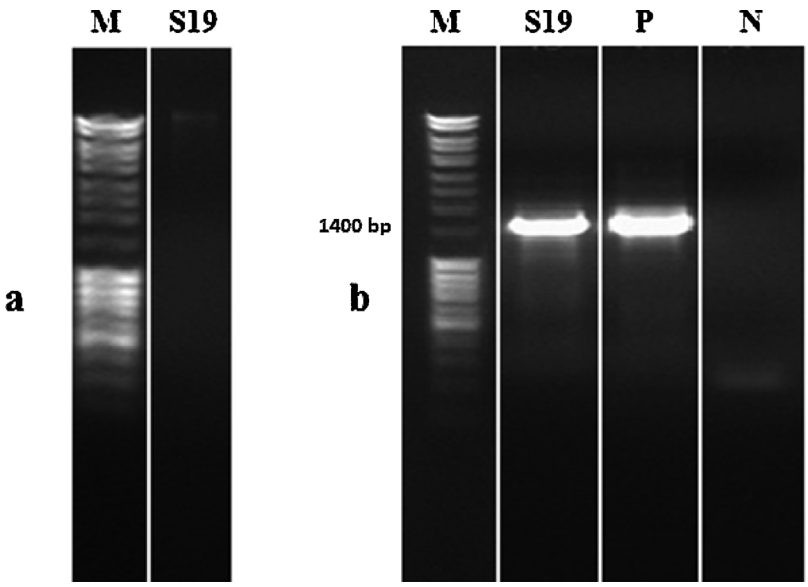

Figure 1. (a) Agarose gel visualization of DNA extraction result by GelDoc™ (BIORAD, USA), M, Marker; S19, Sample 19. (b) Agarose gel visualization of PCR, M, Marker; S19, Bacterial sample 19; P, positive control; N, Negative control.

hydrolyzed starch. It could grow at temperatures and pHs from 30 to 80°C and 5.0 to 10.0, respectively. The DNA was extracted from the bacterial sample numbered as 19. The amount of DNA was measured as 43 μg/mL and the DNA extraction result was visualized by GelDoc™ (Bio-Rad, USA) (Fig. 1a). DNA ladder band sizes are between 10.000 to 100 bp. In addition, the result of PCR which amplifies 16S rRNA gene region by PA/PH primer set was visualized by agarose gel (Fig. 1b). The size of DNA amplicon is 1400 bp. After PCR purification, the amount of DNA was measured as 19.8 μg/mL.

The sequence was compared to related bacteria sequences. The BLAST results showed that the most related species to our bacteria (accession no. KJ095001) based on 16S rRNA gene region sequence is *Anoxybacillus flavithermus* species (accession no. KF952570.1) which has 97% similarity with the bacterium DNA and also has 96% similarity with *Anoxybacillus kestanbolensis* (accession no. AY248710.1), 90% similarity with *Geobacillus stearothermophilus* (accession no. AY608989.1), and 89% similarity with *Bacillus cereus* (accession no. JQ512960.1). Figure 2 shows the phylogenetic tree analysis of six nucleotide sequences which include our bacterial sample nucleotide sequence (accession no. KJ095001), the closest bacteria nucleotide sequences, and four related bacterial sequences.

The DNA of strain S19 had a G + C content of 43.5 mol%. The DNA–DNA hybridization of *Anoxybacillus flavithermus* ssp. *flavithermus* DSM 2641^T (ID 12-1132, ID 13-03) against strain 19 (ID 12-1129) was analyzed, and the DNA–DNA similarity was found to be 52.3%. This result is lower than the recommended threshold value of 70% DNA–DNA similarity for the definition of bacterial species by the ad

