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Characterization of a maltodextrin glucosidase from *Klebsiella variicola* of the GH13_21 subfamily

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ABSTRACT. *Klebsiella variicola* is a Gram-negative bacterium belonging to the *Klebsiella pneumoniae* species complex. It is frequently isolated from many plants, where it is involved in nitrogen fixation and growth. However, it has also been described as associated with disease in humans and animals. *Escherichia coli* and *K. pneumoniae* are related members of the *Enterobacteriaceae* family that metabolize maltose and maltodextrins. However, only isolates of the *K. pneumoniae* species complex metabolize starch. In *E. coli*, four enzymes metabolize maltodextrins, including a periplasmic α-amylase (MalS), a cytoplasmatic amylomaltase (MalQ), a maltodextrin phosphorylase (MalP), and a maltodextrin glucosidase (MalZ). Considering the medical and agricultural importance of *K. variicola* and to better understand its role in maltodextrin degradation, the *malZ* gene was cloned from an isolate of this species, and the coded protein was recombinantly expressed and characterized. The *K. variicola* MalZ protein sequence was 79% identical to the *E. coli* MalZ and is the fifth enzyme and second maltodextrin glucosidase characterized from the GH13_21 CAZy subfamily. Its modeled monomeric structure shows β-stranded amino- and carboxy-terminal domains and a catalytic domain with a $(\beta/\alpha)_8$-barrel. The 70 kDa expressed enzyme could promote glucose release from maltoheptaose and other maltodextrins. The enzyme's optimum temperature was 30°C. The K_M and V_{max} for maltoheptaose were 32.7 mM and 2.58 µmol/min of glucose released, respectively. Considering the characteristics of degrading maltodextrins, the release of glucose from this substrate, and the high profit of recombinant expression, the enzyme expressed in this work could be used in the industrial degradation of starch as a saccharifying enzyme.

Keywords: Maltodextrin glucosidase; *Klebsiella variicola;* Recombinant expression; MalZ.

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Introduction

Klebsiella variicola is a non-motile Gram-negative bacillus that belongs to the *Klebsiella pneumoniae* species complex (Rodríguez-Medina et al., 2019). *K. variicola* is frequently isolated from various plants and is involved in growth promotion and nitrogen fixation (Lin et al., 2015). However, it has also been described to cause pathologies in animals and humans (Rodríguez-Medina et al., 2019).

K. pneumoniae and *Escherichia coli* are related members of the *Enterobacteriaceae* family that metabolize maltose and maltodextrins. *E. coli* has four known coding genes for maltodextrin-metabolizing enzymes. The *malS* gene codifies a periplasmic α-amylase that hydrolyzes maltodextrins from the nonreducing end, producing mainly maltohexaose (Freundlieb & Boos, 1986). The *malQ* gene codes for a cytoplasmic amylomaltase able to release glucose from maltotriose and maltodextrins from longer maltooligosaccharides (Palmer et al., 1976). The *malP* gene codes for a MalP cytoplasmic maltodextrin phosphorylase releases glucose l-phosphate from the nonreducing end of maltopentaose or longer maltodextrins (Schwartz, 1987). At last, the *malZ* gene codes for a cytoplasmic MalZ maltodextrin glucosidase cleaves *p*-nitrophenyl-α-maltoside and releases maltose and glucose from maltotriose to maltoheptaose (Tapio et al., 1991).

In *E. coli* strain K-12, the maltose/maltodextrin regulon contains three operons and an isolated gene. The *malPQ* operon in the *malA* region possesses the *malP*, *malZ*, and *malQ* genes (Bloch & Raibaud, 1986; Schwartz, 1987). The *malS* gene, located in the *malB* region, codes for an α-amylase (Freundlieb & Boos, 1986; Schwartz, 1987). The *malK-lamB* and *malEFG* operons are located in the *malB* region and code for the five components of the maltose and maltodextrin transport system (Puyet, 1993). These four operons are positively controlled by the *malT* gene product, which is located in the *malA* region (Dippel & Boos, 2005; Puyet, 1993).

