Research Article

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Streptococcus infantarius 25124 isolated from pozol produces a high molecular weight amylopullulanase, a key enzyme for niche colonization

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Abstract: Pozol is a beverage made with maize dough that is prepared after boiling the kernels in limewater, causing a decrease in soluble sugars, with starch being the main fermentable carbohydrate in the dough. Previously, *Streptococcus infantarius* ssp. *infantarius* 25124 (Sii-25124) was identified as the most amylolytic bacteria isolated in this product. Analysis of Sii-25124 amylolytic enzymes revealed two amylases, a cytoplasmic α -amylase of 55.7 kDa and an extracellular amylopullulanase of 246.3 kDa, with two catalytic domains, one typical of an α -amylase and another typical of a pullulanase/glycogen debranching enzyme. Characterization of the joint activity of both enzymes using Sii-25124 cell lysate supernatant demonstrated stability between 30 °C and 45°C, and pH stability in a range between 6.8 and 8.0. The joint activity of Sii-25124 amylases showed a fast production of reducing sugars when starch was used as the substrate. In contrast, reducing sugar production from amylopectin was lower, but it steadily increased throughout the reaction time. The amylopullulanase produced by Sii-25124 hydrolyzes the starch in the dough to produce low molecular weight oligosaccharides, which may be transported into Sii-25124 cells, so that intracellular α -amylase hydrolyzes them to

mono- and disaccharides. Amylopullulanase production by Sii-25124 could be an example of a specialized enzyme that successfully dominates starchy food fermentation.

Keywords: nixtamalization; amylolytic lactic acid bacteria; starch; fermentation.

Abbreviations

ALAB, amylolytic lactic acid bacteria; CBM, carbohydrate-binding module; GH, glycoside hydrolase; IRCM, Montreal Clinical Research Institute; MS, mass spectroscopy; MRS, De Man Rogosa and Sharpe culture medium; Sii-25124, *Streptococcus infantarius* spp. *infantarius* 25124.

1 Introduction

Pozol is an acidic, non-alcoholic fermented maize beverage that has been highly consumed in south-eastern Mexico since pre-Hispanic times. To prepare this drink, maize kernels are nixtamalized, where corn is cooked with 1-3% dry maize weight lime [commercial $\text{Ca}(\text{OH})_2$] for 50-90 min and then soaked for 14-18 h in its own cooking solution, which is then removed. Maize kernels are washed thoroughly to obtain the nixtamal and then are coarsely ground into a dough, which is shaped into balls, wrapped in banana leaves and allowed to ferment spontaneously from a few hours up to 1 month or longer. Then, the fermented dough is suspended in water and consumed as a traditional refreshing beverage, sometimes it is flavored with cocoa, horchata or chili [1].

The process of nixtamalization has several effects on maize kernels, with the decrease in the concentration of

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soluble sugars being one of the most important since they are reduced from 2% to 0.1% on a dry weight basis after cooking, soaking and washing processes, leaving starch as the main component of the nixtamalized dough [2].

Amylolytic lactic acid bacteria (ALAB) occur in different amylaceous fermented foods, in which species from *Lactobacillus* are the most frequently reported [3-5], followed by *Lactococcus* and *Streptococcus* species [6]. In previous studies, pozol has been reported as a diverse and dynamic source of ALAB and non-ALAB, with *Streptococcus* being the genus that prevails during the whole fermentation process and *S. infantarius* ssp. *infantarius* 25124 (*Sii*-25124), the most amylolytic bacteria isolated from this product. In this bacterium, low amylolytic activity is observed although it presents a high specific growth rate when grown on starch, suggesting that the observed activity, although low, is enough for *Sii*-25124 to be dominant in pozol fermentation [7].

