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Research Article

Biochemical and Kinetic Characterization of the α-Amylase from *Bacillus amyloliquefaciens* JJC33M, AmyJ33, On Raw Starch

Sarahí Hernández-Heredia and Sandra del Moral*

Instituto de Biotecnología, Universidad del Papaloapan, México

***Corresponding author**

Sandra del Moral, Instituto de Biotecnología, Universidad del Papaloapan, Circuito Central 200, 68400 Tuxtepec, Oaxaca, México, Tel: 52 287 875 9240; Fax: 52 287 875 9240; Email: smoral@unpa.edu.mx

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Abstract

AmyJ33 is an α-amylase from *Bacillus amyloliquefaciens* JJC33M is not calcium dependent and is active on native and raw starch. In this work, a deeper biochemical and kinetic characterization was made. AmyJ33 presented an optimum pH and temperature at 5 and 80°C, respectively, it was stable at pH 5 and a had half-life of 16h (40°C), 2.25 h (50°C) and 0.25 h (60°C). The enzyme produces glucose, maltose and maltopentose traces of potato starch, in 180 min it hydrolyzed 25% of raw starch via exo-corrosion. K_m and V_{max} values were 10.6 mg/mL and 41.0 U/mg, respectively. AmyJ33 could be used in the baking industry or to produce ethanol on raw starch.

ABBREVIATIONS

DP: Degree Of Polymerization; TLC: Thin Layer Chromatography; DNS: 3,5 dinitrosalicylic acid; Rh: Rate Of Raw Starch Hydrolysis; SEM: Scanning Electron Microscopy

INTRODUCTION

Amylases (EC 3.2.1.1) are enzymes that catalyze the hydrolysis of glycosidic bonds α-(1-4) or α-(1-6) of the starch molecule, producing glucose and maltose as products [1,2]. Amylases have applications in several industries: textile, food, cleaning, paper and biofuels, among others. The principal producers of amylases for industrial use are the microorganisms, mainly *Bacillus* and *Aspergillus* genera, specifically *B. licheniformis, B. subtilis* and *B. amyloliquefaciens* [3]. According to the many reports, the molecular weight of the α-amylases varies between 10-120 kDa. Also, α-amylases hydrolyze mainly soluble starch, because granules are very resistant to amylolytic digestion, but some of them are capable of hydrolyze raw starch [4], this is an advantage when saving energy at several processes such as ethanol production or to generate porous starch, a kind of sorbent, which is widely used in the fields of study of food, drugs, animal nourishment, cosmetics, etc. [5]. In a previous research, *B. amyloliquefaciens* JJC33M was isolated from soils cultivated with sugar cane at the Papaloapan region in Mexico and its genome was published [6]. *Bacillus amyloliquefaciens* is a Gram positive bacterium, that produces an α-amylase, AmyJ33. AmyJ33 was partially characterized, resulting non-dependent of Ca^{+2}, it is stable at 40°C and maintained 70% of its activity at acidic pH. According to the analyzed characteristics, AmyJ33 may be suitable for the baking sector´s enzyme [7]. In this work, AmyJ33 was made a more detailed characterization of AmyJ33, including thermal stability, half-life, pH stability, kinetic parameters and hydrolysis on raw starch including how raw starch is degraded.

MATERIALS AND METHODS

Bacterial strain and culture conditions

Bacillus amyloliquefaciens JJC33M was obtained from a collection of strains isolated from soils [7] cultivated with sugar cane and was cultivated on Petri agar dishes on nutritive agar and soluble starch (10 g/L) at 37°C for 24 h. Later, *B. amyloliquefaciens* JJC33M was cultivated in nutritive broth and soluble starch (10 g/L) at 37°C for 16 h at 180 rpm.