The *K. pneumoniae* maltose regulon has been less broadly characterized. However, it is known that this bacterium possesses enzymes involved in maltodextrin degradation, such as a maltodextrin phosphorylase, an amylomaltase, and at least two proteins involved in maltose transport, the MalE and LamB proteins (Dippel & Boos, 2005; Puyet, 1993). Additional maltose-regulated genes in operons *pulAB* and *pulC-0* allow *K. pneumoniae*, but not *E. coli*, to metabolize starch. The *pulA* gene codes for an extracellular and outer membrane enzyme that cleaves α-(1→6) linkages in glycogen and amylopectin (D'Enfert et al., 1987; Puyet, 1993). In *K. pneumoniae*, an *E. coli* MalT homolog can control all these operon expressions (D'Enfert et al., 1987; Puyet, 1993).

The Carbohydrate-Active enZyme (CAZy) database at http://www.cazy.org/CAZY organizes the enzymes acting on starch, glycogen, and related oligo- and polysaccharides within family GH13, which embodies the predominant family of glycoside hydrolases (Janeček & Svensson, 2022; Lombard et al., 2014). Although the GH13 family has been initially divided into 35 subfamilies (Stam et al., 2006) in accordance with the amino acid sequence, the CAZy database currently presents 49 GH13 subfamilies (data from CAZy, September 2025). The *E. coli* MalZ maltodextrin glucosidase is classified as a GH13_21 subfamily protein. This subfamily comprises bacterial α-glucosidases, maltodextrin glucosidases, and α-amylases (Janeček et al., 2014; Janeček & Gabriško, 2016; Stam et al., 2006).

In this work, a maltodextrin glucosidase gene (*malZ*) from a *K. variicola* isolate was cloned. The coded protein was expressed in *E. coli*, and its biochemical traits were identified.

Material and methods

Microorganisms

E. coli strains DH5α™, TOP10™, and BL21 Star™ (DE3) were cultured overnight in Luria-Bertani (LB) medium (1.0 g% NaCl; 0.5 g% yeast extract; 1.0 g% tryptone) at 37°C with orbital agitation (100 rpm) and stocked at −20°C in 50% glycerol.

The isolate of *K. variicola* CTI-79 was obtained from dried maize (*Zea mays*) kernels collected as leftover harvest in August 2015 at the Irrigation Technical Center (CTI) on the State University of Maringá campus, Paraná, Brazil (Santos et al., 2019). This strain was not identified as an amylase producer in a test of hydrolysis halo production in starch nutrient agar (6 g L^{-1} meat peptone; 3 g L^{-1} yeast extract; 5 g L^{-1} NaCl; 2 g L^{-1} starch; 20 g L^{-1} agar), revealed with iodine (2,0 g/% KI; 0,2 g/% I_2).

Genomic DNA extraction

The *K. variicola* CTI-79 genomic DNA had been extracted before (Santos et al., 2019) and was kept in the laboratory.

The *K. variicola* maltodextrin glucosidase *malZ* gene cloning and expression plasmid construction

A maltodextrin glucosidase MalZ (GenBank AN: AHI34565.1) was found in the sequenced and annotated genome of *K. pneumoniae* ATCC BAA-2146 (Hudson et al., 2014). This protein is intracellular and has no signal peptide. The protein gene (GenBank AN: CP006659.2:1150310-1152127 bp) was also found in GenBank, and non-cutter restriction enzymes were selected after in silico analysis in the NEBCutter server. The designed primers (FW 5´-TTGCTAGCATGTTGAAGGCATGGCACCTTC and RV 5´-TTGAATTCggACGGCCGGACCAGACGTTGGC) targeted the entire gene coding region. The FW primer contained a restriction site for *Nhe*I (underlined). A restriction site for *EcoR*I (underlined) was placed at 5´ in the reverse primer, and two additional guanosines were added to keep the His-tag frame in the expression plasmid. The reverse primer had no stop codon. To protect the restriction sites, two additional thymidines were added to the 3´-end of each primer.

