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Characterization of α-Glucosidases from the Non-Conventional Yeast *Spathaspora passalidarum* NRRL Y-27907^T

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ABSTRACT

In the current study, the activity of α-glucosidases from the yeast *Spathaspora passalidarum* NRRL Y-27907^T was determined using permeabilized cells pre-grown on different carbon sources (maltose, sucrose, and α-methyl-glucoside). This yeast was capable of hydrolyzing substrates with α1→1, α1→2, α1→4, and α1→6 linkages, primarily maltose, maltotriose, and sucrose, indicating functional similarities with the α-glucosidases of *Saccharomyces cerevisiae*. Indeed, using various bioinformatics tools, two sequences designated as AG1 and AG2 were identified with 50% identity to maltases (Sc*MALx2*) and isomaltases (Sc*IMAx*). An analysis of the amino acid signature showed that position 216, crucial for substrate discrimination by the enzyme, is occupied by threonine, an amino acid found in maltases and maltase-isomaltases. Additionally, the phylogenetic analysis revealed a closer relationship with *MAL7*, *MAL8*, and *MAL9* from *Scheffersomyces stipitis*, characterized as maltase-isomaltases. Therefore, it is likely that both putative proteins exhibit mixed activity, with specificity for a broad spectrum of substrates.

Keywords: α-glucosidase activity. Maltase. Isomaltase.

1 INTRODUCTION

Yeasts are crucial in converting maltose and maltotriose, the main α-glycosides in brewing wort, into ethanol. To ferment effectively these sugars, this sugars are first transported into the cell and then hydrolyzed by maltases in the cytoplasm, releasing glucose molecules for ethanol production[1,2]. The fermentation of maltose by *S. cerevisiae* requires the presence of at least one of the five *MAL loci* found in the yeast genome. Each *MAL locus* comprises three genes: *MALx1*, encoding a transporter facilitating sugar uptake; *MALx2*, encoding maltase (α-glucosidase); and *MALx3*, which encodes a regulatory protein essential for the effective expression of the *MAL* genes. In *S. cerevisiae*, the α-glucosidases encompass maltases (*MALx2*), capable of breaking down carbohydrates with α1→2, α1→3, and α1→4 linkages, thus exhibiting specificity towards maltose, maltotriose, turanose, maltulose, and sucrose. Moreover, the multigene *IMAx* family encodes isomaltases that hydrolyze carbohydrates featuring α1→2 and α1→6 linkages, including palatinose, isomaltose, α-methyl-glucoside, and sucrose (a substrate with ambiguous specificity)[3,4].

Research on non-conventional yeasts like *Scheffersomyces stipitis*, *Metschnikowia grruessii* and *M. reukaufii* has been performed aiming to genetically and biochemically characterize their use of α-glucosides. These studies explore novel transporters and α-glucosidases, showing maltase-isomaltase activity[5,6,7]. *Sp. passalidarum* belongs to the CUG clade and, like other members of this group, has a unique ability to assimilate and ferment xylose. However, it has also been described as capable of fermenting other carbon sources such as glucose, maltose, α-methyl-glucoside (weakly), trehalose, cellobiose (weakly), and melezitose (slowly)[8].

Therefore, this study aims to explore the capacity of *Sp. passalidarum* to ferment various α-glucosides, evaluating the activity of α-glucosidases and identifying the presence of putative maltases and isomaltases genes in its genome.

2 MATERIAL & METHODS

To assess α-glycosidase activity, permeabilized cells were employed according to the method outlined by Stambuk[9]. In brief, cells were pre-cultured cultivated in rich YP medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone) with 20 g L^{-1} of respective carbon sources (maltose, sucrose, and α-methyl-glucoside) and subsequently permeabilized with toluene/ethanol/Triton X-100 as described by Stambuk[9].

The permeabilized cells were individually tested with final concentrations of 100 mM for each carbohydrate: maltose [glu(α1→4)glu], maltotriose [glu(α1→4)glu(α1→4)glu], melibiose [gal(α1→6)glu], melezitose [glu(α1→3)fru(β2→1)glu], sucrose [glu(α1→2)fru], raffinose [gal(α1→6)glu(α1→2)fru], α-methyl-glucoside [glu(α1→6)CH3], and trehalose [glu(α1→1)glu]). Alternatively, these cells were also tested with the synthetic substrate *p*-nitrophenyl-α-d-glucopyranoside (*p*NPαG) at a final concentration of 2 mM. After being incubated at 30°C for 30 minutes (α-glycoside) or 10 minutes (*p*NPαG), and then boiled at 100°C for 3 minutes, the supernatants separated by centrifugation (2600g for 5min) were used to determine the enzyme activity by measuring glucose released from sugar hydrolysis, measured using a commercial kit (glucose oxidase and peroxidase) (Bioclin, CasaLab, Belo Horizonte, Brazil), as well as by determining *p*-nitrophenol released from *p*NPαG hydrolysis (Abs 400nm). Determinations were performed in triplicate, and negative controls with previously boiled cells were performed in duplicate.