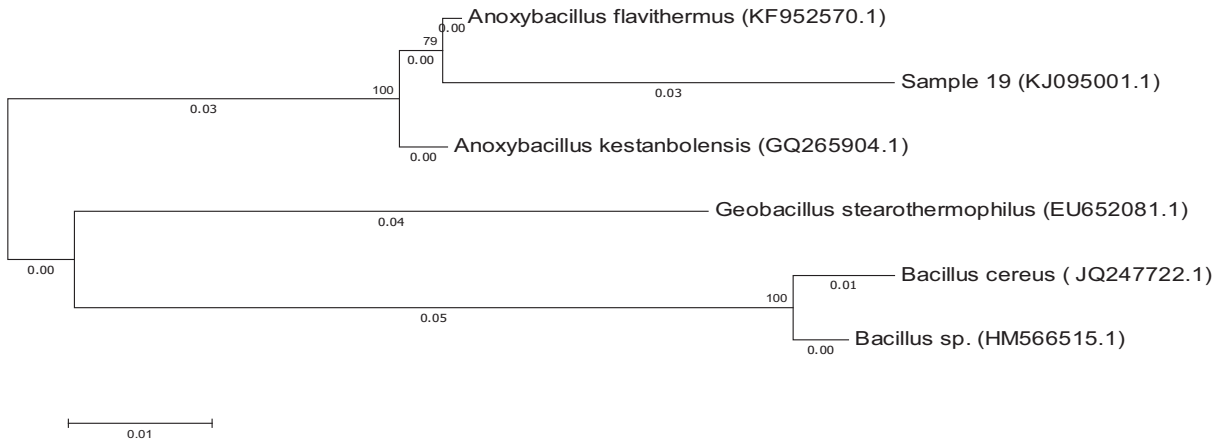

Figure 2. Evolutionary relationships of taxa.

hoc committee considered [31]. With respect to the low similarity of DNA–DNA hybridization between strain 19 and the *Anoxybacillus flavithermus* ssp. *flavithermus* DSM 2641^T (ID 12-1132), phylogenetic analysis, and biochemical differences, we suggest that strain 19 should be placed in the genus *Anoxybacillus* as the type strain for the novel species *Anoxybacillus flavithermus* sp. nov.

3.2 Effect of incubation time on α-amylase production

To determine the optimum incubation time for α-amylase production, it was studied under submerged fermentation conditions at 55°C and pH 7.0. The enzyme activities during the different incubation times such as 0, 4, 8, 12, 16, 24, 36, 48, 72, 96, and 120 h were 0, 115.36, 328.6, 1727.91, 3390.58, 4149.87, 3828.79, 3794.64, 3963.7, 3456.2, and 2917.14 U/mg, respectively. The α-amylase activity was sharply increased from 8 to 24 h. The highest enzyme activity was 4149.87 U/mg at 24 h. The result is incomplete in accordance with the finding of many researchers [11, 28]. After 96 h of incubation, the enzyme activity was decreased. This may be due to the denaturation of α-amylase by the interaction with other components in the fermentation media, change of fermentation media's pH, and consumption of useful nutrient to microorganism [32, 33].

3.3 Effect of temperature and pH on α-amylase production

In this study, the effect of incubation temperature and pH on the production of α-amylase by thermophilic bacteria was experimented. The α-amylase production by newly isolated thermophilic bacteria was affected by various incubation temperatures and pH. The enzyme activities at 30, 40, 50, 55, 60, 70, and 80°C were 347.04, 2446.55, 4197.74, 4149.87, 2155.72, 1358.53, and 261.98 U/mg, respectively. As the incubation temperature was decreased from 40 to 30°C, the enzyme yield was sharply reduced from 2446.55 to 347.04 U/mg. The production of α-amylase was extremely low (261.98 U/mg) at incubation temperature of 80°C. At 30, 70, and 80°C temperature, the production of enzyme was low. The reason of low enzyme yield at these temperatures may be due to the reduction of the bacterial growth (results not given). *A. flavithermus* exhibited the maximum production of α-amylase (4197.74 U/mg) in NB liquid media incubated at 50°C for 24 h at pH 7.0.

