A Novel Raw Starch Hydrolyzing Thermostable α-Amylase Produced by Newly Isolated *Bacillus mojavensis* SO-10: Purification, Characterization and Usage in Starch Industries

Sadin Ozdemir[1]*, Sema Aguloglu Fincan[2], Adem Karakaya[3], Baris Enez[4].

[1]**Mersin University, Technical Science Vocational School, Food Processing Programme, TR-33343 Yenisehir, Mersin, Turkey;*
[2]*Dicle University, Faculty of Science, Department of Biology,21280 Diyarbakir, Turkey;*
[3]*Department of Biology, Faculty of Arts and Science, Siirt University, 56100 Siirt, Turkey;*
[4]*Bingol University, Technical Science Vocational School, Laborant and Veterinary Health Program, 1200, Bingol, Turkey.*

ABSTRACT

The aim of this study is the production, purification, and characterisation of thermostable raw starch hydrolyzing α-amylase produced by Bacillus mojavensis SO-10. The maximum production conditions of α-amylase were found at 36th hour, 35 °C and pH 7.0. We utilized three steps to purify the thermostable α-amylase and as a result, 34-fold and 18% yield were obtained. The molecular weight of purified α-amylase was determined as 73 kD. The K_m and V_{max} rates were detected as 0.010 mM and 3.38 μmol min^{-1}, respectively. This purified α-amylase exhibited the highest activity at pH 5.0-6.0 and 70 ºC and showed stability over a wide variety of pH and temperature at 4.0–8.0, and 40–50 ºC, respectively. The thermostable purified α-amylase exhibited stability in the presence of denaturing agents and heavy metal ions. The purified enzyme hydrolyzed the raw starches of corn and wheat grains in the ratio of 36.7% and 39.2% respectively. The end-yields of soluble starch hydrolysis were analyzed by thin-layer chromatography (TLC). In addition, the usage of purified α-amylase in clarification of apple juice and domestic washing detergent industries were evaluated.

Key words: α-Amylase; Raw starch; Thin-Layer Chromatography (TLC); Detergents; Apple Juice Clarification

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*Author for correspondence: sadinozdemir@mersin.edu.tr

INTRODUCTION

Amylase (EC 3.2.1.1, 1, 4-α-D-glucan-glucanohydrolase) is an extracellular enzyme that hydrolyzes starch and glycogen molecules [1]. It breaks down the α-1, 4 bonds in starch molecules into glucose, maltose, maltotriose and α-limit dextrine [2,3]. Amylase is used primarily in the food industry for the preparation of maltose syrup and clarification of various drinks. In addition, amylase is generally used in other industries like biorefinery, paper, detergents, textiles and pharmaceuticals [4-7].

Starch-hydrolyzing enzymes as amylase play an important role in carbohydrate metabolisms. It is a well-known fact that α-amylases degrade the starches. They can also be utilized to form a variety of major products with various physical and chemical qualities for foods and pharmaceuticals [8].

As native starch is not dissolve in water at low temperature, many conventional processes are carried out at high temperature and pressure for gelatinization of the raw starch. These processes require an extreme-energy input, so the production cost of starch-based products is increased. To decrease the starch processing cost, effective utilization of natural resources and viscosity problems, direct hydrolysis of starch at low temperature is desirable [9]. The significance of enzymatic saccharification of raw starch without heating has become well recognized due to energy savings and low cost of starch processing [10-12]. This has generated the discovery of some raw starch degrading enzymes (RSDE) that can directly hydrolize raw starch below the gelatinization temperature of starch [13]. In recent years, a worldwide interest has been focused on the raw starch digesting amylases, which would be of value to simplify the process of starch conversion.

The most widely utilized thermostable α-amylases are obtained from mesophilic and thermophilic microorganisms which are considered to be potential sources of thermostable α-amylases of industrial significance [14]. They have been indicated from a large range of microorganisms from a few species of genus *Bacillus* and *Streptomyces* [15]. *Bacillus* is usually utilized for the commercial production of thermostable α-amylases.

The present research reports the production, purification and biochemical characterizations of an α-amylase obtained from *Bacillus mojavensis* SO-10. This purified α-amylase displayed attractive characteristics such as temperature and pH stability and chemical agents' resistance. In addition, the purified amylase was utilized in some industrial starch processes like raw starch hydrolyzing, detergents and apple juice clarification.

MATERIALS AND METHODS

Culture conditions

Bacillus mojavensis SO-10 were grown in 250 mL glass bottle containing 50 mL Nutrient Broth (NB) media. The culture media's pH was adjusted with 0.1 M HCl or NaOH to pH 7.0, and glass bottles were autoclaved at 121 ℃ for 15 minutes. After being autoclaved, every bottle was inoculated with 0.5 mL (3.1×10^8 CFU/mL) of cell suspension. The fermentation media was cultured at 35 ℃ on a shaker at 120 rpm.

Enzyme activity

α-Amylase activity was studied by determining the quantity of reducing sugars using 3, 5 dinitrosalicylic acid (DNS) process [16]. According to this process, 0.5% starch solution (200 µL) was incubated with 100 µL crude enzyme solution or 5 µL purified enzyme for 30 min at 70 ℃. The reaction was stopped by adding DNS reagent and reaction solution was boiled for 5 min. After leaving it for cooling at room

temperature, 3 mL of pure water was added to the reaction mixture and spectrophotometric determination was measured at 489 nm. One unit of α-amylase activity was determined as the quantity of enzyme that produced 1 mmol of reducing sugars maltose per minute.