AmyJ33 production and purification

Bacillus amyloliquefaciens JJC33M was inoculated (10% V/V) on flasks containing 1000 mL of yeast extract (5 g/L), K_2HPO_4(6 g/L), KH_2PO_4 (3 g/L), $CaCl_2$ (0.1 g/L), sodium citrate (0.5 g/L), $(NH_4)_2SO_4$ (5 g/L), $FeSO_4$ (0.01 g/L), $MnSO_4$ (0.01 g/L), $ZnSO_4$ (0.001 g/L), starch (10 g/L), MgSO4 (0.2 g/L) [7] and incubated at 37°C and 180 rpm in a Excella E24 Incubator (New Brunswick

Scientific). Supernatant was recovered by centrifugation at the beginning of the stationary phase at 5,580 g during 40 min (HERAEUS Pico 17 Centrifuge, Thermo SCIENTIFIC). Supernatant was precipitated with ammonium sulfate (60%), and the precipitate was collected by centrifugation. The precipitate was diafiltered on 10 kDa Amicon tubes (Millipore) with 50 mM sulfate buffer at pH 7 and $CaCl_2$ (1 mM) during 40 min at 4°C.

Activity assay

The enzymatic activity of AmyJ33 was tested in 50 mM phosphate buffer at pH 7.0 containing 10 g/L raw starch (Eslab) at 45°C, by measuring the release of reducing sugars in 800 µl of volume reactions containing 4.9 µg of purified protein. Samples of 100 µl were withdrawn at 2 min time intervals and reducing sugars were quantified by the 3, 5-dinitrosalicylic acid (DNS) method using a D-glucose standard curve (Sigma). One unit (U) of enzyme activity was defined as the amount of enzyme that releases 1 µmol of glucose equivalents per minute. These conditions were used to evaluate residual activity when thermal and pH stability was performed.

Effect of pH on enzymatic activity and pH stability

The effect of pH on activity of AmyJ33 was determined at 45°C in the range of 4.0 to 9.0 in 1 mM $CaCl_2$, 50 mM acetate buffer (pH 4.0-5.0), phosphate buffer (pH 6.0-8.0), and Glycine-NaOH buffer (pH 9.0) using the activity assay while pH stability was measured from enzyme solutions incubated at 4°C during 24 h in 50 mM buffer at pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. The residual activity was measured as described in the activity assay.

Effect of temperature on enzymatic activity and thermal stability

The effect of temperature on the AmyJ33 was evaluated at pH 5.0 in the range of 40 to 90°C using the activity assay. Thermal stability was measured from enzyme solutions incubated at 40, 50, and 60°C in 50 mM acetate buffer pH 5.0. The residual activity was measured using the activity assay with enzyme samples withdrawn at different time intervals. Half- life was calculated by linear regression of first order.

Kinetic properties

Reaction rates were measured at different concentrations of raw starch in the range of 1 a 14 mg/mL. Reaction conditions were 45°C and pH 5.0, $CaCl_2$ (1 mM). Kinetic parameters K_m and V_{max} were calculated by nonlinear regression of the data to the Michaelis-Menten equation, using the program Origin Pro 8 (version 8.0724). All the measurements were performed twice.

Hydrolysis products of AmyJ33 on raw starch

AmyJ33 (0.15 U/mL) was incubated (45ºC, pH 4.5) on raw starch (10 g/L). Aliquots (100 µL) were with draw at several times: 0, 20, 40, 60, 80, 100, 120, 140,160 and 180 min, centrifuged and boiled for 5 min to inactivate the enzyme. The rate of raw starch hydrolysis (Rh) was calculated according to the equation: Rh (%) = $(A_1/A_0) \times 100$, where A_1 is the amount of reducing sugars in the supernatant after hydrolysis, and A_0 is the amount of raw starch before the reaction. Reducing sugars were determined by duplicate using DNS technique. The samples were observed by Thin Layer Chromatography (TLC) on silica gel (15x10 cm, Merk) with the mobile phase composed of butanol:ethanol:water (30:50:20 V/V/V). The spots were visualized by an α-naphtol solution composed of ethanol: sulfuric acid: water (171:22:13 V/V/V) on the silica, the TLC plates were dried at 70ºC with hot air for 10 min.