In the primary structure classification of glycoside hydrolases (GH, <http://www.cazy.org/Glycoside-Hydrolases.html>; [8]), the enzymes involved in amylolysis are part of the GH families GH13, GH14, GH15, GH31, GH57, GH65, GH70, GH77, GH97, GH119, GH133, which include several specificities, such as: α -amylases (EC 3.2.1.1), glucan 1,4- α -maltohydrolase (EC 3.2.1.133), glucan 1,4- α -maltotetraohydrolase (EC 3.2.1.60), glucan 1,4- α -maltohexaosidase (EC 3.2.1.98), glucan 1,4- α -maltotriohydrolase (EC 3.2.1.116); pullulanases (EC 3.2.1.41); cyclomaltodextrin glucanotransferases (EC 2.4.1.19); cyclomaltodextrinases (EC 3.2.1.54); oligo- α -glucosidases (EC 3.2.1.10); isoamylases (EC 3.2.1.68); glucodextranase (EC 3.2.1.70); α -glucosidase (3.2.1.20); glucoamylases (EC 3.2.1.3), β -amylases (EC 3.2.1.2), amylo-1,6-glucosidases (EC 3.2.1.33); 4- α -D-glucan trehalose trehalohydrolases (EC 3.2.1.141); limit dextrinase (3.2.1.142); limit dextrin α -1,6-maltotetraose-hydrolase (3.2.1.196), 4- α -glucan transferases (EC 2.4.1.25) and 4- α -glucan 1- α -D-glucosylmutases (EC 5.4.99.15) [9]. However, in lactic acid bacteria, although the use of starch as a carbon source has been well documented, very few enzymes have been characterized [10-20]. To understand how *Sii*-25124 predominates in pozol fermentation, the amylolytic system of the bacteria was biochemically studied and characterized.

2 Materials and methods

2.1 Microorganisms and culture conditions

S. infantarius 25124 (*Sii*-25124) isolated from pozol [7] was inoculated in MRS (De Man Rogosa and Sharpe) broth [21]

with 2% soluble starch (J.T. Baker) instead of glucose at a proportion of 1:100 (*Sii*-25124 inoculum: MRS-starch broth) for 24 h at 30 °C, without shaking. This culture was used to inoculate 250 mL of fresh broth (1:50) (*Sii*-25124 culture: MRS-starch broth) that was incubated for 18 h at 30 °C and finally to reinoculate in 250 mL of fresh broth (1:1) that was incubated for 6 h at 30 °C. This final culture was used in all trials.

2.2 Amylase extraction

Sii-25124-6 h cultures were harvested at 10,000 rpm for 15 min at 4 °C. The pellet was resuspended in 25 mL of 20 mM Tris-HCl, 5 mM EDTA, and 25% (w/v) sucrose at pH 8.0 with 2 mg/mL lysozyme (Sigma) and incubated for 1 h at 37 °C. This suspension was centrifuged to recover *Sii*-25124 protoplasts, which were then resuspended in lysis buffer (0.1 M phosphate buffer, 50 mM NaCl, and 5 mM EDTA, pH 6.8) with complete EDTA-free protease inhibitor cocktail (Roche). Cells were disrupted by sonication for 5 cycles of 20 s pulsations/20 s cooling at 60 Hz (Vibra-Cell; Sonics & Materials, Inc.). The obtained proteins were purified from the clarified extract by starch binding [22].

2.3 Purification of *Sii*-25124 amylases by starch adsorption

Three liters of *Sii*-25124-6 h cultures were harvested and sonicated to obtain 30 mL of cell lysate supernatant. Two milliliters of this supernatant were used to resuspend 500 mg of previously washed raw corn starch (Sigma-Aldrich) in 0.1 M phosphate buffer (pH 6.8). The starch/lysate suspension was incubated for 4 h at 4 °C with gentle shaking. The starch-lysate suspension was centrifuged at 12,000 rpm for 20 min and 4 °C to recover both soluble and non-soluble fractions. The latter was washed three times with 10 mL of 0.1 M phosphate buffer (pH 6.8) for 10 min at room temperature and gentle shaking to recover the pellet by centrifugation at 12,000 rpm for 8 min every time. Protein from the pellet was then eluted with three washes of 1 mL of 8 mM β -cyclodextrin solution in 0.1 M phosphate buffer (pH 6.8) and stirring at room temperature for 10 min. The soluble fraction was recovered by centrifugation at 12,000 rpm for 5 min and 4 °C. Eluates were dialyzed extensively against 0.1 M phosphate buffer (pH 6.8) and 10 mM MgCl₂ using a Spectra/Por dialysis membrane of 50 kDa MWCO (Spectrum Laboratories).