Impact of incubation time on α-amylase production
The influence of various time courses on enzyme production was assayed at 35 ℃ in NB at pH 6.0 and 35 ℃ in a shaker at 120 rpm. A 2 mL of fermentation culture media was collected at various incubation times (0-96 h). Then, the media was centrifuged at 7.000 rpm for 10 min. The upper solution was tested for determination of α-amylase activity according to DNS process.

Influence of fermentation temperature and pH on enzyme production
The sterile NB fermentation media was incubated at different temperature between 20 to 55 ℃ at pH 7.0 and 120 rpm in a shaker for 36 hours and enzyme assay was evaluated as mentioned above. The optimum fermentation media's pH for production of α-amylase was examined at pH ranges of 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 on optimum culture conditions. After incubation, fermentation media was centrifuged and the upper solution was tested for enzyme activity.

Purification of α-amylase
The crude enzyme was exposed to $(NH_4)_2SO_4$ up to 80% (w/v) saturation and allowed to precipitate at 4 °C. The precipitates were centrifuged at 10.000 rpm for 15 min. The precipitate was dispersed in 0.1 M potassium phosphate buffer (pH 7.0), and dialyzed overnight at 4 °C. The dialyzed enzyme solution was applied to a DEAE (Diethylaminoethyl) cellulose (DE 32) column (flow rate 15 mL/ h).

Protein analysis on SDS–PAGE
The purity of α-amylase was determined on SDS–PAGE according to Laemmli process utilizing 10% polyacrylamide gel [17].

Determination of kinetic parameters
The purified α-amylase was tested at different starch concentrations from 0.5% to 2% for determination of K_m and V_{max}. The K_m and V_{max} were calculated from the reciprocal plot of starch concentration (S) versus velocity (V).

Influence of temperature on purified α-amylase activity and stability
The purified enzyme activity at various temperatures was studied by incubating the reaction mixture at temperature ranging between 20 ℃ to 90 ℃. The temperature stability of purified enzyme was also experimented by pre-incubating the enzyme between 40-70 ℃ for 30, 60, 90, 120, 150, and 180 min and the remaining purified α-amylase activity was determined.

Influence of pH and pH stability
The optimal pH of the enzyme was experimented by incubating the enzyme reaction solution at pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0). For measuring pH stability of purified α-amylase, the enzyme was pre-incubated between various pH ranges of 4.0 to 8.0 for 30-240 min. The purified α-amylase activity in standard reaction mixture was used as control.

Effects of inhibitors and different metal ions on purified enzyme activity

The effect of different metal ions such as $MnCl_2$, $ZnCl_2$, $MgCl_2$, $CuCl_2$, $CaCl_2$, $FeCl_2$, $HgCl_2$ and various inhibitors such as EDTA (ethylenediaminetetraacetic acid), PMSF (phenylmethanesulfonyl fluoride), β-mercaptoethanol and DTT (dithiothreitol) on purified α-amylase was studied. The purified α-amylase was pre-incubated with 1.5 mM metal ions and 1, 5 and 10 mM inhibitors for 30 min at 37 °C and the standard α-amylase activity assay was carried out.

Thin layer chromatography (TLC)

The purified α-amylase was incubated with 1% soluble wheat starch to determine the end-products of hydrolyzed soluble starches at various incubation time (15, 30 and 240 min). 3 µL of end products of reaction were spotted on TLC plate (Merck). The end products of reaction were examined by pre-coated TLC plate with the solvent system of n-butanol/methanol/H_2O (8:4:3, *v/v/v*). Spots were visualized by spraying a sulfuric acid/methanol solution (1:7, *v/v*), drying and heating at 95 ºC for 10 min.

Industrial applications of Purified α-amylase
Influence of detergents on purified α-amylase activity

Various detergent solutions (Omo, Tursil, and Alo) were prepared at 0.5% concentration. In this test, 50 µL detergent solution, 200 µL starch, and 10 µL α-amylase were mixed and incubated from 15 min to 120 min [8]. The enzyme activity was assayed according to the DNS method as mentioned above.

Raw starch degrading

Wheat and corn raw starch grains were cleaned 3 times with distilled water to eliminate contaminants. The grains were dried and 1% of raw wheat and corn starch grain was suspended in 980 µL of 0.1M buffer (citric acid) at pH 6.0 and 10 µL of purified α-amylase was added. The purified α-amylase was incubated with wheat and corn raw starch grains at 50 ºC for 4 h, and reaction solution was centrifuged. The upper solutions were examined to define released reduce sugars with DNS assay with maltose as the standard. The percentage of degrading of raw starch (Rh) was calculated by the following formula:

$Rh (\%) = (A_1/A_0) \times 100$,

where A_1 refers to the quantity of reducing sugar in the upper after the degrading, and A_0 refers to the quantity of raw starch before the degrading [18]. After 4 h incubation, hydrolyzed starch of wheat and corn grains were cleaned with pure ethanol and dried. The dried starch grains were treated with iodine solution and photographed under the Olympus CX31 light microscope 400 times magnification.

The starch amounts of immature apple juices (green and red) and theirs clarification by purified enzyme

The detection of starch contents in immature apple juice was experimented according to the method defined by Carrín et al. [19], and the usage of purified alpha amylase in the apple juice clarification industry was assayed.

