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Molecular Identification of a *Bacillus mojavensis* UMF29 Producing a Novel Raw-starch Degrading Alpha-amylase

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ABSTRACT

Microbial alpha-amylases demonstrate more compatibility with industrial demands. These industrial enzymes have potential applications in various industrial parts, e.g., starch processing, brewing, baking bread, pharmaceuticals, and detergents. In this study, a mesophyll bacterium, *Bacillus mojavensis* strain UMF29, has been identified based on the 16S rDNA sequence and phylogenetic tree. After 72 h of incubation at 37 °C, the plates were inoculated with Lugol's iodine, and the alpha-amylase-producing isolates were distinguished by clear zones in the blue background of starch agar plate around the colonies. Biochemical characterization of this enzyme was also investigated. Results showed that the optimal activity of this enzyme was at 50 °C, and pH of 7.0. In addition, the alpha-amylase exhibited optimum stability at 40 °C, and pH of 7.0. Some metal ions including Mn^{2+}, Cu^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+}, Hg^{2+}, and Mg^{2+} stimulated the alpha-amylase activity by about 122, 105, 61, 47, 46, 23 and 16%, respectively. The best activity of this enzyme was achieved in 0.5 M of KCl (81% enhancement), and 1.5 M of NaCl (9% enhancement). This alpha-amylase hydrolyzed a wide range of raw-starch granules (1.0 %, w/v) including potato, corn, grain, rice, and wheat and optimally was effective on wheat starch (16%, w/v) at 45 °C for 6 h with relative hydrolyses of ~10 U/ml. To our knowledge, it was also found that the alpha-amylase was a Ca^{2+}-dependent enzyme for hydrolyzing higher concentrations of raw wheat starch (90-180 mg/ml) after 6 h of incubation at 45 ℃. Finally, these results indicated that UMF29 alpha-amylase showed high capacity in the degradation of the raw starch.

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Introduction

Alpha-amylases are enzymes that randomly hydrolyze the internal α-1→4-glycosidic bonds in starch. Microbial alpha-amylases demonstrate more compatibility with industrial demands (Pandey *et al.,* 2000; Sudan *et al.,* 2018; Sangngern *et al.,* 2020). These industrial enzymes have potential applications in various industrial portions, for example, brewing, starch processing, pharmaceuticals, baking bread, and detergents (Kammoun *et al.,* 2008; Kohli *et al.,* 2020). Determination of the biochemical properties of alpha-amylases is very important to their appropriate applications in the related industries (Gangadharan *et al.,* 2009). Despite the wide range of alpha-amylase applications, starch processing is the most common industry for this purpose (Souza *et al.,* 2010). Among carbohydrates found in plants, starch is a significant source of carbon and energy. Furthermore, its hydrolysis gives high-value compounds such as glucose, fructose, or maltose syrups, and ethanol (Roy, 2004; Fincan *et al.,* 2021). Inter and intra-molecular bonds between the constructive molecules of starch granules resulted in a polycrystalline state of the carbohydrate. The traditional conversion of starch granules in the course of gelatinization needs heating the high temperatures of 100 °C, consuming high energy, and causing cost

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production (Goyal *et al.,* 2005; Božić *et al.,* 2017). That's why searching for the digesting enzymes with a direct effect on raw starch granules below gelatinization temperature is very important to facilitate the conversion process and reduce the energy requirement (Robertson *et al.,* 2006; Sun *et al.,* 2009; Shofiyah *et al.,* 2020). Based on the previous reports, alpha-amylase of some fungi and yeasts were mostly capable of efficient digestion of raw starch (Morita and Fujio, 2000; Matsubara *et al.,* 2004; Božić *et al.,* 2017), although few alpha-amylases from *Bacillus* sp. have been described to be raw starch degrading. From the point of view of the alpha-amylase ability for digesting raw starch, two groups of bacterial alpha-amylases are known including, raw-starch adsorbing alpha-amylases and raw-starch non-adsorbing alpha-amylases (Božić *et al.,* 2017; Shivlata and Satyanarayana, 2017; Božić *et al.,* 2020; Shofiyah *et al.,* 2020).
Bacillus raw-starch digesting amylases generally hydrolyze starch granules over a longer time but cannot effectively hydrolyze all types of raw starches (Bozia *et al.,* 2011; Du *et al.,* 2018; Božić *et al.,* 2020). Behinds this, a high concentration of starch (around 15%) is used in the starch industry (Goyal *et al.,* 2005). Therefore, finding high concentration raw starch digesting alpha-amylase is much more important. However, there have been few reports on the decomposition of high concentration raw starches by amylases.
In this research, we introduce and characterize a mesophyll bacterium from Kerman farmlands, Iran, which produced a halophilic and very effective alpha-amylase. This alpha-amylase digested different raw starch granules such as wheat, corn, potato, rice, and barley, especially wheat at the higher concentrations of starch, usually applied in the starch handling industry.