2.4 Electrophoresis analyses

SDS-PAGE (resolving gel with 8% acrylamide/N,N'-methylbisacrylamide) was performed according to the method of Laemmli [23]. Proteins were visualized by Coomassie blue staining as described by Blakesley and Boezi [24]. Activity staining was performed in the gel after renaturation of the enzymes using a modification of the method described by Lacks and Springhorn [25]. Briefly, acrylamide gels were washed three times with 0.1 M phosphate buffer (pH 6.8) for 10 min with constant shaking. Then, the gels were incubated overnight with a 1.0% soluble starch solution in 0.1 M phosphate buffer (pH 6.8) at 37 °C. Finally, the gels were stained with a 4% iodine solution until the appearance of clear zones under a dark background.

2.5 Sequencing and identification of *Sii-25124* amylases

Candidate electrophoretic protein bands were excised from acrylamide gels and sent to the Montreal Clinical Research Institute (IRCM) for sequencing. At IRCM proteins were hydrolyzed using trypsin; resulting peptides were analyzed with a nanoLC-2D system (Eksigent) and coupled to the LTQ Orbitrap Velos (ThermoFisher Scientific).

The sequences of the obtained peptides were compared with the NCBI bacterial protein database using the BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>; [26]). Comparisons between other bacterial amylases were made using BioEdit Sequence Alignment Editor software [27]. For prediction of protein localization and motif identification, PSORTb v.3.0 (<http://psort.org/>; [28]), the Conserved Domain database (<https://www.ncbi.nlm.nih.gov/cdd/>; [29]), and the Pfam search engine version cdd.v.3.18 sequence database (<http://pfam.xfam.org/>; [30]) with the preset options were used.

2.6 Amylolytic activity assay

Amylase activity was determined toward soluble potato starch by measuring the residual substrate after the enzymatic reaction. Starch is complexed with iodine using the method described by Giraud *et al.* [15]: 0.1 mL of cell lysate supernatant with 0.8 mL of a solution containing 1.2% of Panreac soluble starch in 0.1 M phosphate buffer (pH 6.8) and 10 mM MgCl₂ was incubated at 37 °C for 10 min. One enzyme unit was defined as the amount of enzyme capable of hydrolyzing

10 mg of starch in 30 min under the conditions described in the following equation:

$$\text{Amylolytic Activity} \left(\frac{U}{mL} \right) = \frac{(Abs_0 - Abs_{t10})}{m} \times 3$$

where Abs_0 and Abs_{t10} are the absorbances of the sample at zero reaction time and after 10 min of reaction, respectively, and m is the slope of the starch concentration calibration curve.

Protein concentration was assessed using a Bio-Rad Protein Assay kit using bovine serum albumin as a standard.

Initial hydrolysis rates were studied on soluble starch and amylopectin (Sigma) by the release of reducing sugars [31]. To this end, previously dialyzed *Sii-25124* cell lysate supernatant was incubated in 0.1 M phosphate buffer (pH 6.8) and 10 mM MgCl₂ at 37 °C for 0-90 min. Reducing sugar production was determined colorimetrically by absorbance at 540 nm.

2.7 Effect of temperature on *Sii-25124* amylases stability

Sii-25124 cell lysate supernatant was subjected to 8 different temperatures (15, 25, 30, 37, 45, 50, 55 and 60 °C) for 10 min and 1 h, respectively. After the incubation time, the residual amylase activity of the samples was determined at 37 °C, pH 6.8 and 10 mM MgCl₂. Triplicate samples from three independent experiments were assayed. The control assay consisted of the amylolytic activity assay at 37 °C, pH 6.8 and 10 mM MgCl₂ (standard conditions), regarded as 100%.

2.8 Effect of pH on *Sii-25124* amylases stability

Sii-25124 cell lysate supernatant was subjected to 10 different pH values (3.0, 4.0, 5.0, 5.5, 6.0, 6.8, 7.0, 7.5, 8.0 and 9.0) using 0.1 M phosphate-citrate buffer (dibasic sodium phosphate and monohydrated citric acid from J.T. Baker) for pH 3.0-5.5; 0.1 M phosphate buffer for pH 6.0-8.0 and 0.1 M Tris-HCl buffer for pH 9.0. Each lysate (at each pH value) was incubated for 24 h at 37 °C and subjected to the amylolytic activity assay under standard conditions. Assays were performed three times from three independent experiments. The control was the amylolytic activity assay of *Sii-25124* cell lysate supernatant at a pH value of 6.8, and it was regarded as 100%.