RESULTS AND DISCUSSION

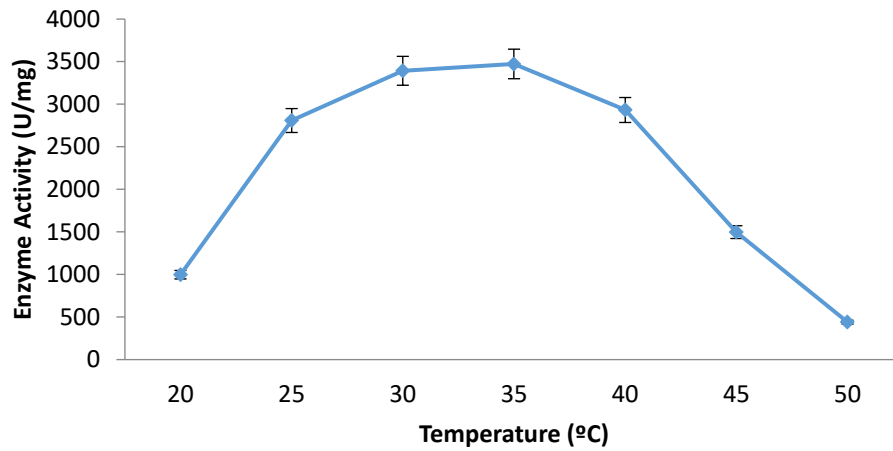**Impact of time courses, temperature, and pH on α-amylase production**

We tested the impact of fermentation course, temperature, and pH on α-amylase production. It is well known that short incubation time is important for cheap production of enzyme in fermentation industry. The production of α-amylase is demonstrated in Fig. 1. The production of the enzyme increased when the incubation time increased up to 36th. The maximum α-amylase production was achieved with 36th

(Fig. 1a) and the enzyme production decreased after 36th. After this incubation period, there was a decrease in the production of α-amylase. This is because the cells have reached the decline phase and displayed low α-amylase synthesis. The decrease in the enzyme production may be due to consumption of the nutrients in the fermentation culture or due to accumulation of toxic by-products. The fermentation temperature and pH were also significant parameters, which clearly affect enzyme production. The fermentation temperature and pH were tested between 20 to 55 °C and 4.0 to 10.0 respectively in an incubator shaker to define the temperature and pH influences on α-amylase production. The highest α-amylase production was determined at 35 °C. When the fermentation temperature went up higher than 35 °C, α-amylase production diminished (Fig. 1b). The enzyme yield reduced 87.3%, while the fermentation temperature increased from 35 to 50 °C. The α-amylase activity found to be increasing as the fermentation pH rose from 4.0 to 7.0 (Fig. 1c). This was followed by a slight reduction in α-amylase activity above the 7.0. The highest enzyme activity was recorded at pH ranges of 6.0-7.0. The optimum fermentation conditions for α-amylase production were recorded at 36th hour, 35ºC and pH 6.0-7.0 by *B. mojavensis* SO-B11.

(a)
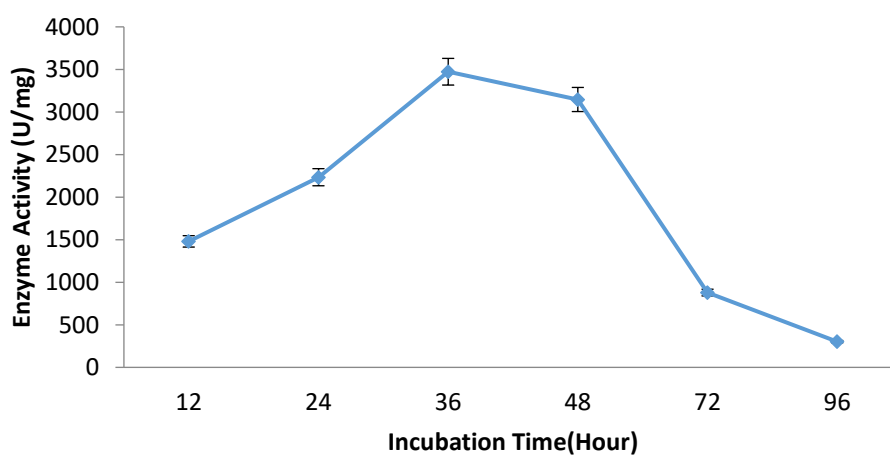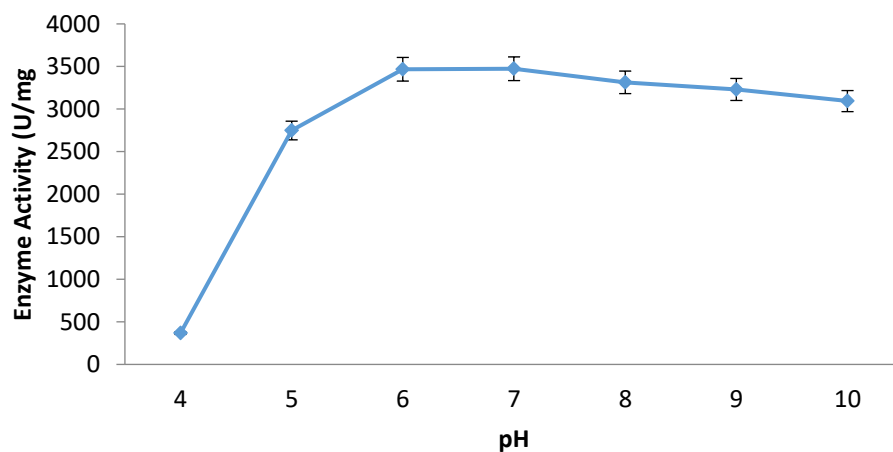
Figure 1 Impact of time cources (a), temperature (b), and pH (c) on α-amylase production.