Material and Methods

Enzyme production

Kerman farmlands were chosen for the isolation of the alpha-amylase-producing bacteria (30.2760° N, 57.1382° E). First, a total of 50 microbial isolates capable of starch hydrolysis were isolated on the starch agar containing soluble starch (1.0 %), yeast extract (0.2 %), $MgSO_4.7H_2O$ (1.0 %), NaCl (1.0 %), and $CaCl_2.6H_2O$ (0.02 %) (pH 7.0) (Oziengbe and Onilude, 2012). After 72 h of incubation at 37 °C, the plates were inundated with Lugol's iodine, and alpha-amylase-producing isolates were distinguished by clear zones in the blue background of starch agar plate around the colonies. In the end, among 50 isolates, 5 isolates namely UMF2, UMF13, UMF24, UMF29, and UMF42 appeared the clearest zones around their colonies and then were maintained as frozen stocks in the nutrient broth media at -80 °C. Productivity level of alpha-amylase was further verified after inoculating 20 ml preculture, prepared from 100 µl glycerol stocks of the five isolates in nutrient broth medium, into alpha-amylase production medium containing KH_2PO_4 (1.0 g/l), $Na_2HPO_4.2H_2O$ (2.9 g/l), tryptone (2.0 g/l), $(NH_4)_2SO_4$ (1.8 g/l), $MgSO_4.7H_2O$ (0.04 g/l), $CaCl_2.6H_2O$ (0.04 g/l), soluble starch (1.0 g/l) (pH 7.0) (Samie *et al.,* 2012). Subsequently, the UMF29 isolate, which showed a higher ratio of alpha-amylase production in the liquid medium, was selected for the following experiments in this research. Some biochemical tests were employed to introduce the isolate selected. Molecular and physiological identification of this strain has been performed based on our previous reports (Badoei-Dalfard and Karami, 2013; Afrisham *et al.,* 2016). All chemical materials used in this study were purchased from Merck Company (Germany).

Optimization of alpha-amylase production

Enzyme production of the isolated strain was investigated in certain time intervals. For enzyme production, 5% of the bacterial pre-cultures were inoculated in Erlenmeyer flasks with 50 ml of production culture shaking at 140 rpm, 37 °C, and for 24-72 h. After regular intervals of 24 h, 10 ml of the culture medium was gathered and then centrifuged at 1000 rpm for 20 min at 4 °C. The supernatants were applied for alpha-amylase activity.

Enzyme assay

UMF29 enzyme was assayed based on the Bernfeld process to determine the value of reducing sugars at 37 °C (Bernfeld, 1955). In a typical assay, 0.5 ml alpha-amylase solution (10 U/ml) mixed with 0.5 ml of (1.0 %, w/v) starch

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dissolved in 100 mM $Na_2HPO_4.2H_2O$ buffer (pH 7.0) was maintained at 37 °C for 20 min. The value of reducing sugars released was calculated at 540 nm, followed by stopping the enzymatic reaction with 1 ml 3.5-dinitrosalicylic acid. After boiling for 10 min, the value of alpha-amylase, which produced 1 μmol of reducing sugars to glucose per min at 50 °C, was defined as one unit of this enzyme. The assay tests in the absence of enzyme were used as negative controls.

Characterization of the alpha-amylase

To determine the effect of different pH values on the enzyme activity, a concentration of 100 mM of the buffer solutions, including glycine buffer (pH 2.0- 3.0), CH_3COONa buffer (pH 4.0- 5.0), $Na_2HPO_4.2H_2O$ buffer (pH 6.0-7.0), Tris/HCl buffer (pH 8.0-10.0), and glycine buffer (pH 11.0- 12.0) were used (Shafiei *et al.,* 2010). PH activities tests were performed in described buffer solutions at 37 °C, using starch soluble in $Na_2HPO_4.2H_2O$ buffer (100 mM, pH 7.0), as described in the “enzyme assay” section. For enzyme stability, the enzymes were pre-incubated in the presence of different pH values at room temperature for 60 min, and then the residual activity was measured as described above.

The optimal temperature was obtained by measuring the enzyme activity over the range of 20-90 °C under assay conditions (Prakash *et al.,* 2009). Maximum thermal stability was also performed by enzyme pre-incubating at 20-90 °C followed by measuring the remaining activity at the standard condition (Karakaş *et al.,* 2010).

The impact of some metal ions and chemical additives was considered by incubating 0.5 ml of amylase and 0.5 ml of soluble starch with 500 μl of 15 mM solution of various metal ions (Fe^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+}, Hg^{2+}, and K^+) and chemical additives including EDTA, 2-mercaptoetanol, H_2O_2, SDS, and Triton X100, separately, at standard assay conditions (Shafiei *et al.,* 2010; Asoodeh *et al.,* 2013).