Raw starch characterization

Eslab starch and residual starch of hydrolysis reaction were analyzed by SEM. Residual starch of the hydrolysis via AmyJ33 was withdrawn at 0, 80 and 160 min. The samples were boiled for 5 min, after, they were centrifuged at 10 000 g for 2 min (Hereaus pico 17 Thermo Scientific), the supernatant was discharged and the pellet was dried at 60ºC for 24 h (New Brunswick Scientific, Excella E24). Dry pellet and Eslab starch were placed on a metal plate, previously covered with a carbon double sided adhesive tape, submitted to a 20 nm gold layer application, and observed with a scanning electron microscope, model DSM 960 ZEISS - "Digital Scanning Microscope". Amylose content was analyzed through Megazyme kit, following the specifications of the supplier.

RESULTS AND DISCUSSION

AmyJ33 is a α-amylase which has activity in raw starch [7], in this work, a deeper characterization on raw starch was made that included the hydrolysis products of the raw starch and how starch granules are degraded.

The influence that pH has on AmyJ33 activity and stability

In previous work, AmyJ33 was partially characterized [7]. The pH effect on AmyJ33 activity was evaluated at the pH range between 4-8 using several buffers (Figure 1A). AmyJ33 was active at the same range of pH which showed the highest activity at pH 5 (24.61 U/mg) and at pH 4 and 6 it showed 94 and 77% of residual activity, respectively. AmyJ33 showed the lowest activity at pH 7 and 8, 61 and 62%, respectively. However, AmyJ33 at pH 9 showed 67% of residual activity. According to statistical analysis, the activity between pH 7-9 do not show significant difference. AmyJ33 is more active in acidic pH. This behavior has been observed in other amylases, for example amylases produce from *B. amyloliquefaciens* ATCC 23849 and *B. amyloliquefaciens* showed their pH optimum at 5. However, amylases from *Bacillus* sp. BKL20 [8], *Bacillus* sp [9], *B. subtilis* KIBGE HAS [10] presented the highest activity between pH 6-8. While amylase from *Bacillus* sp BMM1 showed the optimum between pH 5-7 [11]. Amylases from most bacteria have optimum pH in the acidic to neutral range [12]. This comportment could be related with the change in the active site and consequently in the amino acid sequence [13]. The pH stability was measured after 24 h at 4ºC in the corresponding pH. AmyJ33 retained 100% of activity at pH 5 (24.61 U/mg). In the other pHs, AmyJ33 was less stable, at pH 4 only presented 45% of initial activity, at pH 6, 7, 8 and 9 it showed 79, 67, 47 and 30% of residual activity, respectively. In contrast, the amylase from *Bacillus* sp. AB68 was stable at a pH range between 5-8 (80% of residual activity), at a pH greater its activity was diminished [14]. Comparing the effect and pH stability on AmyJ33, it can observe that AmyJ33 does not change

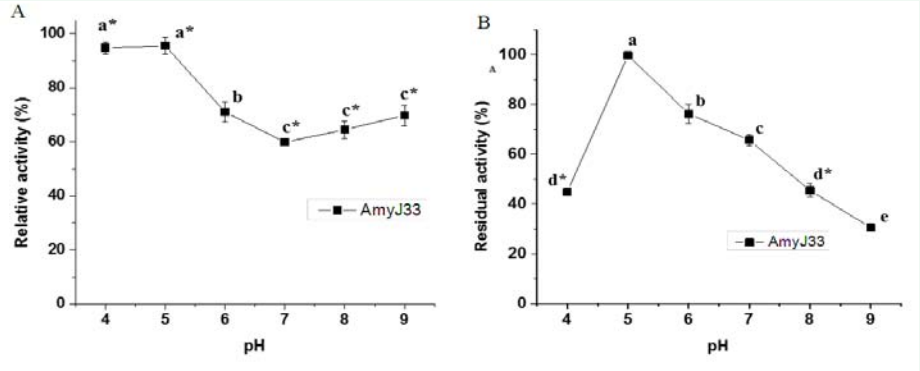

Figure 1 Effect of pH (A) and pH stability (B) on the activity of AmyJ33. Conditions of the experiment: raw starch as substrate (10 g/L), 0.15 U/mL of AmyJ33, 45ºC. Tukey P < 0.05, n=2.