Purification of α-amylase

The enzyme purification steps were (NH$_4$)$_2$SO$_4$ precipitation (80%), dialyzed and DEAE-cellulose ion-exchange chromatography, respectively. The purification fold and yield were determined as 34% and 18 % respectively completion of purification steps. The purification results are represented in Table 1. The α-amylase secreted by *Geobacillus stearothermophilus* was purified to homogeneity (65-fold and 46% yield) through a series of steps [20]. Recently, Xie et al. [15] have indicated 13.1 purification fold and 7.0% yield, Abdel-Fattah et al. [21] have pointed out 59.3 purification fold and 12.6 % yield, and Shukla and Singh [22] have reported 37 purification fold and 16.8% yield using chromatography methods.

Table 1 Purification of amylase from *B. mojavensis*.

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Fold
Crude extract	283.374	188.444	1.504	100	1
NH$_4$)$_2$SO$_4$ precipitation/Dialysis	106.705	8.746	12.201	38	8
DEAE-Cellulose	50.929	0.982	51.870	18	34

Molecular mass determination on SDS–PAGE

The molecular mass of the purified enzyme which appeared as a single band on SDS-PAGE was found to be 73 kDa (Fig. 2). Murakami et al. [23] reported α-amylases having molecular masses of 105 and 75 kDa, respectively. Asoodeh et al. [24] determined the molecular weight of α-amylase as 68.9 kDa. Michelin et al. [25] demonstrated amylase as a single band of about 75 kDa by SDS-PAGE. Another α-amylase with molecular weight of 70 kDa was indicated by Zafar et al. [26]. Different molecular masses of the α-amylases from various *Bacillus* sp. ranging from 42 to 150 kDa have been reported [27].
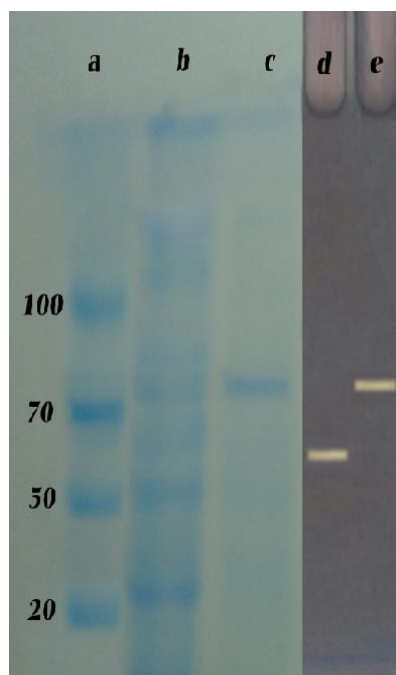

Figure 2 Molecular mass determination on SDS–PAGE. Lane a: standard proteins; Lane b: after dialyze c: purified enzyme from DEAE cellulose; Native-PAGE (using iodine stain) Lane d: standard a-amylase; Lane e: purified enzyme from DEAE cellulose.