Effect of different salt concentrations

The activity of the UMF29 amylase was measured in 0.0-4.0 M NaCl under normal conditions to conclude the influence of salt values on the enzyme activity (Samie *et al.,* 2012). Enzyme activity of the untreated sample (no salt) was considered as 100%.

Raw starch hydrolysis

To examine the activity of UMF29 alpha-amylase on the different raw starches, 1 ml of the enzyme solution was supplemented with different starch granules ((20 mg) (potato, corn, grain, rice, and wheat)) and 1 ml $Na_2HPO_4.2H_2O$ buffer (100 mM, pH 7.0). It then was incubated in a rotary shaker at 37 °C and 110 rpm. After 5 h, the assay reaction mixture was centrifuged at 10,000 rpm, at 4 °C for 10 min, and the amount of reducing sugar in the supernatants was measured using the dinitrosalicylic acid method under standard conditions.

Raw-starch-digesting process

For optimization of the raw-starch-digesting process, the enzyme solution was incubated with various values of the raw starch from 1 to 20% in the same buffer at 37 °C for 5 h, and the reducing sugars in the supernatants were assayed (Shafiei *et al.,* 2010). In further investigations, raw wheat starch at an optimal concentration was used to evaluate the digestibility of raw starch at 25-55 °C during 2.0-12.0 h (Bai *et al.,* 2012).

Kinetic properties

The kinetic factors, K_m and Vmax, were calculated by using the different values of raw wheat starch as substrate. The extent of starch hydrolysis was measured by incubating enzyme solution with the mixtures of raw wheat starch at the concentrations of 20-180 mg/ml and 100 mM phosphate buffer treated and untreated with 5 mM of $CaCl_2$ at 45 °C for 6 h, using the DNS method with glucose as standard (Mehta *et al.,* 2013).

Results

Bacterial identification

In this study, a mesophyll bacterium, UMF29, producing a raw-starch digesting alpha-amylase, was isolated from Kerman farmlands. This strain was gram-positive, catalase-negative, gelatin and casein hydrolyzing, rod-shaped, and aerobic according to morphological and biochemical investigations. It was identified as *Bacillus mojavensis* according to the 16S rDNA sequence of this strain (Fig. 1).

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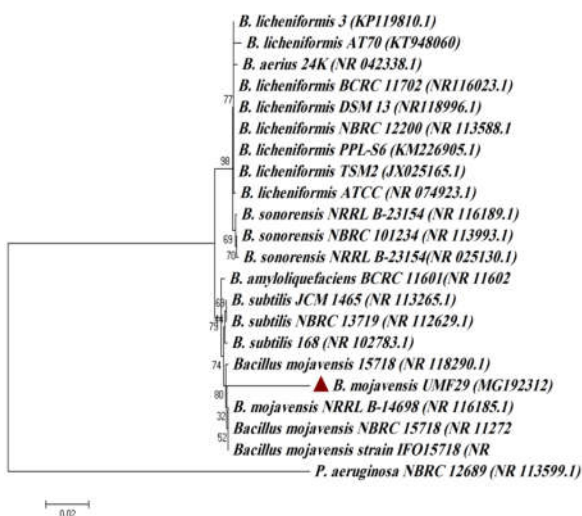

Fig. 1. Phylogenetic tree of *Bacillus mojavensis* UMF29 based on 16S rDNA sequences of related strains. Scale bar, 0.02 represents nucleotide substitutions per site.

The phylogenetic tree of this isolate was created by the blast of the various 16S rDNA sequences with an online tool (MEGA 6). The 16s rDNA sequence was submitted in Gene Bank with an accession number of MG192312. The isolated strain showed the highest clear zone around the colony on the starch agar plate (Fig. 2) and produced a maximum yield of 597 U/ml of alpha-amylase after 72 h of incubation at 37 °C (Fig. 3).

Enzyme characterization

The pH activity assay showed that the UMF29 alpha-amylase had optimum activity and stability at a pH 7.0, and the alpha-amylase also showed good activity and stability at the pH range of 4.0-9.0 (Fig. 4a).

Enzyme activity and stability assay on different temperatures (20-90 °C) reflected that the temperatures required for maximum activity and stability were 50 and 40 °C, respectively (Fig. 4b). The enzyme was well active and stable over a lower temperature range of 20-40 °C. The enzyme also illustrated 89 and 74% of its maximum activity at temperatures of 60 and 70 °C.

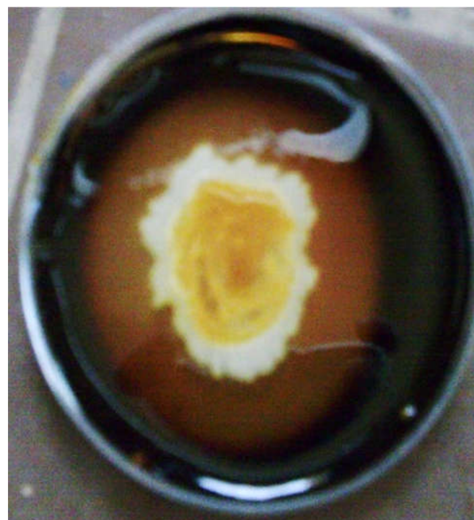

Fig. 2. The clear zone around the colony of strain UMF29 on the starch agar plate.