The 25-µL PCR reaction contained 1 U High Fidelity Platinum® *Taq* DNA Polymerase (ThermoFisher Scientific, USA), 1X enzyme buffer, 25 pmol of each primer, 0.2 mM of each dNTP, 2.0 mM $MgSO_4$, and 2 µL of the obtained *K. variicola* DNA. The cycling conditions included an initial denaturation incubation of 94°C for 5 min; 25 cycles of 1.0 min at 94°C, 1.0 min at 56°C, and 1.0 min and 30 sec at 68°C; and a 68°C 10-min final incubation.

The 1,833 bp PCR-obtained product was cloned into the pCR2.1® vector (Thermo Fisher Scientific, USA). The obtained recombinant plasmid was used to transform *E. coli* DH5α™ (Chung et al., 1989) and was recovered from the bacteria by alkaline lysis (Sambrook & Russell, 2001).

The cloned gene (*malZ*) was sequenced using M13 primers directed for the plasmid and primers designed for the *K. pneumoniae* ATCC BAA-2146 maltodextrin glucosidase gene (GenBank AN: CP006659.2:1150310-1152127). The sequencing was carried out at the Center for Human Genome Studies and Stem Cells at the University of São Paulo, Brazil. The contig was generated using the BioEdit program (Hall, 1999), and the obtained sequence was deposited in GenBank.

To construct the expression plasmid, the cloning plasmid was digested with *Nhe*I and *Eco*RI restriction enzymes. The digestion fragments were separated by electrophoresis in agarose gel, and the released gene was gel-purified using the Wizard® SV System (Promega, USA). The pET21a(+) (Novagen, Germany) plasmid was restrict-digested and purified similarly. The gene fragment was ligated with the linearized plasmid with T4 DNA ligase. Then, DH5α™ *E. coli* was transformed with the ligation mixture (Chung et al., 1989). The recombinant plasmid was recovered by alkaline lysis (Sambrook & Russell, 2001).

Structural modeling and phylogenetic tree construction

The cloned gene-derived protein sequence was found using the Translate platform. Protein sequence alignments and identity analysis among the *K. variicola* protein and other maltodextrin glucosidases were performed in the Clustal Omega server. Conserved Protein Domain Database (CDD) and HMMER Pfam were used to search for structural domains.

The *K. variicola* maltodextrin glucosidase monomeric structural model was built by homology using the Phyre2 program (Kelley et al., 2015) in normal mode. The template automatically chosen by the modeling program was the *E. coli* maltodextrin glucosidase MalZ protein structure (PDB 7VT9 chain A) (Ahn et al., 2022), a CAZy GH13_21 subfamily protein. The servers Procheck and VADAR version 1.8 were used to evaluate the model's stereochemical quality and the secondary structure content, respectively. The modeled PDB structure was visualized and colored in the CCP4MG program (McNicholas et al., 2011). Alternatively, the protein dimeric model was obtained in the SwissModel platform, using the *E. coli* MalZ maltodextrin glucosidase (PDB 7VT9) as a model (Ahn et al., 2022).

For the phylogenetic analysis, maltodextrin glucosidase sequences from the subfamily GH13_21 were obtained in the CAZy and GenBank databases. In addition, GH13_20 subfamily proteins, which group cyclic α-1,6-maltosyl-maltose hydrolases, α-amylases, maltogenic α-amylases, neopullulanases, pullulanases, and cyclomaltodextrinases were also procured. Furthermore, protein sequences from the GH13_30 subfamily, which includes α-glucosidases, and from the GH13_26 subfamily, which comprises malto-oligosyltrehalose synthases, were also added as outgroups.