Table 1: Summary of starch binding assay purification steps of *Sii*-25124 amylases.

Purification step	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg of protein)	Purification fold	Yield (%)
Initial cell culture	25	342	1520	4.4	1	100
Cell lysate supernatant	30	166	2018	12.1	2.7	132.7
Cell debris	15	73	296	4	0.9	19.5
Affinity chromatography elution	3	0.27	27	99.6	22.6	1.8
Unbound protein	30	157	1866	11.9	2.7	122.7

2.9 Effect of metal ions on *Sii*-25124 amylases activity

Sii-25124 cell lysate supernatant was subjected to the presence of 10 different metal cations (CaCl_2 , BaCl_2 , CuCl_2 , FeCl_3 , HgCl_2 , KCl , MgCl_2 , MnCl_2 , NaCl and ZnCl_2). Each metal cation solution was added to a final concentration of 10 mM and incubated at 37 °C for 30 min. Each sample was subjected to the amylolytic activity assay under standard conditions with no addition of MgCl_2 . The relative activity of the enzyme was reported as compared in 0.1 M phosphate buffer (pH 6.8) at 37 °C, which was considered 100%.

2.10 Statistical analysis

All tests were performed in triplicate, and data are expressed as the mean $\pm$ SD. Statistical significance was determined using Student's *t*-test. A difference at $P < 0.05$ was considered significant.

3 Results and discussion

3.1 Purification of *Sii*-25124 amylases by starch binding assay reveals the presence of two proteins with amylolytic activity

In the zymogram, amylolytic activity of approximately 250 kDa was observed in all purification steps, activity was recovered in both soluble and cell debris fractions, indicating that the protein likely has affinity for insoluble cell components or its possible aggregation. The enzyme was difficult to recover since only a small amount of

protein was bound to insoluble starch. As shown in Table 1, practically all the amylolytic activity remained in the supernatant, and very little activity was detected in the fractions. Amylases are enzymes with molecular weights of approximately 50 or 60 kDa [32], but unfrequently, the existence of high molecular weight amylases has been reported, such as those α -amylases extracted from *L. amylovorus* (105 kDa) [11], *L. plantarum* A6 (95 kDa) [10], *L. manihotivora* (98.4 kDa) [13], *L. plantarum* S21 (95.3 kDa) [33], and *Eubacterium rectale* (150 kDa) [34]. In all the aforementioned cases, the presence of more than one carbohydrate-binding module (CBM) that boosts the binding of amylase to starch was demonstrated, which is particularly important for the hydrolysis of raw starch [22].

On the other hand, SDS-PAGE showed a second protein with amylolytic activity but low molecular weight (approximately 50 kDa) and was only present on cells, protoplasts and cell lysate supernatant fractions, indicating the possible intracellular localization of this second amylase (Fig. 1). Likewise, when compared with the initial cell culture fraction, the inner localization of this amylase may be the reason for the increased activity found in the cell lysate supernatant (Table 1). Despite the fact that intracellular and extracellular amylases of *Streptococcus* strains have been reported [17, 35-38], to our knowledge, this study is the first to report the production of about 250 kDa amylase by an ALAB strain, which compared to the amylases produced by its most closely related enzymes, is likely to be a different carbohydrate-active enzyme.

Figure 1: Analysis of purified amyolytic enzymes by SDS-PAGE (8% acrylamide/N,N'-methylbisacrylamide) stained with 4% iodine solution for zymography. M: molecular weight marker; lane 1: *Sii*-25124 initial cell culture; lane 2: protoplasts; lane 3: *Sii*-25124 initial cell lysate supernatant; lane 4: residual *Sii*-25124 cell lysate supernatant; lane 5: 1st eluate; lane 6: 2nd eluate; lane 7: 3rd eluate; lane 8: *Sii*-251245 cell debris. Arrows indicate the excised bands used for MS peptide mapping.