The results demonstrated that the effects of incubation pH on enzyme production by thermophilic *A. flavithermus* in submerged fermentation at 50°C for 24 h were 672.89, 4546.2, 4197.74, 3874.18, 2746.23, and 1189.85 U/mg for pH 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0, respectively. The highest α-amylase activity (4546.2 U/mg) was observed at pH 6.0. The enzyme production was sharply declined at pH 5.0. As pH was increased or decreased from 6.0, there was gradual decrease in growth of the organisms. The growth of thermophilic bacteria was obtained very low in culture media at pH 5.0 and 10.0 (data now given). The alkalinity and acidity of culture media caused low reproduction of bacteria. So it can be concluded that the pH of the fermentation media is an important factor for production of α-amylase by thermophilic *A. flavithermus*. Similar results were obtained by many researchers [34, 35].

3.4 Effect of carbon and nitrogen sources on α-amylase production

The effect of different carbon sources (1%) such as glucose, galactose, maltose, lactose, fructose, sucrose, wheat, and maize starch on production of amylase in NB media was examined. When enzyme production was compared with control, among the studied carbon sources, galactose, wheat, and maize starch induced the enzyme production. The results are in good agreement with our previous study [28]. The maximum enzyme production was obtained with maize starch as 4836.21 U/mg. On the other hand, enzyme yield was greatly decreased as the carbon sources of glucose, maltose, lactose, fructose, and sucrose were added to the fermentation media. Similar results were reported by Lin et al. and Asghar et al. [36, 37]. It is well known that especially glucose and sucrose may cause catabolic repression [38].

The various nitrogen sources such as glycine, tryptone, urea, ammonium sulfate, ammonium nitrate, natrium nitrate, yeast extract, and bacto liver were used for the amylase production at the concentration of 1%. When we compared it with control, it was observed that urea, natrium nitrate, yeast extract, and bacto liver diminished the enzyme activity. When we added urea in culture medium at concentration of 1%, bacterial growth decreased 39.3% (results were not given). For this reason, the α-amylase production might be reduced. The maximum α-amylase production was achieved with ammonium sulfate (4896.32 U/mg) following by ammonium nitrate (4612.89 U/mg). These results are in agreement with the studies of other researcher who indicated the maximum α-amylase yield when ammonium sulfate was utilized for nitrogen sources [32, 39].

3.5 Effect of surfactant on α-amylase production

This study was applied to test the influence of SDS and Tween 40 on α-amylase production at different concentrations (0.025–0.1%). It is well known that surfactants enhance production of enzymes by increasing the cell membrane permeability and the surface tension in the fermentation culture [40]. At the concentration of 0.025% SDS and Tween 40, the enzyme production was 4738.21 and 4764.38 U/mg, respectively. The highest amylase production was obtained with Tween 40 as 5032.15 U/mg at 0.5%

concentration. The secretion of enzyme yield decreased when the concentration was higher than 0.05%. At the concentration of 0.1% SDS, the production of α-amylase by newly isolated thermophilic *A. flavithermus* is decreased. It was reported that the bacterial growth and production of amylase were inhibited in the presence of SDS [41].

3.6 Effect of temperature on α-amylase activity and stability

The influence of temperature on the extent of hydrolysis of different starches is a very important factor [42]. From that point of view, the effect of temperature on α-amylase activity was experimented by applying the enzyme at various temperatures in the range of 30–90°C at pH 7.0. The α-amylase activity at various temperatures such as 50, 60, 70, 80, and 90°C was found as 68, 89, 100, 97, and 71%, respectively. The optimum temperature of thermophilic amylase was found as 70°C. Similar finding was reported by Goyal et al. [43]. Higher than 80°C, the enzyme activity then steeply decreased as a result of denaturation of enzyme. To determine the thermal stability of partially purified enzyme, the enzyme preincubated for 30, 60, 90 120, 240, and 720 min at the temperature of 50°C and the original enzyme activity retained was 100, 100, 100, 98, 93%, and 62%, respectively. The enzyme was entirely active (100%) after heat treatment 70 and 60°C for 60 min. The enzyme showed to have remarkable thermostability, so it can be suitable for starch-processing industries [44, 45].