For *in silico* analyses, the BLASTp tool (https://www.ncbi.nlm.nih.gov/) was used to identify sequences with similarity to α-glycosidases of *S. cerevisiae* (MALx2p and IMAxp). The presence of a signal peptide was verified using SignalP 5.0 (http://www.servicesheathtech.dtu.dk/SignalP-5.0). Additionally, the ClustalOmega software (http://www.ebi.ac.uk/Tools/msa/clustalo/) was employed to perform multiple sequence alignment. Finally, the maximum parsimony phylogenetic tree was constructed using MEGA11 software (https://www.megasoftware.net/).

3 RESULTS & DISCUSSION

S. cerevisiae cells, when cultivated in glucose —the main carbon and energy source— cease to synthesize the permeases and α-glucosidases necessary for fermenting other carbon sources. This occurs due to glucose repression, which inhibits the transcriptional expression of genes[10]. Consequently, to determine the activity of α-glucosidases in permeabilized cells of the yeast *Sp. passalidarum* NRRL Y-27907^T, the α-glycosides maltose, sucrose, and α-methyl-glucoside (α-MG) were used in the pre-culture to induce the expression of both maltases and isomaltases. As illustrated in Figures 1A and 1B, the yeast was capable of hydrolyzing maltose > maltotriose > sucrose > α-MG > trehalose, as well as the synthetic substrate *p*-nitrophenyl-α-D-glucopyranoside (*p*NPαG). Conversely, low enzymatic activity was reported for the substrates melibiose, melezitose, and raffinose.

These results suggest the presence of enzymes primarily capable of cleaving α1→4 (maltose, maltotriose, and *p*NPαG), α1→2 (sucrose), and α1→6 (α-MG) linkages, like the maltases and isomaltases of *S. cerevisiae*. Despite the hydrolytic activity on different glycosidic bonds, a higher cleavage was observed in α1→4 linkages, evidenced by the higher activity on maltose and maltotriose substrates. Pre-cultivated cells in maltose, sucrose, and α-MG showed values of 252, 183, and 475 nmol of glucose released mg cell^{-1} min^{-1}, respectively. This finding is particularly interesting, as maltose and maltotriose are the main sugars present in wort, comprising 50-60% and 15-20% of fermentable sugars, respectively[11]. The ability to hydrolyze the synthetic substrate *p*NPαG (Figure 1B) further supports this preference, as it also contains an α1→4 linkage. Conversely, the substrate α-MG (with an α1→6 linkage), although hydrolyzed, showed values of 23, 17, and 45 nmol of glucose mg cell^{-1} min^{-1} when the cells were cultivated in maltose, sucrose, and α-MG, respectively. Is important to note that higher activities were obtained after growth in α-MG.

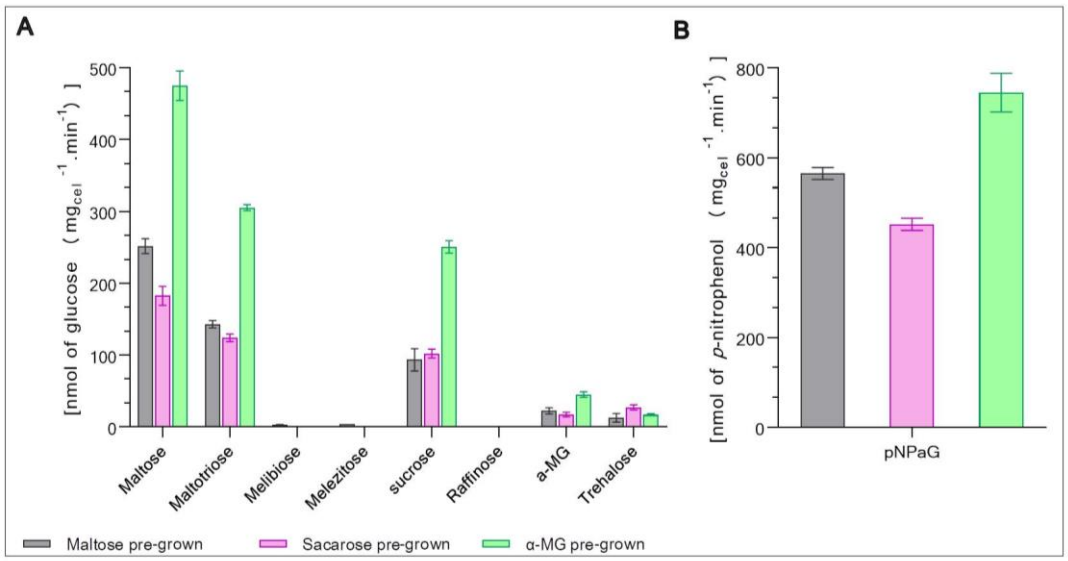

Figure 1: α-Glucosidase activity in permeabilized cells for **(A)** maltose, maltotriose, melibiose, melezitose, sucrose, raffinose, α-methyl-glucoside, and trehalose at a concentration of 100 mM, and **(B)** *p*-nitrophenyl-α-D-glucopyranoside at a concentration of 2 mM, by the yeast *Sp. passalidarum* NRRL Y-27907^T pre-cultivated in rich YP medium with maltose, sucrose, or α-methyl-glucoside, as indicated.