Determination of kinetic parameters

Kinetic studies of α-amylase were determined using soluble starch as substrate. The K_m and V_{max} values were estimated from a Lineweaver–Burk plot (Fig. 3). Values of V_{max} and K_m for the purified enzyme were 0,007964 mM and 2, 77 µmol min^{-1} respectively. Aguilar et al. [28] declared the K_m value of α-amylase as 3.44 mg/mL. Aguloglu Fincan et al. [29] determined K_m and V_{max} as 0.005 mM and 3.5 µmol min^{-1}, respectively.
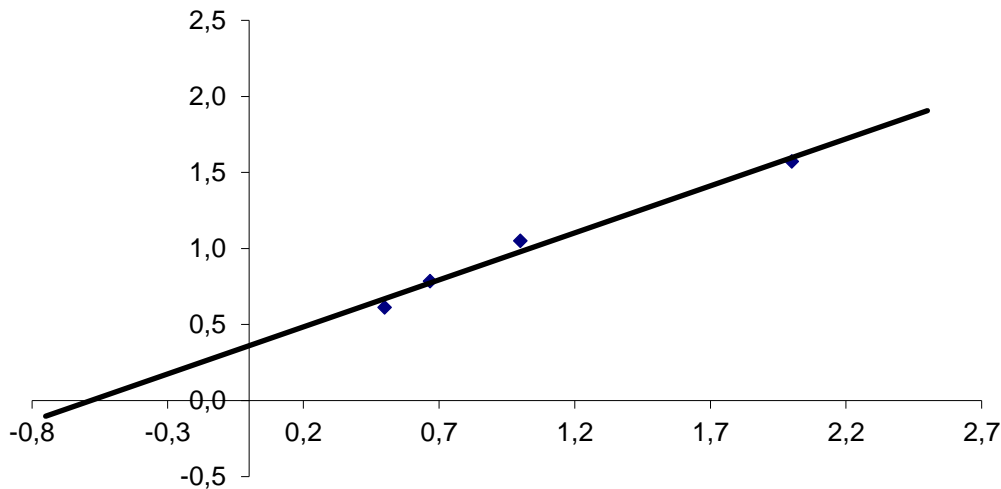
Figure 3 Lineweaver–Burk plot of purified α-amylase from *B. mojavensis*.
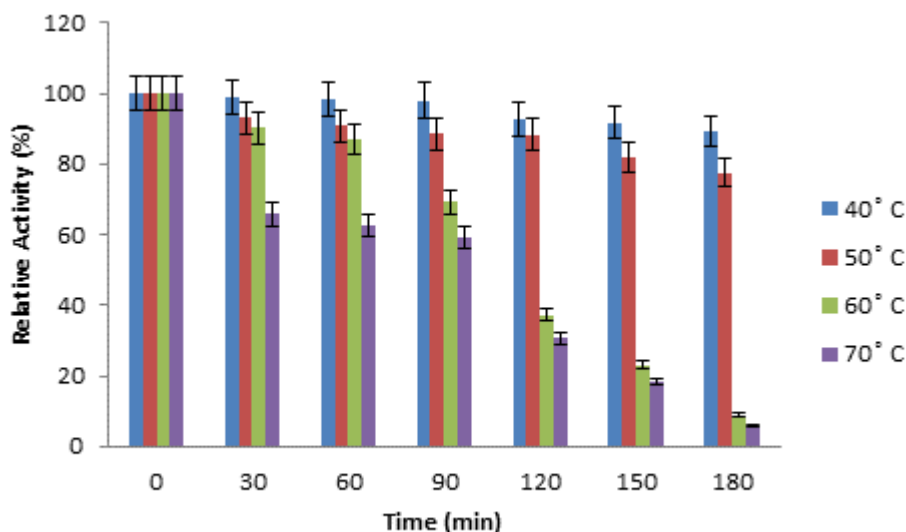
Impact of temperature on purified amylolytic activity and stability
The impact temperature on amylolytic activity is shown on Fig. 4a, and the residual activities of purified enzyme were found as 37.1%, 70.9%, 82.7%, 90.3% and 97.6% at 20, 30, 40, 50, and 60 °C, respectively. The purified amylase produced from *B. mojavensis* SO-B11 exhibited optimal activity at 70 °C. The results revealed that the relative amylolytic activity sharply declined from 82.3% to 24.4% with increasing temperature from 80 °C to 90 °C, respectively. The optimum temperature for α-amylase from *Bacillus* sp. *ferdowsicou* [24], *Bacillus subtilis* [8] and *Anoxybacillus flavithermus* [14] were found as 70, 60, and 70 °C, respectively. This reveals that the optimum temperature of purified amylase obtained from *B. mojavensis* SO-B11 showed well parallelism with these studies. Thermal stability of the purified enzyme produced by *B. mojavensis* was examined by incubating the enzyme at different temperatures (30-180 min). The results of thermal stability are represented in Fig. 4b. The purified enzyme was steady at temperature of 40 °C and 50 °C up to 180 min. The 87.1% and 85.7% of residual enzyme activity was retained after 30 min of incubation at the temperature of 60 °C and 70 °C, respectively. According to the results obtained from thermal stability of purified α-amylase, the enzyme can be applied in brewing and food processing.
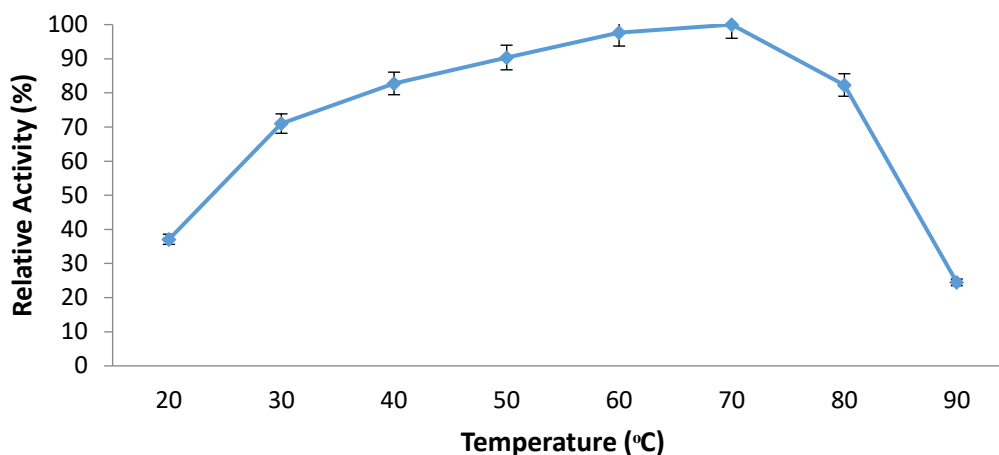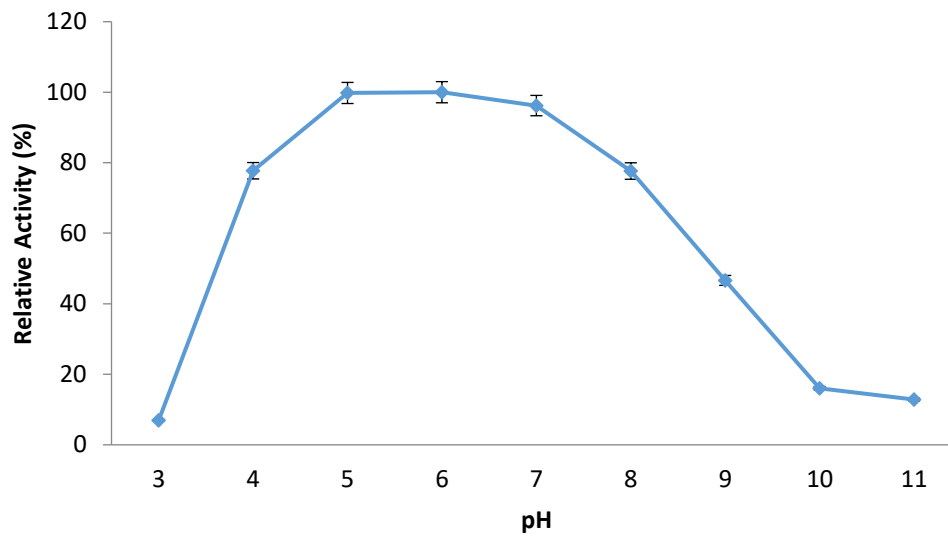
(a)