Peptide sequences predicted in the SignalP-5.0 platform were removed. The neighbor-joining method (Saitou & Nei, 1987) was used to conduct the phylogenetic analysis in the MEGA 11 program (Tamura et al., 2021). The branching confidence limits were evaluated using Bootstrap analyses with 1000 heuristic replicate trees (Felsenstein, 1985), and values higher than 70% proved reliable grouping. Finally, the evolutionary distances were computed considering amino acid substitutions using the Poisson correction method (Zuckerkandl & Pauling, 1965).

Expression and purification of the recombinant maltodextrin glucosidase enzyme

For the expression analysis, the strain *E. coli* BL21 Star™ (DE3) was transformed (Chung et al., 1989) using 50 ng of the pET21a(+)-*malZ* vector, and the entire transformation reaction was mixed with 10 mL of LB-ampicillin (50 μg mL^{-1}) and incubated overnight at 37°C and 100 rpm of orbital shaking. Then, a 500-μL aliquot of this culture was transferred to an Erlenmeyer containing 50 mL of LB-ampicillin (50 μg mL^{-1}) and incubated at 37°C and 100 rpm for 4 h before the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. The culture was then incubated overnight at 20°C and 100 rpm. After that, cells were recovered (2,000 g, 10 min) and resuspended in 10 mL of lysis buffer (50 mM NaCl; 1 mM PMSF; 50 mM Tris, pH 7.0). Cell sonication was performed with 60 cycles of 5 seconds on and 5 seconds off with 40% amplitude. After sonication, a centrifugation was performed (10,000 g, 5 min).

The recombinant protein in the supernatant was purified using a HisTrap™ HP column (GE Healthcare Life Sciences, USA), previously equilibrated with 50 mM Tris (pH 7.0), 50 mM NaCl, and 20 mM imidazole. The column was washed with the same equilibration buffer and eluted with 50 mM Tris (pH 7.0), 50 mM NaCl, and 300 mM imidazole. Fractions of 2.0 mL were collected, and fractions with increased absorbance readings (280 nm) were pooled and dialyzed three times in the dialysis buffer (50 mM Tris, pH 7.0, 50 mM NaCl). Glycerol (20%) was added to the dialyzed protein and kept at -20°C.

Enzyme assay

The maltodextrin glucosidase activity was evaluated using the glucose oxidase method (Trinder, 1969) using a commercial kit (Gold Analisa, Brazil). For that, an enzyme aliquot (50 µL) was incubated (1 h at 37°C) with the substrate (Maltodextrin Sigma cat. Nº 419672, dextrose equivalent 4.0-7.0; or Maltoheptaose, Dp7, Sulpelco Cat. Nº 4-7872) in a final volume of 100 µL. After that, 200 µL of 0.3 M trichloroacetic acid were added for protein precipitation, and a centrifugation was performed for 5 min at 12,000 g. Aliquots (triplicates) of 25 µL of the supernatant were added to 1.0 mL of the Trinder reagent, and an incubation of 15 min at 37°C was performed before absorbance reading at 505 nm. A standard curve was performed with glucose. One international enzyme unit consists of the enzyme's amount that releases one µmol of glucose/min in the assay conditions.

Electrophoretic analysis

Protein concentration was estimated by the Bradford test (Bradford, 1976) using albumin as a standard. A 4-µg aliquot of the purified protein and aliquots (20 µg) of the pellet and supernatant obtained after sonication of the pCR2.1-*malZ* plasmid harboring cells were mixed (1:1) with the sample elution buffer (Laemmli, 1970). The samples were boiled (10 min) in a water bath and then loaded on a discontinuous denaturing 7.5% polyacrylamide gel (SDS-PAGE), pH 8.9, with a 4.5% stacking polyacrylamide gel, pH 6.8, in the Tris-glycine buffer system (Laemmli, 1970). The broad-range marker from BioRad (USA) was treated in the same manner before loading into the gel. Coomassie brilliant blue R-250 was used to stain the gel. The apparent molecular mass of the purified protein, as estimated from the gel, was compared with the theoretical mass calculated using the Compute pI/MW tool.