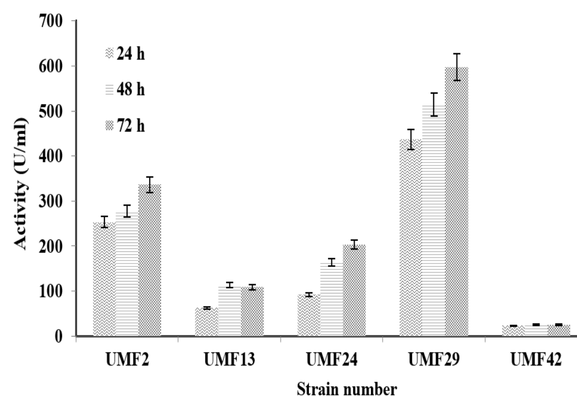

Fig. 3. Effect of incubation time on the alpha-amylase production of the five isolated strains: UMF2, UMF13, UMF24, UMF29, and UMF42. Enzyme samples were retrieved every 24 h to 72 h.

The activity of UMF29 alpha-amylase was studied in various metal ions and additives. Several metal ions well increased UMF29 alpha-amylase activity, while Co^{2+} inhibited alpha-amylase activity by 18%. Partial inhibition of enzyme activity was seen on EDTA by about 10%. Incubation with protein denaturant, SDS, also inhibited the enzyme by 36%, but Triton X-100 improved the enzyme activity by about 18% (Table 1).

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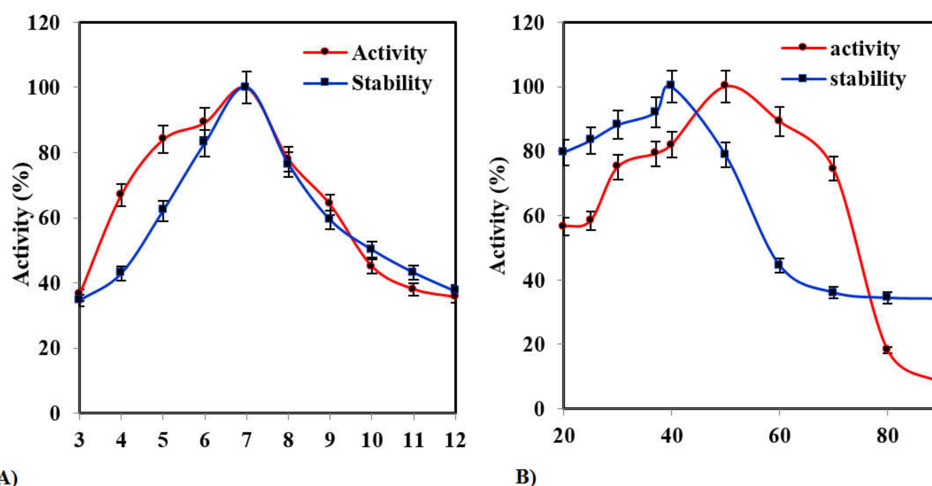

Fig. 4. The effect of various pH values and temperatures on the activity and stability of UMF29 alpha-amylase: Effect of various pH values (A); Effect of various temperatures (B); The effect of pH and temperature on enzyme activity was assayed at various pH values (3.0-12.0) and temperatures (30-90 °C), respectively, using 1% soluble starch under standard conditions. The stability profile of the enzyme was determined by measuring residual activity after any treatment at 37 °C.

Table 1: The effect of different metal salts and additives on UMF29 alpha-amylase activity

Metal ions and chemical additives	Activity	Metal ions and chemical additives	Activity
Fe^{2+} (5 mM)	147	K^{+} (5 mM)	102
Cu^{2+} (5 mM)	205	Mg^{2+} (5 mM)	116
Co^{2+} (5 mM)	82	T.X.100 (5 mM)	117
Zn^{2+} (5 mM)	146	SDS (5 mM)	64
Mn^{2+} (5 mM)	222	H_2O_2 (5 mM)	169
Ca^{2+} (5 mM)	161	EDTA (5 mM)	90
Hg^{2+} (5 mM)	123	Control	100

Effect of different salt values

The enzyme activated at various values of NaCl and KCl between 1.0 and 4.0 M (Table. 2). The best activity of this enzyme was achieved in 0.5 M of KCl (81% enhancement), and 1.5 M of NaCl (9% enhancement). The enzyme retained 42 and 88% of its maximum activity at 4.0 M KCl and NaCl, respectively. Therefore, it required less NaCl concentration for optimum activity.