its activity for 24 h at pH 5-7, but in the rest of the pHs its activity decreased.

The influence of temperature on AmyJ33 activity and stability

The activity of AmyJ33 was evaluated in a range of temperature between 40-90°C, with an optimum activity at 80°C (Figure 2A). The relative activities at 40, 50, 60 and 70°C were 27, 43, 54 and 83%, respectively. At 90°C only 2% of the optimum activity was detected. These results differ slightly from those reported by [7], the optimum temperature is the same (80°C), but the residual activity at 40-60°C varies lightly. The results are compared with the information that was reported for the amylase from *B. licheniformis* CUMC305 [15], *Bacillus* sp US100 [16], *Bacillus* spp MK8 [17] which presented their optimum temperature between 80-90°C. The thermal stability of AmyJ33 was evaluated at 40, 50 and 60°C where its half-life was 16.5, 2.31 and 0.25 h, respectively (Figure 2B). These results are according to amylase from *B. licheniformis* MTCC1483 and *Bacillus* sp UEB-S, they did not change their activity at 40°C (1 h), however at 60°C they totally lost their activity [18]. Based on these results, AmyJ33 is not a thermo stable enzyme, nevertheless at 40°C is stable for 16 h and at 50°C it maintains 80% of residual activity, for this reason, AmyJ33 should be used in these conditions.

Kinetics parameters of AmyJ33 on raw starch

The effect of the substrate in AmyJ33 was evaluated for 1-14 mg/mL and the kinetic parameters were obtained (Figure 3). The kinetic behavior of AmyJ33 was adjusted to the Michaelis-Menten model and the kinetic parameters were determined by Origin Lab software (v9.0). The K_m and V_{max} values were 10.6 mg/mL and 41 U/mg, respectively. K_m value indicates that AmyJ33 has low affinity to raw starch. In general, the most of amylases only hydrolyse soluble starch; therefore, they show more affinity to substrate and higher velocities (Table 1). Hydrolysis on raw starch is difficult due to a high level of compacting of the grain [19].

Partial characterizing of raw starch

The raw starch (Eslab) was characterized partially, percentage of amylose, amylopectin and morphology of the grains through SEM were determined. The starch contains 27.8% amylose and 72.2% amylopectin. The most starches contain 20-30% amylose depending on the botanical source [20]. [21], reported amylose contents varying from 24.4 to 27.3% for four potato cultivars. According to SEM images, Eslab raw starch is composed by granules spherical, oval shape and smooth surface (Figure 5A). All these characteristics are similar to the granules of potato starch [4, 22], therefore, Eslab starch is of potato.