Enzyme characterization

The enzyme's initial velocities were determined with different concentrations of maltoheptaose (1 to 50 mM) and a constant amount of the enzyme (50 µL; 6.8 µg), in a final volume of 100 µL. The glucose oxidase method evaluated the glucose released (µmol/min). Non-linear regression was used to calculate K_M and V_{max} with the Solver tool of the Microsoft Excel software.

To obtain the optimum temperature, the enzyme was incubated with 1% maltodextrin for 10 min at temperatures varying from 30 to 70°C in increments of 10°C. Then, the glucose oxidase assay was used to estimate the released glucose.

The recombinant enzyme's calcium dependence was evaluated by adding 2, 5, or 10 mM to the enzyme's reaction and subsequently evaluating the glucose release in the glucose oxidase test.

The product analysis

A 25-µL aliquot (3.4 µg) of the purified enzyme was added to 25 µL of 0.1 M maltoheptaose in 50 mM Tris buffer, pH 7.0, and incubated for 18 h at 40°C with rotatory agitation (10 rpm). In addition, 25 µL of 0.1 M maltoheptaose was incubated accordingly with 25 µL of 50 mM Tris buffer, pH 7.0, and used as a control reaction.

For the sample and control reactions, 10-µL aliquots were applied to a Silica gel 60 thin-layer chromatography (TLC) plate (Sigma-Aldrich, Germany). The standard consisted of 10 µL of 100 mM D-(+)-anhydrous glucose. The solvent system was butanol/ethanol/water (5:3:2). After air-drying the plate, the spots were developed by spraying them with H_2SO_4 and methanol (1:9) containing 0.2% orcinol, followed by heating at 80°C (Benassi et al., 2014).

Statistical analysis

When indicated, averages and standard deviations (SD) of triplicates were submitted to ANOVA and compared using the Tukey test in the SASM–Agri program (Canteri et al., 2001).

Results and discussion

Sequence analysis of the *K. variicola* maltodextrin glucosidase

The entire gene of a maltodextrin glucosidase from *K. variicola* CTI-79 genomic DNA was amplified, cloned, and sequenced. The obtained gene sequence was deposited in GenBank (Accession Number PP756683.1). The 1,833-bp amplified gene (without a stop codon) encodes a 605-amino acid protein without a signal peptide, indicating its intracellular localization. The *K. variicola* CTI-79 cloned gene protein sequence

shares 98.84% identity with the *K. pneumoniae* putative maltodextrin glucosidase (GenBank AHI34565.1) and 79.14 % with the GH13_21 subfamily *E. coli* MalZ maltodextrin glucosidase (GenBank CAD6014888.1) (Figure 1). Therefore, it can be inferred that the cloned gene coded protein is the *K. variicola* MalZ maltodextrin glucosidase, which fits inside the GH13_21 subfamily. This enzyme is the fourth protein and the second maltodextrin glucosidase characterized within this subfamily.

In addition, the *K. variicola malZ* gene is the third GH13 family gene cloned from isolates of the *K. pneumoniae* complex. The first cloned gene codes for a *K. pneumoniae* extracellular cyclodextrin-glycosyltransferase (Binder et al., 1986). The second cloned gene codes for a *K. pneumoniae* maltohexaose-producing amylase, corresponding to the periplasmic alpha-amylase MalS from *E. coli* (Momma, 2000). The third enzyme, characterized in this work, may be involved in starch metabolism, for instance, degrading maltodextrins produced by other enzymes.