Table 2. Effect of various concentrations of NaCl and KCl on enzyme activity.

Salt [M]	Activity (%)	Salt [M]	Activity (%)
	KCl		NaCl
0	100	0	100
0.5	181	0.5	101
1	160	1	104
1.5	147	1.5	109
2	124	2	106
2.5	114	2.5	103
3	104	3	82
3.5	101	3.5	59
4	88	4	42

Raw-starch hydrolysis

UMF29 alpha-amylase showed the potential digestibility of various raw starch granules at the concentration of 1.0%, during 5 h of incubation at 37 °C (Fig. 5).

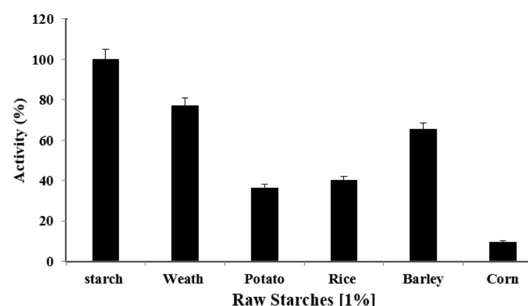

Fig. 5. The digestibility of UMF29 alpha-amylase for 1% (w/v) concentration of various raw starches after 5 h of incubation.

During direct hydrolyses of different raw starches, raw wheat starch (77 % digestion) was the best substrate for UMF29 alpha-amylase. In

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comparison, the enzyme showed minimum hydrolyzing activity on raw corn starch (9 % digestion). Relative hydrolyses of 65, 40, and 36% were obtained for barley, rice, and potato.

Optimization of raw-starch digesting process

Different concentrations of the raw starches (wheat, rice, potato, corn, and barley) at a range of 1-20% were tested for direct hydrolyses by UMF29. The highest hydrolysis rate of raw starch acceded on the raw wheat starch at a value of 16% (9.8 U/ml) with effective hydrolysis in the concentration range of 1-20%.

In the case of other raw starches, the most ability of UMF29 alpha-amylase for hydrolyses occurred with 8% barley (7.5 U/ml), 8% rice (6.8 U/ml), 16% potato (6.1U/ml), and 16% corn (4.4 U/ml.) (Fig. 6A).

The role of time and temperature of incubation on the raw starch degradation by UMF29 alpha-amylase was verified by measuring the hydrolysis value of 16% wheat starch in the temperature range of 25-55 °C after 2-10 h of incubation.

The enzyme demonstrated good affinity in the digestion of raw wheat granules at temperatures between 35 and 55 °C with optimum hydrolyses at 45 °C (Fig. 6B).

The enzyme was also sufficient in good digestion of raw wheat granules at a short time of 6 h and showed hydrolyses degrees of 70-90% at time durations of 4-10 h (Fig. 6C).

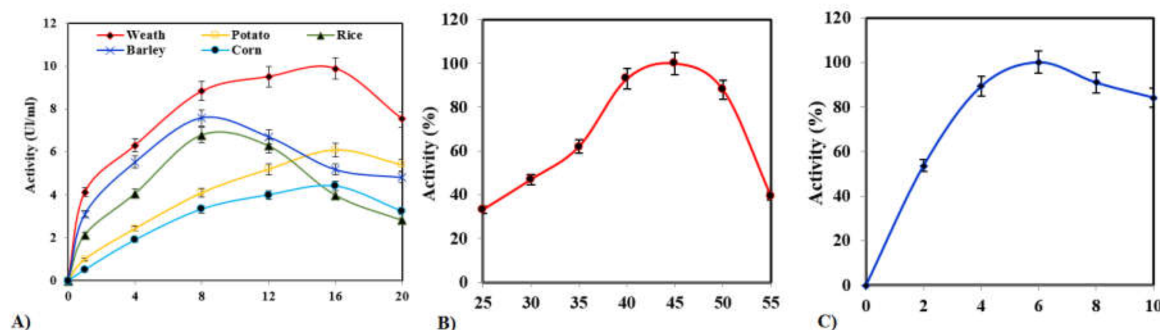

Fig. 6. A) Hydrolyses rate of the various concentrations of raw starches by UMF29 alpha-amylase for 5 h. Hydrolysis extent was calculated as the percent of maximum hydrolyzing activity according to the standard method. B) The effect of incubation temperature on the rate of hydrolyses of 16% raw wheat starch with the UMF29 alpha-amylase. C) Determination of enzymatic hydrolyses extent of raw wheat starch (16%) during different periods. Raw wheat starch was incubated in the alpha-amylase of UMF29 at 45 °C for 2-10 h and hydrolyses degree measured every 2 h at standard assay conditions.