(b)

Figure 4 Impact of temperature on purified amylolytic activity (a) and stability (b).
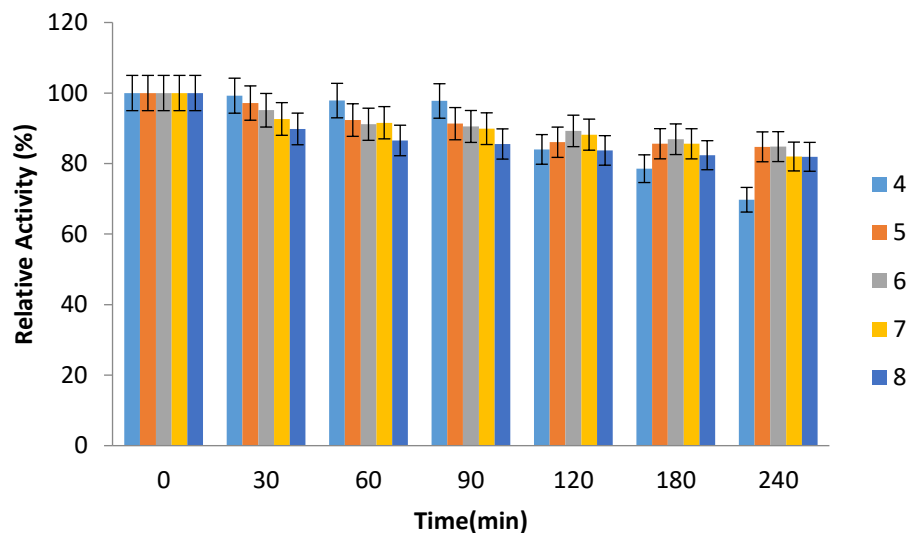
Effect of pH on purified α-amylase activity and stability
The pH profile of the purified enzyme activity is depicted in Fig. 5a. As shown in Fig. 5a, the amylolytic activity is nearly the same at pH 5.0-7.0. The amylolytic activity sharply decreased from pH 4.0 to 3.0 and from pH 8.0 to 9.0. The purified α-amylase showed optimum activity at pH 5.0-6.0. Parallel results were found by some researchers such as Hamilton et al. [30], Sarikaya and Gürgün [31] and Kikani and Singh [32]. The retained enzyme activity was about 96.2% at pH 7.0. The purified enzyme pH stability experimental results are depicted in Fig. 5b. The amylolytic activities did not significantly change between pH ranges of 4.0-8.0 for 120 min. The retained α-amylase activities obtained from *B. mojavensis* SO-B11 were defined as 84.1%, 86.1%, 89.2%, 88.2% and 83.8% at pH 4.0, 5.0, 6.0, 7.0, and 8.0 respectively

for 120 min. These results indicated that purified novel enzyme can be utilized in starch saccharification and detergent industries.

(a)

(b)

Figure 5 Effect of pH on purified amylolytic activity (a) and stability (b).

Effect of Different Cations and Inhibitors on Purified α-Amylase Activity
The influences of Zn^{2+}, Mg^{2+}, Hg^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+} and Fe^{2+} and different inhibitors on purified α-amylase activity are depicted in Table 2. When compared with the control group, the enzyme activities in presence of Zn^{2+}, Mg^{2+}, Hg^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+}, and Fe^{2+} were determined as 82%, 86%, 38%, 119%, 121%, 79%, and 94% respectively. The purified enzyme was activated by Ca^{2+} and Mn^{2+}, but slightly inhibited by Fe^{2+}, Zn^{2+}, Mg^{2+}, and Cu^{2+}. Lin et al. [33] suggested that enzyme was activated with Ca^{2+}, while it was inhibited in the presence of Hg^{2+}. The effect of various metal ions on purified α-amylase activity was reported by Mamo and Gessesse [34], Aguloglu Fincan and Enez [20], and Liu et al. [35]. Our results are very close

to their studies. The highest inhibitory effect was obtained with EDTA. This inhibition can reveal that the purified α-amylase produced by *B. mojavensis* SO-B11 is a metalloenzyme. Our results completely match with the results obtained by other investigations on the α-amylase [15, 36, 37]. PMSF and β-mercaptoethanol did not show important influence on the purified α-amylase activity. This can make it obvious that serine residues and disulfide bonds are not essential for the amylolytic activity [38].