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PP756683.1      -MLKAWHLPVAPFIKVQQDRLFITLWLSGESLPQRITLRAEEDNEELSLPMQRLRQAPQPGVVAWRGEIS   69
AHI34565.1      -MLKAWHLPVAPFIKVQQDRLFITLWLSGESLPQRITLRAEEDNEELSLPMQRLRQAPQPGVVAWRGEIS   69
CAD6014888.1    MMLNAWHLPVPPFVKQSKDQLLITLWLTGEDPPQRIMLRTEHDNEEMSVPMHKQRSQPQPGVTAWRAAID   70
                 **:****** **:* .:*:*:*****:**. **** **:*.****:*:**:: *. *****.***. *.
                           CSR VI
PP756683.1      LASGQPRRRYSFKLLWADHQRWFTPQGFTRFPPARLEQFAIDLPDAGPQWVADQVFYQIFPDRFARSAAR   139
AHI34565.1      LASGQPRRRYSFKLLWADHQRWFTPQGFTRFPPARLEQFAIDLPDAGPQWVADQVFYQIFPDRFARSAAR   139
CAD6014888.1    LSSGQPRRRYSFKLLWHDRQRWFTPQGFSRMPPARLEQFAVDVPDIGPQWAADQIFYQIFPDRFARSLPR   140
                *:************** *:*********:*:*********:*:** ****.***:************  *

PP756683.1      DADQDAVYYHHAAGREIVRKAWDEPLTGEAGGSTFYGGDLDGISEKLPYLKQLGVTALYLNPVFAAPSVH   209
AHI34565.1      DADQDAVYYHHAAGREIVRKAWDDPLTGEAGGSTFYGGDLDGISEKLPYLKQLGVTALYLNPVFAAPSVH   209
CAD6014888.1    EAEQDHVYYHHAAGQEIILRDWDEPVTAQAGGSTFYGGDLDGISEKLPYLKKLGVTALYLNPVFKAPSVH   210
                :*:** ********:**: : **:*:*.:**********************:************ *****
                                          CSR I    ♦H251                    D279♦
PP756683.1      KYDTEDYRRVDPQFGGDAALLRLRHNTQRAGMRMILDGVFNHTGDSHPWFDRHQQGSGGAGHYPDSPWRD   279
AHI34565.1      KYDTEDYRRVDPQFGGDAALLRLRHNTQRAGMRMILDGVFNHTGDSHPWFDRHQQGSGGAGHDPDSPWRD   279
CAD6014888.1    KYDTEDYRHVDPQFGGDGALLRLRHNTQQLGMRLVLDGVFNHSGDSHAWFDRHNRGTGGACHNPESPWRD   280
                ********:********.**********: ***::*******:**** *****::*:*** * *:*****
                CSR V                                        CSR II  D336▲ ♦H339
PP756683.1      WFTFSEEGQAHNLLGYASLPKLDYRSTSLVNEIYAGEDSIVRHWLKAPWSMDGWRLDVVHMLGEGGGARN   349
AHI34565.1      WFTFSEEGQAHNWLGYASLPKLDYRSTSLVNEIYAGEDSIVRHWLKAPWSMDGWRLDVVHMLGEGGGARN   349
CAD6014888.1    WYSFSDDGTALDWLGYASLPKLDYQSESLVNEIYRGEDSIVRHWLKAPWNMDGWRLDVVHMLGEAGGARN   350
                *::**::* * : ***********:* ******* **************.***************.*****
                          CSR III ▲E373
PP756683.1      NLQHIAGITQAAKQTQPEAFVFGEHFGDARQWLQADAEDAAMNYRGFTFPIWGFLANTDISYDPQKIDAQ   419
AHI34565.1      NLQHIAGITQAAKQAQPEAFVFGEHFGDARQWLQADAEDAAMNYRGFTFPIWGFLANTDISYDPQKIDAQ   419
CAD6014888.1    NMQHVAGITEAAKETQPEAYIVGEHFGDARQWLQADVEDAAMNYRGFTFPLWGFLANTDISYDPQQIDAQ   420
                *:**:****:***::****::.**************.*************:***************:****
                                  CSR IV  ▲D448                          CSR VII
PP756683.1      TCMAWMDNYRAGLSHQQQLRMFNQLDSHDTARFKSLLGKDVARLPLAVVWLFSWPGVPCIYYGDEVGVDG   489
AHI34565.1      TCMAWMDNYRAGLSHQQQLRMFNQLDSHDTARFKSLLGKDVARLPLAVVWLFSWPGVPCIYYGDEVGVDG   489
CAD6014888.1    TCMAWMDNYRAGLSHQQQLRMFNQLDSHDTARFKTLLGRDIARLPLAVVWLFTWPGVPCIYYGDEVGLDG   490
                ************************************:***:*:***********:**************:**