3.2 Identification of a surface-attached amylopullulanase and a cytosolic α -amylase

To determine what kind of amylases were produced by *Sii*-25124, amyolytic activity bands generated through SDS-PAGE and revealed by zymography (Fig. 1) were excised from the gel and sequenced in IRCM. Protein bands were named arbitrary as Amy1 (low molecular weight protein band) and Amy2 (high molecular weight protein band). The amino acid sequences of the peptides found for each of the analyzed proteins are shown in Figure 2.

BLAST comparison of the eight peptides found for Amy2 against the non-redundant protein sequence database in NCBI exhibited 100% identity with peptides from an extracellular pullulanase from *S. infantarius* subsp. *infantarius* ATCC BAA-102 (Accession No.: EDT46783.1) with a theoretical molecular weight of 246.3 kDa (ProtParam: <https://web.expasy.org/protparam/>; [39]). Referring to Amy1 BLAST analysis, all five peptides identified (Fig. 2) showed 100% identity with a cytosolic α -amylase of the same bacterium ATCC BAA-102 (Accession No.: EDT47408.1), with a theoretical molecular weight of 55.7 kDa (according to ProtParam tool), a similar molecular weight to that observed on the zymogram (Fig. 1).

Analysis of the conserved domains of the protein recognized as Amy2 identified two catalytic domains, one

Figure 2: Sequence of analyzed amyolytic protein bands of *Sii*-25124. Peptides found by LC-MS/MS are highlighted and most accurate identification is annotated. (A) Band: Amy2; % coverage: 6%; 100% identity with *Streptococcus infantarius* subsp. *infantarius* ATCC BAA-102 amylopullulanase (according to UniProt Bacteria Database; BLASTp version 2.2.31+ was used); calculated MW: 246,320 Da (according to ProtParam tool provided by the ExPASy Bioninformatics Resource Portal). (B) Band: Amy1; % coverage: 26.5%; 100% identity with *Streptococcus infantarius* subsp. *infantarius* ATCC BAA-102 α -amylase (according to UniProt Bacteria Database; BLASTp version 2.2.31+ was used); calculated MW: 55,763.4 Da (according to ProtParam tool provided by the ExPASy Bioninformatics Resource Portal).

typical of an α -amylase and a second one of a pullulanase/glycogen debranching enzyme (PulA) (Fig. 3A). This finding clearly indicates that this enzyme is an amylopullulanase, and more strictly an α -amylase-pullulanase [40] that does not belong to the extracellular pullulanases previously described in the genus *Streptococcus* [41-44]. Other conserved domains in *Sii*-25124 amylopullulanase were identified: four CBM41s, a CBM family with α -glucan binding function (e.g. amylose, amylopectin and pullulan) typically found in pullulanases, as well as in glycogen degrading enzymes, amylopullulanases and α -amylase-pullulanases [45,46]; and one CBM48 with glycogen binding function in glycogen-kinases [47,48]. The presence of all these domains indicates that the enzyme must be able to accommodate α -glucans containing glucose residues linked by α -1,6-linkages and to hydrolyze them, as well as the α -1,4-glycosidic linkages.

Figure 3: (A) Conserved domains of *Sii*-25124 amylopullulanase identified using Conserved Domain Database (NCBI). From left to right – YSIRK-type signal peptide; two tandem family CBM41s; α -amylase catalytic domain; two tandem family CBM41s; family CBM48; α -amylase catalytic domain; C-terminal β -sandwich domain found in *Streptococcus pneumoniae* Spu4 multimodular cell wall-attached enzyme (SpuA_C); LPXTG cell wall anchor motif. AmyA, family of glycosidases related to carbohydrate metabolism and transport; AmyAc, α -amylase catalytic domain found in pullulanase family; PuA, Pullulanase/glycogen debranching enzyme family. Conserved catalytic residues and cation binding residues are indicated by black and dotted lines, respectively. (B) Conserved domains of *Sii*-25124 α -amylase identified using the Conserved Domain Database (NCBI). From left to right – α -amylase catalytic domain; domain of unknown function found in some amylases (DUF1939). AmyA, family of glycosidases related to carbohydrate metabolism and transport; PRK09941 cytoplasmic α -amylase family. Conserved catalytic residues and cation binding residues are indicated by black and dotted lines, respectively.