Determination of kinetic properties

Kinetic properties of the UMF29 alpha-amylase were evaluated by enzyme activity assay on various concentrations of raw wheat starch at optimal conditions of hydrolyses reaction, 45 °C for 6 h. These values were obtained in the presence and absence of 5mM Ca^{2+}. In each of the two states, with or without Ca^{2+}, K_m and Vmax values were found from Line weaver–Burke and Michaelis-Menten plots (Afrisham *et al.,* 2016; Badoei-Dalfard and Karami, 2013). UMF29 alpha-amylase displayed increased alpha-amylase activity with a rise in the value of raw substrate from 20 mg/l to 180 mg/l. K_m and *Vmax* values obtained in the presence of Ca^{2+} at a concentration of 5 mM were 423 mg/ml and 1428.57 U/ml, which were superior to the values of K_m (111.7 mg/ml) and V*max* (526 U/ml) in the absence of Ca^{2+} (Fig. 7).

Discussion

Despite multiple reports on the raw starch digesting amylases production from fungi, some recent studies long with our study indicate the production of bacterial alpha-amylases with the capacity of raw starches decomposing (Lakshmi *et al.,* 2020; Sadeghian Motahar *et al.,* 2020).

As the results show, 60-80% of UMF29 alpha-amylase activity was retained at the pH range of 4.0-9.0 with optimal activity and stability at the pH of 7. This optimum pH range is close to the reported pH of the other raw-starch digesting alpha-amylases (Demirkan *et al.,* 2005; Goyal *et al.,* 2005; Demirkan *et al.,* 2005; Goyal *et al.,* 2005; Shafiei *et al.,* 2010; Demirkan *et al.,* 2005; Goyal *et al.,* 2005).

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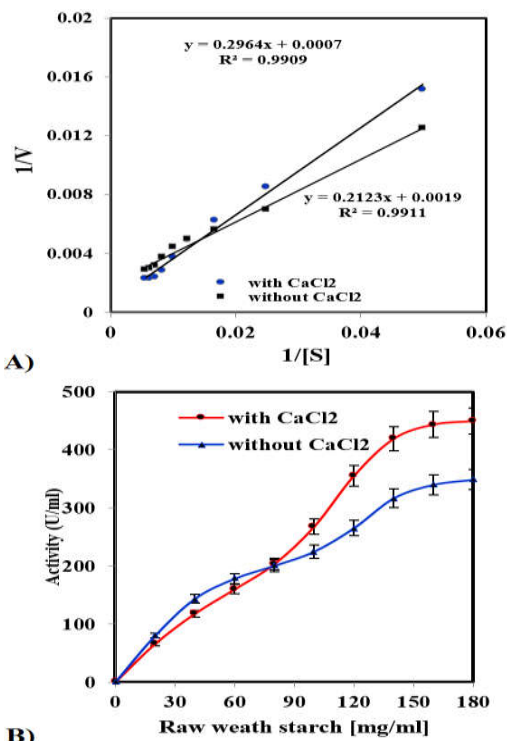

Fig. 7. Effect of Ca^{2+} (5 mM) on the enzymatic degradation of different concentrations of raw wheat starch (20-180 mg/ml) as substrate, after 6 h at 45 °C. In each of two states, with or without Ca^{2+}, K_m and Vmax values were found from Line weaver–Burke and Michaelis-Menten plots.

It is shown that the best pH value in the starch liquefaction is 7.0. Therefore, this enzyme can be used as a potential candidate in starch manufacturing. Furthermore, the alpha-amylase activity at a low pH value leads to the diminished formation of several by-products, including maltulose, which is normally synthesized at high pH.

The temperature profile of UMF29 alpha-amylase indicated that its maximum activity and stability were at 50 and 40 ℃, respectively. Based on these results, the UMF29 alpha-amylase is moderately thermostable, suggesting that this enzyme can be used in industrial processes i.e., customary brewing and food handling (Stamford *et al.,* 2002; Jiang *et al.,* 2015; Ferreira *et al.,* 2021). Maximum activity of UMF29 alpha-amylase was found at 40-55 °C, similar to the other raw starch degrading alpha-amylases (Demirkan *et al.,* 2005; Gangadharan *et al.,* 2009; Shafiei *et al.,* 2010). However, higher temperature activity was described for the alpha-amylases from *Bacillus* sp. I-3 (Goyal *et al.,* 2005).

Results showed that the alpha-amylase activity was evaluated by the metal ions except for Co^{2+}. However, EDTA insignificantly decreased the enzyme activity by about 10%, suggesting that the UMF29 alpha-amylase is a metalloenzyme. EDTA generally exhibits a potent inhibitory effect on the alpha-amylases at low concentrations (Gupta *et al.,* 2003) as *Bacillus* sp. IMD 370 alpha-amylase decreased its activity at 1 mM EDTA (Asgher *et al.,* 2007). However, some alpha-amylases were found not to be sensitive in the presence of EDTA (Kiran *et al.,* 2008; Peng *et al.,* 2015).