Table 2 Effects of inhibitors and some metal ions on amylase activity

Agent	Concentration (mM)	Relative activity of purified α-amylase (%)*
PMSF	1	77
	5	74
	10	65
DTT	1	97
	5	93
	10	92
β-mercaptoethanol	1	96
	5	95
	10	92
EDTA	1	78
	5	78
	10	64
Mn^{+2}	1.5	119
Fe^{+2}	1.5	94
Zn^{+2}	1.5	82
Mg^{+2}	1.5	86
Hg^{+2}	1.5	38
Cu^{+2}	1.5	79
Ca^{+2}	1.5	121
Control	0	100

*Activity remaining after incubation for 30 min at 37 ∘C.

Determination of the end products of soluble starch by TLC

The purified α-amylase was incubated with soluble starch for 15, 30 and 240 min. It is seen from the Fig. 6 that the purified α-amylase produced by *Bacillus mojavensis* SO-10 hydrolyzed the soluble starch. There were various end products after hydrolysis of soluble starch by amylase. Maltose (G2), maltotriose (G3), maltotetraose (G4), and maltooligosaccharides occurred after 15 min and glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), and maltooligosaccharides after 30 min. After 240 min, whole soluble starch hydrolyzed into (G1). Parallel finding was described by Takasaki [39]. These results described that purified α-amylase can be used in starch liquefying industry or food industries because of the end products of (G1), (G2), (G3), (G4), and maltooligosaccharides.
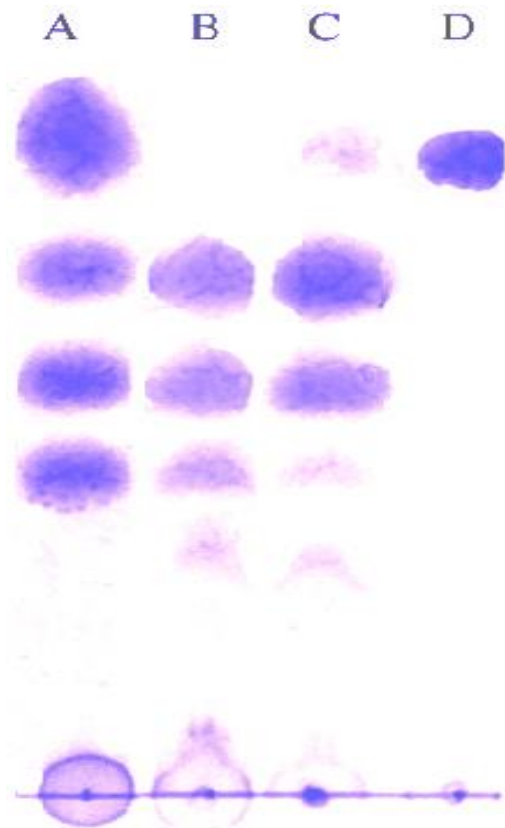
Figure 6 End products of soluble starch by Thin-layer Chromatography TLC. Lane A, glucose (G1), maltose (G2), maltotriose (G3) and maltotetraose (G4); Lane B-D, reaction time for 15 min, 30 min and 240 min, respectively.

Industrial applications of purified α-amylase
The capable of raw starch hydrolyzing of corn and wheat by purified α-amylase
Purified α-amylase capable of hydrolyzing raw starch was examined by defining the percentages of hydrolyzing of corn and wheat starch grains. After 4 h of incubation, the purified α-amylase hydrolyzed the raw wheat and corn grains in the ratio of 36.7% and 39.2% respectively. Parallel results were reported by Božić et al. [40] and Hayashida et al. [41]. Considering the literature, this is the first study to hydrolyze the raw starch by α-amylase obtained from *B. mojavensis* SO-B11. It is too important to degrade the raw starch grains under the gelatinization temperature of starch due to the economic approach. α-Amylase produced by *B. mojavensis* SO-B11 can be utilized for hydrolyzing of raw wheat and corn starch in various industries such as food, fermentation and gelatinization of starch. The hydrolyzed raw corn and wheat starch granules were monitored with Olympus CX31 light microscope (Fig. 7). The rate of corn and wheat starch grains decreased after 4 h of degrading, while the structure of starches grains were moderately damaged.
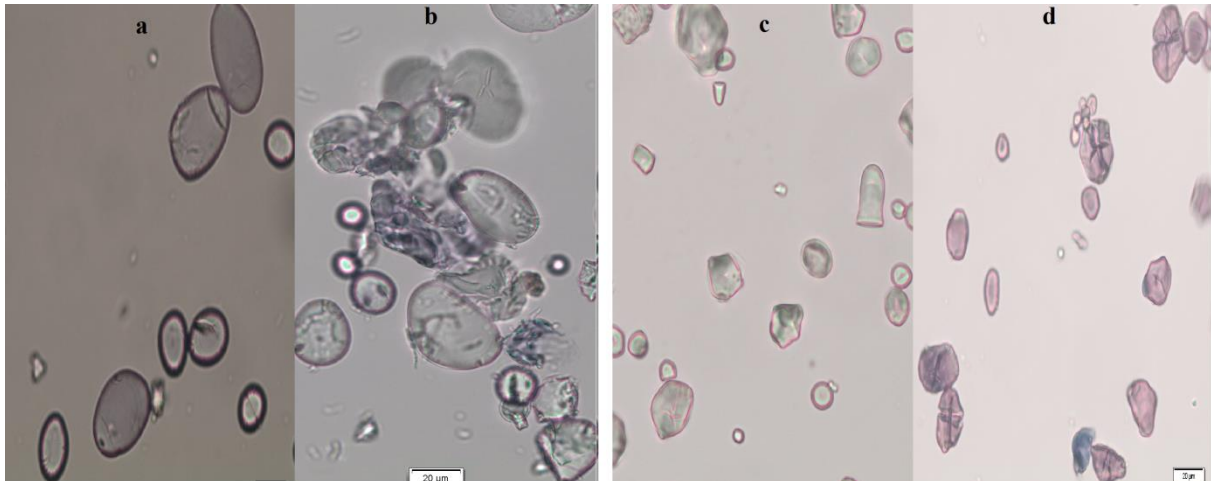
Figure 7 Raw starch hydrolyzing. (A) Corn starch before hydrolyzing; (B) Corn starch after hydrolyzing; (C) Wheat starch before hydrolyzing; (D) Wheat starch after hydrolyzing.
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Influence of some commercial detergents and surfactants on purified enzyme activity