However, very weak binding of Amy2 to starch granules was observed even though key binding platform residues are present (data not shown), consequently we cannot explain this phenomenon; nevertheless, this kind of weak binding has been previously reported in a *Lactobacillus acidophilus* cell-attached pullulanase that contains two CBMs, one CBM41 and one CBM48 [49]. Further, CBM41 from *Thermotoga maritima* pullulanase was found to be able to bind preferentially to substrates with α -1,4-glycosidic linkages over those with both α -1,4- and α -1,6-linkages [50].

Other important regions that indicate the location of the enzyme are the YSIRK-type signal peptide and the LPXTG cell wall anchor motif at the N- and C-terminal end of the protein, respectively. The prediction of the location of the enzyme by PSORTb [28] clearly marks it as cell wall-anchored (Fig. 3A). Both motifs have been found as part of surface proteins from Gram positive bacteria, such as *Streptococcus*, *Enterococcus* and *Staphylococcus* [51].

Amylopullulanases are classified as part of families GH13 or GH57, included in the sequence-based classification of Carbohydrate Active enZymes, the CAZy database (<http://www.cazy.org/>; [8]). Regarding *Sii*-25124 α -amylase-pullulanase, CAZy database

classifies *S. infantarius* amylopullulanase as a member of subfamilies GH13_12 and GH13_41 [8,52]. Their presence is common in aerobic and anaerobic bacteria, such as *Bacillus*, *Geobacillus*, *Thermoanaerobium*, *Thermoanaerobacterium*, *Thermoanaerobacter*, *Thermobacteroides* and *Thermotoga*; as well as in aerobic and anaerobic archaea like *Thermococcus* and *Pyrococcus* [40,53]. However, it has been reported the presence of putative genes encoding pullulanases in the genome sequence of several strains of *Bifidobacterium* [54], and the production of an extracellular amylopullulanase of 174.98 kDa by *B. breve* UCC2003 has been demonstrated [55]. Likewise, there are few reports of pullulanase-producing ALABs, such as amylopullulanases from *L. amylophilus* GV6 [56], *L. paracasei* B41 [57] and *L. plantarum* L137 [19], the latter having a molecular weight of 216 kDa. In addition, several non-lactic acid species from *Streptococcus*, such as *S. pneumoniae* (150 kDa) [40] and *S. suis* JS14 (230 kDa) [42], are well-known to produce pullulanases. Furthermore, surface-attached amylopullulanases are rare in nature because of the advantage of secreting enzymes into the extracellular space to hydrolyze polysaccharides and to internalize small oligosaccharides by sugar transporters. However,

nixtamalized dough can be a different environment, a solid fermentation in which the water content (40-60%) [58] is so low that the enzymes cannot diffuse. The presence of the amylopullulanase *Sii-25124* anchored to the cell surface could explain the high amyolytic activity found in the cellular debris fraction during the purification assay (Table 1 and Fig. 1). The expression of amylopullulanase by *Sii-25124* could be an example of specific adaptation to nixtamalized dough, given the requirement of enzymes as well as microorganisms, to successfully bind and to hydrolyze starch.

In the case of intracellular Amy1, CAZy database classifies α -amylases as part of clan GH-H (GH13, GH70 and GH77), as well as part of GH57 and GH119 families [8]. *S. infantarius* intracellular α -amylase is classified as part of GH13_5 subfamily, according to CAZy database [8,52]. Analysis of its conserved domains showed the presence of an α -amylase catalytic domain and a domain of unknown function (DUF1939), which was identified in the C-terminal portion (Fig. 3B). This unknown domain has a secondary structure consisting of an eight-stranded antiparallel β -sheet having a Greek key motif, and although the function has not been elucidated, in conjunction with the catalytic domain, it has been identified as a PRK09441 member, which has been previously characterized as part of many cytosolic α -amylases from bacterial species, such as *Streptococcus*, *Bacillus* and *Thermococcus* [59]. Therefore, it is likely that the low molecular weight amylase produced by *Sii-25124* is an intracellular α -amylase, which probably hydrolyze oligosaccharides to produce mono- and disaccharides, as starch cannot pass through the cell membrane; therefore, Amy1 was only found in cell and cell lysate supernatant fractions (Fig. 1).