3.7 Effect of pH on α-amylase activity and stability

To examine the pH activity of α-amylase, various buffers at the concentration of 0.1 M were used in a pH range of 4.0–11.0. As shown in Fig. 3a, the α-amylase activity was around 86, 74, 63, 48, and 31% at the pH ranges of 7.0, 8.0, 9.0, 10.0, and 11.0, respectively. The enzyme activity declined sharply, as the pH level was lower than 6.0. The optimum pH of partially purified enzyme activity was found to be 6.0. In comparison to other *Bacillus* α-amylases, our results showed good accordance with many investigators [28, 46, 47].

The pH stability of partially purified enzyme was experimented by preincubating at the optimum temperature for 30, 60, 90, 120, 360, and 240 min at five various pHs (5.0, 6.0, 7.0, 8.0, and 9.0) without substrate, and the residual activity of enzyme was assayed by the method mentioned above. The partially purified enzyme exhibited about 91% (at pH 5.0), 100% (at pH 6.0, 7.0, and 8.0), and 97% for 60 min and about 67% (at pH 5.0), 85% (at pH 6.0), 82% (at pH 7.0), 80% (at pH 8.0), and 73% (at pH 9.0) for 240 min retained enzyme activity after treatment under standard enzyme process condition (Fig. 3b). The results indicated that partially purified thermotolerant α-amylase can be used in detergents industries because of its stability at alkaline pHs.

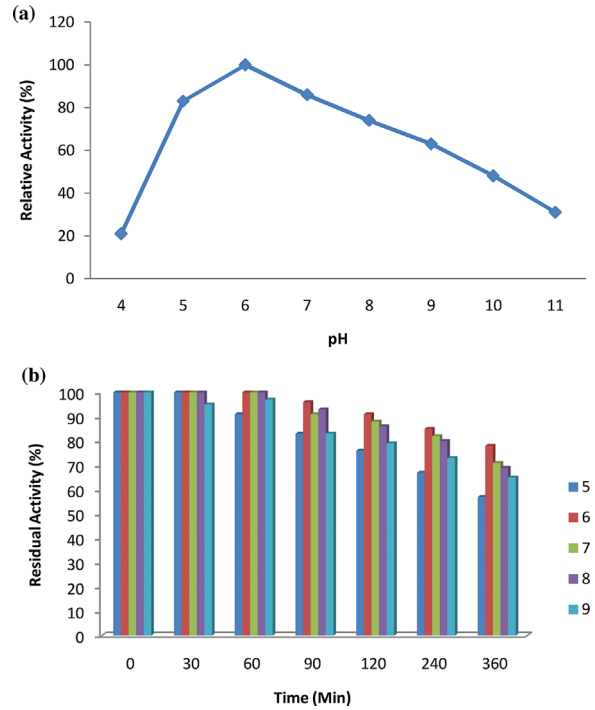

Figure 3. Effect of pH on α-amylase activity and stability. (a) Optimum pH; (b) pH stability.

3.8 Effect of detergents and surfactants on α-amylase activity

The various detergents such as Omo, Ariel, Alo, SDS, and Tween 40 on partially purified α-amylase were tested at different incubation times. The results of effect of detergents on enzyme activity are shown in Fig. 4. The thermostable α-amylase from *A. flavithermus* exhibited superior stability with studied detergents. It was most compatible with "Alo" remaining 85% activity after 60 min incubation, and it kept 90 and 83% its original activity after 90 and 120 min incubation, respectively. Carvalho et al. [48] and Özdemir et al. [28] examined the influences of commercial detergents, and our results showed good agreement with their studies.