The influence of some surfactants and trading domestic washing detergents on the purified *B. mojavensis* α-amylase was experimented. As demonstrated in Fig. 8, the purified *B. mojavensis* α-amylase exhibited significant stability with surfactants and domestic washing detergents such as Omo (44.7%), Tursil (51.9%), Alo (63.1%), SDS (60.9%), Tween-10 (97.2%), Tween-40 (99.5) and Triton X-100 (98.2%) for 60 min and Omo (41.3%), Tursil (53.2%), Alo (73.8%), SDS (69.7%), Tween-10 (102.9%), Tween-40 (99.5%) and Triton X-100 (101.3%) for 120 min. Parallel findings were indicated by Goyal et al. [42] and Pathak and Rekadwad [43].
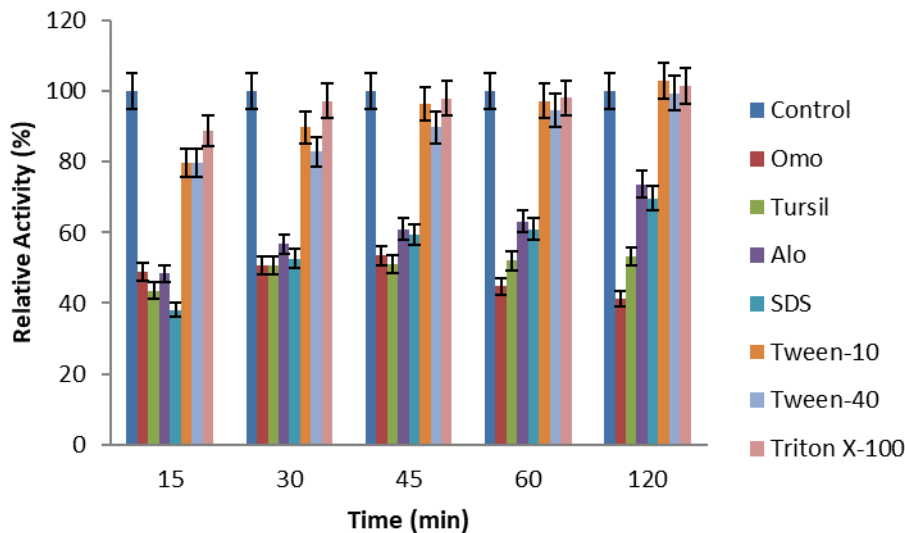

Figure 8 Influence of some commercial detergents and surfactants on purified enzyme activity.

The starch amounts of immature apple juices (green and red) and theirs clarification by purified enzyme

Immature starch amounts of red and green apple were tested by using process of Carrín et al. [19]. The starch amounts of immature red apple were determined as 0.59 g/L for soluble starch and 3.87 g/L for insoluble starch and the starch amounts of immature green apple were determined as 0.52 g/L for soluble starch and 3.68 g/L for insoluble starch. Digesting of soluble starch of immature red and green apple was experimented for 30, 45, and, 60 min. The degrading percentages of soluble starch contents of red apple and green apple were found as 61% and 58%, 74% and 71%, 85%, and 80% for 30, 45, and 60 min, respectively. The finding results demonstrated that purified α-amylase should be applied in apple juice industry.

CONCLUSIONS

A thermo and pH stable α-amylase were produced, purified, characterized and utilized for various starch industries. The amylolytic activity did not meaningfully change between pH ranges of 4.0-8.0 and temperature degrees of 40, 50, and 60 °C for 120 min. The purified enzyme exhibited stability in the presence of surfactants and metal ions. The purified enzyme obtained from *B. mojavensis* SO-B11 was capable of hydrolyzing wheat and corn raw starch granules. G1, G2, G3, G4, and maltooligosaccharides were obtained after TLC analysis. In addition, the newly purified alpha amylase should be used in domestic washing detergents and apple juice clarification industries.

ACKNOWLEDGEMENTS

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