The presence of a cytosolic amylase could be relevant to improving *Sii-25124* carbohydrate consumption and growth rate, as seen in *S. bovis* WI-1, where inactivation of this gene resulted in a decrease in growth rate, indicating that the intracellular enzyme plays an important role in rapid cell growth [60].

3.3 Characterization of *Sii-25124* amyolytic cell lysate supernatant revealed neutral pH stability and apparently no activation by Ca^{2+}

As both *Sii-25124* amylases likely work together in their natural environment, the synergistic activity of both enzymes using *Sii-25124* cell lysate supernatant was analyzed. A previous work [7] reported the optimal temperature and pH of *Sii-25124* amyolytic activity as 37 °C and 6.8, respectively. The joint activity of the amylases

Figure 4: Temperature and pH stability of joint activity of *Sii-25124* amylases in cell lysate supernatant. (A) Thermostability for 10 min (■) and 60 min (◆) of incubation at pH 6.8 and 10 mM Mg^{2+} . (B) pH stability at 37 °C and 10 mM Mg^{2+} . Values represent the average $\pm$ standard deviation of three independent experimental replicates.

had more stability at temperatures from 30 °C to 45 °C as well as a higher amyolytic activity when incubated for short periods (10 min), rather than long periods (60 min). At 60 min of incubation, stability dropped from 100% at 30 °C to 36% at 45 °C (Fig. 4A). The combined *Sii-25124* amylases had a thermostability similar to that of *S. bovis* JB1 and *L. amylophilus* extracellular amylases, both with thermostabilities from 30 °C to 40 °C [36, 61]. ALAB are found in amylaceous raw materials that undergo spontaneous fermentation at room temperature in the tropics. Therefore, their amylases usually show stability at temperatures between 30-55 °C [6].

On the other hand, compared to the control, pH stability was found at 6.8-8.0, with a relative amyolytic activity of almost 100% (Fig. 4B). Moreover, pH stability is an attractive feature of ALAB amylases, since ALAB have developed tolerance mechanisms to withstand the acidic environment produced by themselves to colonize and to dominate their niche [62,63]. Most ALAB amylases, such as *S. bovis* JB1, *L. plantarum* A6 and *L. amylovorus* ATCC 33620 extracellular α -amylases, show pH stability between 4.5-6.0 [15,36,61]. Furthermore, the *Sii-25124* cell lysate supernatant displayed high amyolytic activity (58%) under alkaline pH conditions (9.0) (Fig. 4B), an unusual characteristic among ALAB amylases, since

Figure 5: Effect of the presence of metal ions on the amyolytic activity of the joint activity of *Sii-25124* amylases in cell lysate supernatant at 37 °C and pH 6.8. Values represent the average $\pm$ standard deviation of three independent experimental replicates. Different letters indicate significant differences between treatments (*t*-test, $p < 0.05$).

Figure 6: Effect of the joint activity of *Sii-25124* amylases in cell lysate supernatant at pH 6.8, 10 mM Mg²⁺ and 37 °C on the release of reducing sugars from 8 mg of starch and 8 mg of amylopectin. Values represent the average $\pm$ standard deviation of three independent experimental replicates.

they are a group of organic acid-tolerant and organic acid-producing bacteria [6,64]. Additionally, there are no reports about other ALAB amylases showing tolerance to alkaline pH; for instance, extracellular α -amylase from *S. bovis* JB1 inactivates at pH 8.5 [36]. It is likely that neutral pH conditions (7.0-7.8) of nixtamalized dough [1] favor *Sii-25124* amylases and promote hydrolysis of starch at the initial stages of pozol fermentation.