Moreover, the stimulatory effect of Ca^{2+}, as a divalent metal ion, showed that this enzyme was Ca^{2+}-dependent in which this ion caused the rigid structure of the alpha-amylase (Burhan *et al.,* 2003). The presence of Ca^{2+} also stimulated a halophilic and raw-starch digesting alpha-amylase from *Nesterenkonia* sp. strain F by about 127.5 % (Shafiei *et al.,* 2010). Denaturant reagent SDS decreased the activity of UMF29 by about 36%, while Triton X-100 increased the enzyme activity by about 18%. These effects of SDS and Triton X-100 were in line with the outcomes of Wang et al, in which these detergents inhibited and activated this enzyme activity, respectively (Wang *et al.,* 2011).

A strong stimulatory effect of the UMF29 alpha-amylase activity was detected by different concentrations of KCl (81% enhancement by 0.5 M KCl) and NaCl (9 % enhancement by 1.5 M NaCl). Such behavior on KCl and NaCl concentrations (0.0-4.0 M) was reported for many halophilic and raw-starch digesting amylases (Coronado *et al.,* 2000; Deutch, 2002; Prakash *et al.,* 2009; Shafiei *et al.,* 2010).

The enzymatic activity of amylases is mainly influenced by metal ions as activators or inhibitors. Generally, metal ions act as electrophiles that hold the functional groups in three-dimensional orientations, and form enzyme-substrate interaction by coordinate bond formation. Metal ions also stabilize the catalytic active site of enzymes (Palmer and Bonner, 2007). Therefore, metal ions play an essential role in the activation and stabilization of enzymes (Li *et al.,* 2013). UMF29 alpha-amylase

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showed the highest hydrolyzing activity in the raw wheat starch and the raw barley starch by about 77% and 65%, respectively. Similarly, *Nesterenkonia* alpha-amylase exhibited the highest rate of raw starch digesting for raw wheat starch (Shafiei *et al.,* 2010). However, incubation of the *Bacillus* alpha-amylase in raw potato starch followed by raw corn starch efficiently elevated the hydrolysis percentage than the raw wheat and rice starches (Gangadharan *et al.,* 2009). Enzymatic hydrolysis percentage of 1.0 % of corn, wheat, and potato starch granules were 57.5, 53, 45.1%, respectively, by *Bacillus* sp. YX-1 alpha-amylase after 8 h of incubation.

Digesting characterization of UMF29 alpha-amylase was examined in 1-20% of several raw starches. The results showed that 16% of raw starch wheat cussed to the highest hydrolyses rate. The comparison of the hydrolyses rate of wheat starch by UMF29 alpha-amylase to *Nesterenkonia* alpha-amylase showed good ability for the direct hydrolysis of 1-4% of wheat starch, while the hydrolyzed starch sharply dropped at ranges of 10-15% (Shafiei *et al.,* 2010). However, strong digestibility of raw starches was observed in the case of the alpha-amylase from *Bacillus* sp. YX-1 for 20 % of raw corn starch and alpha-amylase from *Anoxybacillus flavothermus* for 31% raw starch suspension (Tawil *et al.,* 2012). Degrading alpha-amylase capacity on the raw wheat starch (16%) was optimized at 45 ℃ after 6 h of incubation. Goyal et al. obtained a temperature range of 60–90 ℃ for good digestion of the potato starches (1%) with an optimal temperature of 70 ℃ (Goyal *et al.,* 2005). Enzymatic hydrolyses percentages of wheat and potato starches by Gs4j-amyA were the highest when temperature increased to 80 and 90 ℃, respectively. Similarly, some alpha-amylases indicated robust degradation ability on the various raw starches in a short time (Bai *et al.,* 2012; Peng *et al.,* 2015; Mukherjee *et al.,* 2019; Ferreira *et al.,* 2021). In contrast, the digestion of the different starch granules such as corn, rice, wheat, sweet, and potato in the presence of *B. amyloliquefaciens* alpha-amylase was kept constant through 12-24 h (Demirkan *et al.,* 2005). This finding is in agreement with the extended time durations (at least 24 h) reported by the most starch-degrading alpha-amylases (Mitsuiki *et al.,* 2005; Wong *et al.,* 2007; Hasan *et al.,* 2008; Hostinová *et al.,* 2010; Puspasari *et al.,* 2011; Mukherjee *et al.,* 2019; Ferreira *et al.,* 2021).