3.9 Effect of some metal ions on α-amylase activity

In this research study, the effect of metal ions ($MnCI_2$, $ZnCI_2$, $MgCI_2$, $CoCI_2$, $CuCI_2$, $CaCI_2$, $FeCI_2$, $CdCI_2$, and $HgCI_2$) on the extracellular thermostable α-amylase was studied and the results are shown in Fig. 5. When the influences of metal ions were tested on partially purified enzyme, the enzyme activity demonstrated a dramatic reduce to 35% with $ZnCI_2$, 48% $CuCI_2$, 86% $CdCI_2$, and 95% $HgCI_2$. The results showed that partially purified enzyme may be a metalloenzyme that could be inhibited

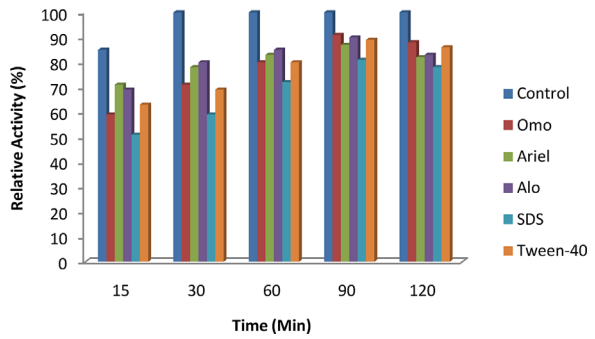

Figure 4. Effect of detergents and surfactants on α-amylase activity.

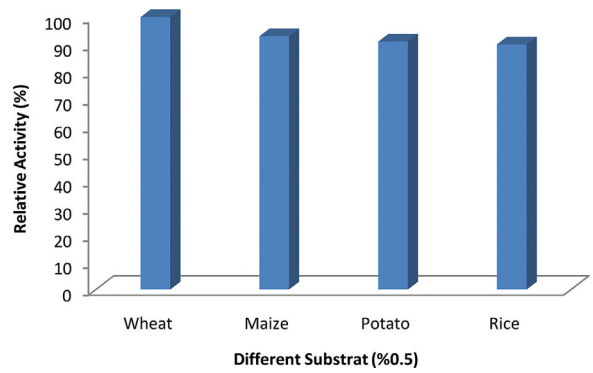

Figure 6. The investigation of the different starches hydrolysis by partially purified α-amylase.

by heavy metals reason irreversible inhibition of enzyme by binding vigorously to their amino acid backbone [49]. On the other hand, many substances chance the activity of an enzyme by combining it in a way that effects the binding of the substrate. These substances can be called as effectors either being activators or inhibitors. Metal ions can be considered as good examples, different metals exhibiting different behaviors in their ability to act as effectors. The enzyme was induced by $MgCI_2$ and $CoCI_2$. The highest enzyme activity was obtained with $CoCI_2$ (212%). In our study, $CoCI_2$ can be an activator for alpha amylase. This result exhibits good agreement with the previous work [37].

3.10 The investigation of the different starches hydrolysis by partially purified α-amylase

Figure 6 exhibits the hydrolysis extent of wheat, maize, potato, and rice starches by partially purified α-amylase at optimum conditions. The hydrolysis rates of the wheat, maize, potato, and rice starches were found to be 100, 93, 91, and 90%, respectively. Results indicated that thermostable α-amylase can be used for different starch hydrolysis industries.

3.11 The starch contents of unripe green apple juice and its degradation by partially purified α-amylase

The starch contents of unripe green apple juice were tested. The amount of insoluble and soluble starch in apple juice was obtained as 5.99 and 0.38 g/L, respectively. Carrín (2004) found the amount of insoluble and soluble starch in unripe apple juice as 7.68 and 0.51 g/L. The partially purified enzyme was incubated with pasteurized juices for degradation at different incubation times (Fig. 7). The starch contents were found to be 92, 87, 75, 52, 43, and 18% after 5, 10, 15, 20, 25, and 30 min of incubation, respectively. The results of degradation of apple juice by α-amylase showed also good agreement with the Carrín previous report [29].