Compared with the control, the effect of divalent ions on amyolytic activity showed that the presence of Mg²⁺ increased amyolytic activity by 146%, while Mn²⁺, Fe³⁺, Ca²⁺ and Na⁺ increased amyolytic activity to 141%,

116%, 112% and 111%, respectively; though none of these increases were significant (Fig. 5). However, given the presence of both amylases in the cell lysate supernatant, it is not possible to know the contribution of each amylase to the activity found, although it is clear that together, *Sii-25124* amylases have good amyolytic performance alone or in the presence of Ca²⁺, Mg²⁺, Mn²⁺, Fe³⁺ and Na⁺. Regarding the results from this study, it is worth considering that nixtamalized maize dough contains an important amount of Ca²⁺ (138 mg per 100 g of nixtamalized dough) [65]; consequently, it is possible that *Sii-25124*, as well as other amyolytic strains from nixtamalized dough,

may be found on a suitable substrate for their amylolytic enzymes to function. It is known that most amylases are considered metalloenzymes, which are enzymes that require metal ions (usually Ca^{2+}) to maintain their stable native state; however, there are metal-activated amylases that require Ca^{2+} only during catalytic activity as well as Ca^{2+} -independent amylases [66]. Regarding ALAB amylases, it has been reported that *L. plantarum* A6 and *S. bovis* JB1 extracellular amylases are not activated by Ca^{2+} [15,36], while others are activated and stabilized by other divalent metal ions, such as *L. amylophilus* NRRL B-4437 and *B. licheniformis* 2618 amylases, which require Ba^{2+} and Mg^{2+} , respectively [61,67]. It would be worth determining whether either one or both *Sii*-25124 amylases contains structural Ca^{2+} or Mg^{2+} as well as other metal ions needed to activate and to stabilize each of them.

3.4 *Sii*-25124 amylases on cell lysate supernatant hydrolyze starch and amylopectin substrates to different extents

The hydrolytic activities of soluble starch and amylopectin were evaluated to determine the catalytic activity of *Sii*-25124 amylases on two different substrates. Reducing sugar production from amylopectin, a substrate branched at every 20-30 residues, was lower than reducing sugar production from soluble starch (Fig. 6). While *Sii*-25124 cell lysate extract hydrolyzed starch generating 7.74 μmol of glucose at 25 min, the same amount of enzyme hydrolyzed amylopectin producing 2.48 μmol of glucose at the same time of reaction. Total production at 90 min of reaction was 13.3 μmol of glucose from starch and 7.5 μmol of glucose from amylopectin (Fig. 6). Interestingly, reducing sugar production seemed to go on beyond 90 min, which was probably due to the presence of both amylases in the *Sii*-25124 cell lysate supernatant. However, based on our results it is not possible to decipher the contribution of each amylase to the hydrolytic pattern observed. It is likely that fast hydrolysis of starch was largely because of both enzymes, while slow hydrolysis of amylopectin was probably due mainly to amylopullulanase. This low hydrolysis capacity was reported for extracellular cell-attached pullulanase from *Lactobacillus acidophilus*, whose catalytic efficiency was observed to be 14-times lower for amylopectin than for β -limit dextrin and 155-times lower than for pullulan, indicating a preference for short α -1,6-glucosidic branches [49]. These results demonstrate the ability of *Sii*-25124 amylases to utilize starch and amylopectin, the main components of nixtamalized dough. However, it is necessary to corroborate the

functionality of *Sii*-25124 amylopullulanase on pullulan, amylose and insoluble starch and to continue studying and investigating the individual characteristics of each *Sii*-25124 amylase to better understand the dynamics and development of spontaneous fermentation of pozol and other native products.

4 Conclusions

A 246.3 kDa membrane-attached amylopullulanase and a 55.7 kDa cytoplasmic α -amylase are the two *Sii*-25124 pozol strain enzymes responsible for the starch hydrolysis of nixtamalized maize dough during the initial stages of pozol fermentation. At this stage, membrane-attached amylopullulanase is likely to hydrolyze α -(1-4) and α -(1-6) glycosidic linkages in starch, producing low molecular weight oligosaccharides, which could be easily transported into *Sii*-25124 cells, so that intracellular α -amylase hydrolyzes them to mono- and disaccharides; likewise, oligosaccharides produced by *Sii*-25124 amylopullulanase are likely to contribute to dough colonization by other lactic acid bacteria, which may allow fermentation to continue. The joint effect of these two enzymes could be the reason for the high specific growth rate of *Sii*-25124 previously reported.

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