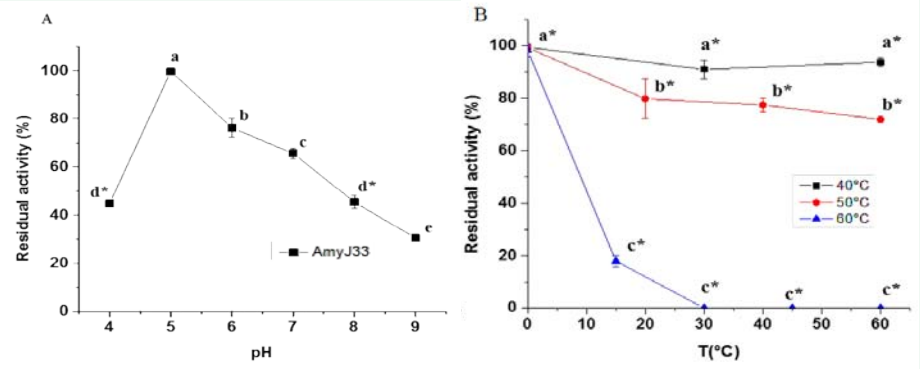

Figure 2 Effect of temperature (A) and thermal stability (B) on the activity of AmyJ33. Conditions of the experiment of pH stability: raw starch as substrate (10 g/L), 0.15 U/mL of AmyJ33, pH 5. Tukey, p < 0.05, n=2.

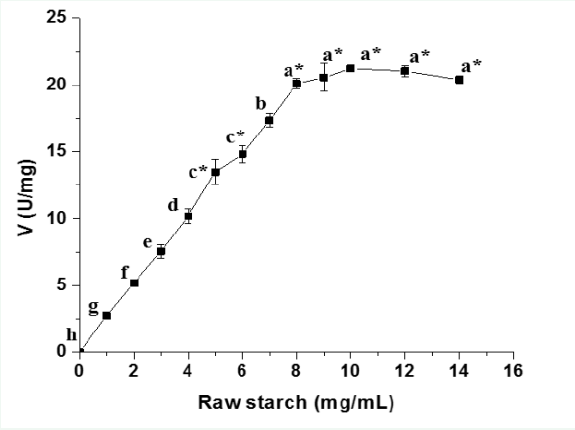

Figure 3 Effect of substrate concentration on the activity of AmyJ33. Conditions of the experiment 45°C, raw starch as substrate, 0.15 U/mL of AmyJ33, pH 5. Tukey $p < 0.05$, n=2.

Table 1: Biochemical properties of microbial α–amylase.

Microorganism/ enzyme	MW (kDa)	Optimum pH	Optimum temperature	pH stability	Termal stability	V_{max} and K_m	Type of starch	Reference
B. subtilis	48	6.5	50°C	≤ 7.0	≤ 50°C	V_{max} = 581.5 U/mg K_m = 3.84 mg/mL	---	[28]
B. amyloliquefaciens (AmyJ33)	50	5.0	80°C	5.0 (24 h)	40°C	V_{max} =41 U/mg K_m = 10.6 mg/mL	Raw	This work
B. subtilis KCC 103	53	5-7	65-70°C	---	---	V_{max} =909 U/mg K_m =2.6 mg/mL	---	[29]
B. subtilis KIBGE HAS	56	7.5	50°C			K_m =2.68 mg/mL	ND	[10]
B. halodurans MS-2-5 (α-amilasa E)	58	10.5-11	60-65°C	5.0-11.0	55°C	---	Soluble	[30]
B. caldolyticus	70	5.5	70°C	---	70°C	---	---	[31]
Bacillus sp. BBM1	77.6	5.0-7.0	60°C	---	60°C	---	Soluble	[11]
B. licheniformis MTCC 1483	---	8.0	40°C	8.0	40°C	---	Soluble	[18]
Bacillus sp BKL20	---	7.0	60-70°C	---	---	---	ND	[8]
Bacillus sp. UEB-S	130	5.0	70°C	5-10 (24 h)	30-50°C	---	raw	[24]
B. subtilis AX20	149	6.0	60°C	---	---	K_m = 3.3 mg/mL	Soluble	[33]

Abbreviations: ND: Not Determinate, MW: Molecular Weight.

Hydrolysis and degradation of raw starch via AmyJ33

To examine the hydrolysis of raw starch using AmyJ33, the enzyme was stored with raw starch for 180 min (Figure 4A). At the start of the reaction, 0-40 min (0.0125 g/L min) the production of reducing sugars was lower than in the period between 40-100 min (0.025 g/L min), where the production of reducing sugars was increased by twice the amount. Subsequently, productivity decreased and when reaching 140 min the production of reducing sugars had no changes. During the process, AmyJ33 hydrolyzed 25% of raw starch, hydrolysis percentage was similar to the amylose percentage therefore, AmyJ33 could hydrolyze part of amylose polymer.