Sp. passalidarum also demonstrated the ability to hydrolyze trehalose, a sugar composed of two glucose molecules linked by an α1→1 bond, suggesting that this strain possesses other enzymes, such as trehalases. Considering the intracellular location and the pH 6.8 used in this assay, it is likely a neutral trehalase, which in *S. cerevisiae* is encoded by the *NTH1* gene[12]. However, the hydrolysis of trehalose was comparatively lower than that of other substrates, which may have been caused by factors such as lower expression of the genes encoding trehalases under the experimental conditions used. Additionally, considering that melezitose and raffinose contain galactose molecules in their structures, it is suggested that *Sp. passalidarum* lacks enzymes capable of cleaving α-galactosides. It is worth noting that a previous study showed that this yeast is also unable to grow on these two carbon sources[13].

Given its ability to hydrolyze numerous α-glycosides, a search in its genome for sequences with identity to maltases (*MALx2*) and isomaltases (*IMAx*) of *S. cerevisiae* was performed. Two putative proteins, designated as AG1 and AG2, were identified, with lengths of 571 and 568 amino acids, respectively. The SignalP software was used to confirm the localization of both, and therefore, they were predicted to be intracellular, as no signal peptide was detected. The identity of the two sequences with Sc*MALx2* and Sc*IMAx* was approximately 50%. Additionally, a set of amino acids near the binding site constitutes the "amino acid signature," important for substrate specificity by α-glycosidases. The amino acid signature for ScIMAx was determined as Y158/V216/G217/S218/L219/M278/Q279/D307/E411, while for ScMAL62 it is F158/T216/A217/G218/L219/V278/A279/E307/D411[14,15]. Thus, maltases and isomaltases have distinct spatial forms, so that, due

to steric hindrance, they are selective for maltose or isomaltose[5,6,15]. A valine residue (V216) has particular importance, as it is essential for discriminating the α1→6 bond by isomaltases. In contrast, in maltases and mixed-character enzymes (maltase-isomaltases), this position is occupied by threonine (T) or alanine (A) [14].

Therefore, AG1 and AG2 were aligned with ScMALx2p and ScIMAxp to verify which amino acid residue is present at this position. Sequence alignment showed that the predicted proteins can be maltases or maltase-isomaltases, as a T216 is present. These data are corroborated by analyzing the phylogenetic tree, as the possible α-glycosidases of *Sp. passalidarum* are related to characterized enzymes of *S. stipitis*, identified as maltase-isomaltases (Figure 2)[5]. α-Glycosidases with this characteristic have a binding pocket capable of accommodating sugars similar to maltose, as well as those similar to isomaltose[5,6,15]. However, as two possible maltase-isomaltases were identified in the *Sp. passalidarum* genome, the α-glycosidase activities measured in this work do not allow us to determine the exact substrate specificity for each of the two predicted proteins, requiring cloning and heterologous expression studies for this evaluation.

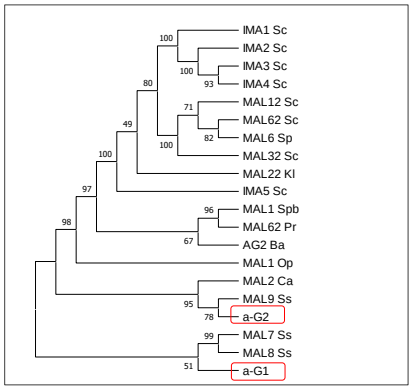

Figure 2: Maximum parsimony phylogenetic tree generated using MEGA11 with sequences of α-glucosidases (AG1 and AG2) found in the yeast *Sp. passalidarum* NRRL Y-27907^T with others characterized in the literature.

4 CONCLUSION

Based on the results obtained so far, the yeast *Sp. passalidarum* has demonstrated the ability to cleave carbohydrates with α1→4, α1→2, and α1→6 linkages. This ability suggests the presence of maltases and/or isomaltases in this yeast, which can be corroborated by phylogenetic alignment performed with genes known to encode maltases (*MALx2*) and isomaltases (*IMAx*) in *S. cerevisiae*. However, for the complete characterization of these potential enzymes, it is suggested to clone and heterologous express them in *S. cerevisiae*, as the yeast *Sp. passalidarum* has shown promise as an organism for carbohydrate bioconversion.

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