3.12 Electrophoresis

Molecular weight of partially purified α-amylase was determined by SDS–PAGE electrophoresis as described in Section 2.17. Molecular weights of partially purified α-amylase were found to be approximately 96 kDa (Fig. 8). Activity staining of amylases indicated white

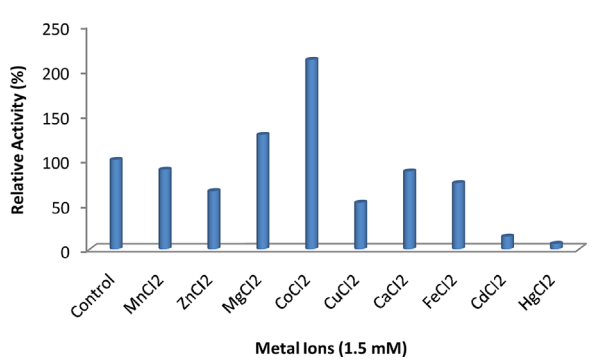

Figure 5. Effect of some metal ions on α-amylase activity.

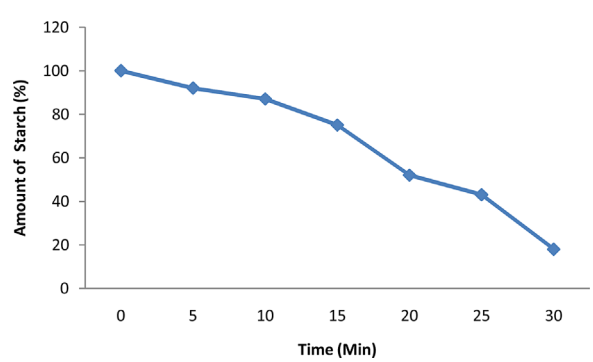

Figure 7. Degradation of apple juice starch by partially purified α-amylase.

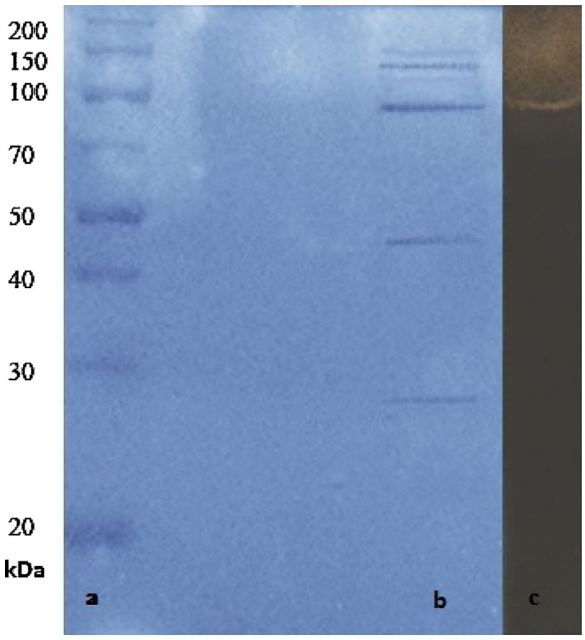

Figure 8. The α-amylase showed a molecular mass. Lane (a) Standard protein, SDS-PAGE; Lane (b) dialyse; Lane (c) native-PAGE (using iodine stain).

bands in the dark-colored gel, which confirmed the amylolytic activity.

4 Conclusions

In conclusion, isolated thermophilic strain should be a novel *Anoxybacillus flavithermus* according to the biochemical and molecular analyses. The highest amylase production was achieved as 5032.15 U/mg when 0.05% Tween 40 was added to the NB media at 50°C and pH 6.0 for 24 h. The partially purified α-amylase was characterized. The optimum temperature and pH activity were 70°C and 6.0, respectively. Thermostable and thermoactive α-amylase can be used in industries such as detergents, starch, and fruit juice.

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The authors have declared no conflicts of interest.

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