The enzymatic reaction was monitored through TLC (Figure 4B). At the early stage of hydrolysis (0-80 min of reaction), glucose (DP1) and maltose (DP2) were released in low concentration, however at 80 min, the concentration of DP1 and DP2 increased and traces of maltopentose (DP5) appeared. This correlates with the increase in the production of reducing sugars. Nevertheless, DP1, DP2 and DP5 disappeared at 180 min. These results are similar to those reported for the α-amylase from *B. subtilis* AX20 and *Bacillus* sp UEB-S, where glucose and maltose were the end products of starch hydrolysis [23,24]. However, other amylases from *Bacillus* sp. 18, *Bacillus* sp. BL3, *Bacillus* sp. 12B produced mainly glucose, maltotriose (DP3) and maltopentose (DP5) [25]. The saccharifying enzyme from *B. subtilis* generates largely glucose and maltose from starch while the liquefying amylase from *B. amyloliquefaciens* yields predominantly maltosaccharides [4,26], based on this, it is possible to classify AmyJ33 as an endoamylase saccharifying enzyme.

At the same time, the pattern of hydrolysis of the raw starch was also examined for SEM at 0, 80 and 160 min (Figure 5B-5D). The raw starch granules only showed degradation on their surface, and it did not show any degradation pattern of pin-holes

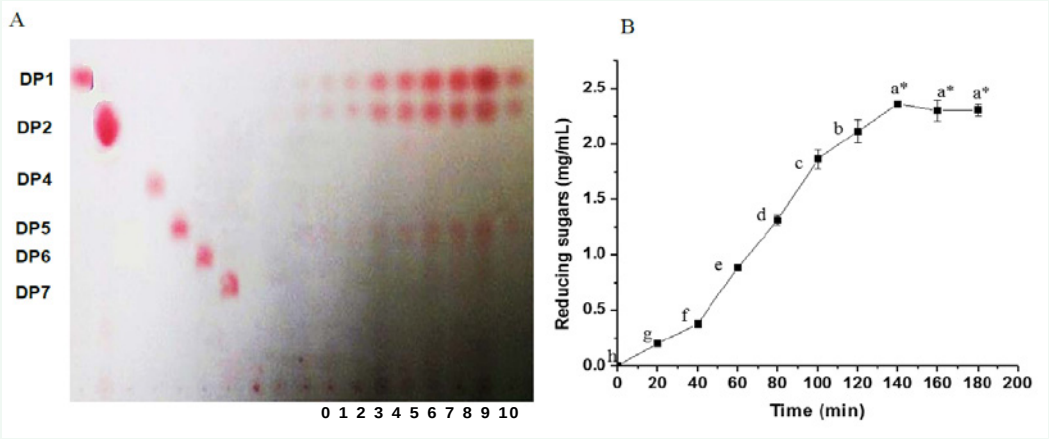

Figure 4 Hydrolysis of raw starch by AmyJ33.
A. Production of reducing sugars by AmyJ33 on raw starch.
B. Thin-layer chromatography (TLC) of hydrolysis products of AmyJ33 on raw starch (10 g/L).
System: Butanol /ethanol /water (30:50:20 V/V/).
Standards: DP1, DP2, DP4, DP5, DP6 and DP7, line 0: raw starch (negative control), lanes 1-10: hydrolysis products between 0-180 min