K_m and Vmax values of UMF29 alpha-amylase were gained to be 111.7 mg/ml and 526 U/ml for raw wheat starch that was increased to 423 mg/ml and 1428.57 U/ml in the presence of Ca^{2+} (5 mM). Owing to the use of the various substrates and reaction conditions, it is hard to compare the K_m and V*max* values of different alpha-amylases (Gangadharan *et al.,* 2009). The presence of Ca^{2+} usually had no inhibition effect on the kinetic of amylases (Mehta *et al.,* 2013). However, Ca^{2+} revealed the inhibitory effect on the Vmax value of alpha-amylase from *Geobacillus thermoleovorans*, indicating the Ca^{2+} binding to the enzyme-substrate complex. However, the K_m value of the alpha-amylase was increased by Ca^{2+} (Mehta *et al.,* 2013). The elevated value of K_m was reflected in reducing enzyme affinity toward the substrate. Similar to UMF29 alpha-amylase, Ca^{2+} (5 mM) led to the significant increase of the activity of *Aeromonas salmonicida* ssp. *Salmonicida* alpha-amylase on rice (Peng *et al.,* 2015). This study was the first report on the efficient digestion of high concentrations of raw starch mashes by halophilic alpha-amylases.

Conclusion

Recently, raw starch degrading alpha-amylases have been more considered by researchers. The aim of this investigation was the isolation and biochemical characterization of raw starch degrading alpha-amylase from *Bacillus mojavensis* strain UMF29. The results showed the potential ability for degradation of several raw starches, including wheat, rice, potato, corn, and barley. The highest hydrolytic affinity of this enzyme was obtained for 16% wheat starch, after 6 h of incubation at 45 °C. Furthermore, the enzyme activity improved about 81 % against 0.5 M KCl. Results showed that the optimal activity of this enzyme was at the temperature of 50 °C, and the pH of 7.0. Mn^{2+} and Cu^{2+} improved the alpha-amylase activity by about 122 and 105 %, respectively. Taken together, these results indicated that UMF29 alpha-

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amylase has high potential in starch manufacture.

Conflict of interests

The authors declare that they have no conflict of interest.

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شناسایی مولکولی باسیلوس باسیلوس موجاونسیس سویه UMF29 مولد آلفا آمیلاز جدید تجزیه کننده نشاسته خام

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چکیده

آلفا آمیلازهای میکروبی با حوزه های صنعتی انطباق پذیری بالایی را نشان می‌دهند. این آنزیم‌های صنعتی در بخش‌های صنعتی مختلف از جمله نوشیدنی‌ها، پخت نان، دارویی و شوینده‌ها کاربردهای بالقوه‌ایی را نشان داده‌اند. در این مطالعه یک باکتری مزوفیل، باسیلوس موجاونسیس سویه UMF29 بر اساس توالی 16S rDNA و درخت فیلوژنی رسم شد. بعد از ۷۲ ساعت انکوباسیون در دمای°C ۳۷ پلیت‌ها با محلول لوگول یدی انکوبه شدند و سویه‌های مولد آلفا آمیلاز بر اساس ناحیه شفاف در اطراف کلونی‌ها روی زمینه آبی رنگ پلیت نشاسته آگار شناسایی شدند. تعیین خصوصیت بیوشیمیایی این آنزیم نیز انجام شد. نتایج نشان داد که فعالیت بهینه آنزیم در دمای°C ۵۰ و اسیدیته ۷ می‌باشد. علاوه بر این آلفا آمیلاز پایداری بهینه را در در دمای °C ۴۰ و اسیدیته ۷ می‌باشد. برخی یون‌ها از جمله، Mg^{2+}،Mn^{2+}، Cu^{2+}، Ca^{2+}، Fe^{2+}، Zn^{2+} و Hg^{2+} فعالیت آلفا آمیلاز را حدود ۱۲۲، ۱۰۵، ۶۱، ۴۷، ۴۶، ۲۳ و ۱۶ درصد افزایش می‌دهند. بهترین فعالیت این آنزیم در حضور غلظت نیم‌مولار KCl (۸۱ درصد افزایش) و ۱/۵ مولار NaCl (۹ درصد افزایش) به‌دست آمد. این آلفا آمیلاز یک محدوده وسیعی از گرانول‌های نشاسته خام (۱ ٪، w/v) از جمله سیب زمینی، ذرت، جو، برنج و گندم را هیدرولیز کرد و روی هیدرولیز نشاسته گندم (۱۶ ٪، w/v) در دمای °C ۴۵ به‌مدت ۶ ساعت با هیدرولیز نسبی به میزانU/ml ۱۰ موثر بود. بر اساس دانش ما، این آنزیم یک آلفا آمیلاز با فعالیت وابسته به کلسیم می‌باشد که توانایی هیدرولیز غلظت‌های بالای نشاسته گندم (۹۰ تا ۱۸۰ میلی گرم در میلی‌لیتر) بعد از ۶ ساعت انکوباسیون در ۴۵°C را دارا می‌باشد. در پایان این نتایج خاطرنشان ساخت که آلفا آمیلاز UMF29 ظرفیت بالایی را در تجزیه نشاسته خام نشان داد.

واژگان کلیدی: آلفا آمیلاز؛ باسیلوس موجاونسیس؛ تعیین خصوصیت بیوشیمیایی؛ شناسایی مولکولی؛ تجزیه نشاسته خام

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