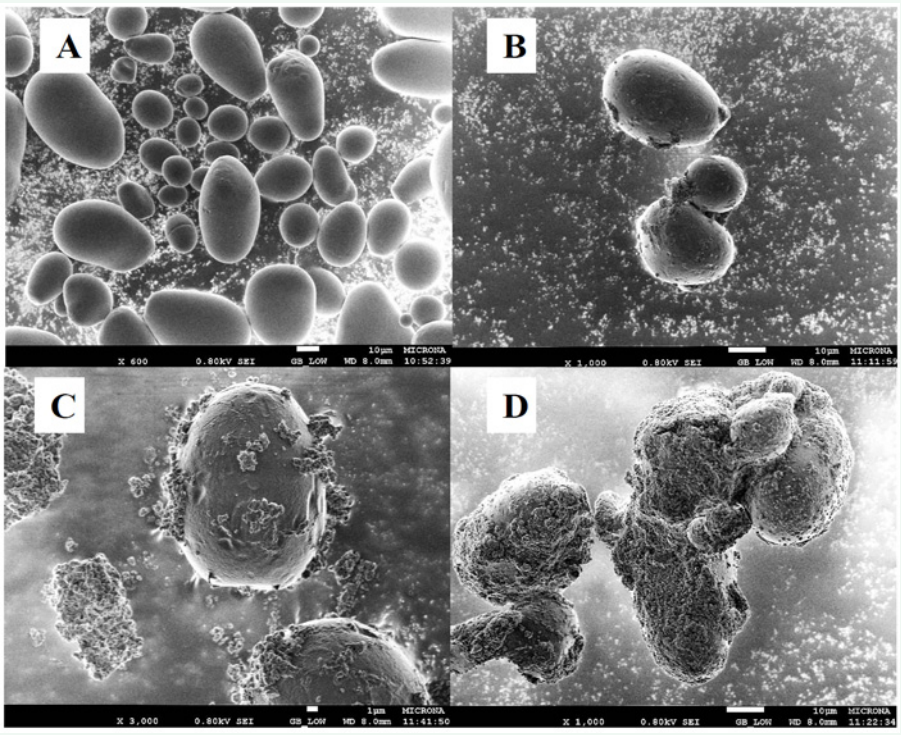

Figure 5 Scanning electron microscopy of potato starch untreated (A) and treated with AmyJ33 during 0 (B), 80 (C) and (D) 180 min.

or sponge like. This indicates that AmyJ33 hydrolyzes raw starch of potato by exo-corrosion. Potato starch is the most resistant starch to hydrolysis, which is only eroded when α-amylases at low concentrations are used [20,27], as here (0.15 U/ml). Other starches, such as, cassava, maize, potato sweet are more easily hydrolyzed when α-amylases are used.

The hydrolysis by exo-corrosion exhibited by α-amylases on potato starch granules has been incipient [4,20,27], in contrast, AmyJ33 at 160 min has eroded all the surface of the granule starch (Figure 5D). This behavior could be taken advantage to produce ethanol or syrups from raw starch, avoiding the energy costs by starch gelatinization.

These findings would indicate why AmyJ33 has a high Km value in potato starch which could be due to the difficult process of potato starch hydrolysis. Could be interesting analyze hydrolysis behavior of starch of other sources through AmyJ33, such as: wheat, banana, maize.

CONCLUSION

Amylases are the most important enzymes in the industry. AmyJ33 is able to hydrolyze 25% of raw starch of potato in 180 min via exo-corrosion mechanism on reaction conditions: pH 5, 45°C. AmyJ33 was stable at pH 5 for 24 h at 4°C, the half-life presented at 40°C was 16 h, at 50°C was 2.35 h and 60°C was 0.25 h, K_m and V_{max} values on raw starch were: 10.6 mg/mL and 41.0 U/mg respectively, it produces mainly glucose, maltose and maltopentose traces, for this reason it is a saccharifying enzyme and should be used on the bakery, ethanol production and syrup industries. In this moment, we are doing experiments to analyze the AmyJ33 behavior in soluble starch and the degree of hydrolysis on raw and soluble starch grains also bettering the reaction conditions to increase the percentage of hydrolysis of raw starch.

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