PP756683.1      NNDPFCRKPFPWDPALQDTQLLALYQRMAKLRKAQQALRYGGCQVIYAEDNVVVFVRVYKQQRVLVAINR   559
AHI34565.1      NNDPFCRKPFPWDPALQDTQLLALYQRMAKLRKAHQALRYGGCQVIYAEDNVVVFVRVYKQQRVLVVINR   559
CAD6014888.1    KNDPFCRKPFPWQVEKQDTALFALYQRMIALRKKSQALRHGGCQVLYAEDNVVVFVRVLNQQRVLVAINR   560
                :***********:   *** *:******  ***  ****:*****:************ :******.***

PP756683.1      GEACEVVIEDSPLLNVAGWTLLEGAGAFQDGVLTLPAISANVWSGR   605
AHI34565.1      GEACEVVIEDSPLLNVAGWTLQEGAGAFQDGVLTLPAISANVWSGR   605
CAD6014888.1    GEACEVVLPASPFLNAVQWQCKEGHGQLTDGILALPAISATVWMN-   605
                *******:  **:**.. *   ** * : **:*:******.** .
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Figure 1. Sequence alignment. Alignment of the amino acid sequences from the *K. variicola* CTI-79 maltodextrin glucosidase derived from the cloned gene (GenBank PP756683.1) and maltodextrin glucosidase sequences from *K. pneumoniae* (HI34565.1) and *E. coli* (CAD6014888.1). There is no secretion sequence in these proteins. The catalytic amino acid residues in the active site are in bold green and have a black triangle at the top. The seven relatively conserved sequences among α-amylases (Janeček et al., 2014; Janeček & Gabriško, 2016) are highlighted in bright yellow. The calcium-binding residues are in bold magenta and have a black diamond on the top. Underlined in the *K. variicola* CTI-79 maltodextrin glucosidase sequence is the catalytic domain in blue, the *N*-domain in green, and the *C*-domain in red.

The three invariably conserved catalytic residues (Asp336, Glu373, and Asp448, given positions in our protein) in all α-amylases (Janeček et al., 2014) were identified (Figure 1). Amylases comprise a large variety of enzymes with highly variable sequences. However, seven short sequences are conserved (Janeček, 2002; Janeček et al., 2014; Janeček & Gabriško, 2016; Nakajima et al., 1986), which are inside the *K. variicola* CTI-

79 MalZ maltodextrin glucosidase sequence (Figure 1).

The *K. variicola* CTI-79 expressed maltodextrin glucosidase has the *maltodextrin_glucosidase* family domain (PRK10785, IPR017069, and PIRSF036918), as indicated by the CDD and Pfam platforms.

Structural modeling and phylogenetic tree construction

The *K. variicola* CTI-79 maltodextrin glucosidase was modeled with 100% residue coverage and 90% confidence (Figure 2). The Procheck program validated this model with 78.3% of the amino acids in most favored regions in the Ramachandran plot. A VADAR server analysis of the obtained model showed a secondary structure of β-conformation (34%), α-helix (19%), turns, and coils (Figure 2A).

The structural model of the *K. variicola* cloned gene-coded maltodextrin glucosidase demonstrates a three-domain structure (Figure 2A and Figure 1). It has a $(\beta/\alpha)_8$-barrel catalytic domain, corresponding to the A-domain of α-amylases. Proteins containing this domain are classified into the GH13 family, according to CAZY (El-Fallal et al., 2012; Janeček et al., 2014; Lombard et al., 2014). The catalytic domain contains the three highly conserved catalytic residues, and the active site has a predominantly negatively charged environment (Figure 2A). The antiparallel β-stranded *C*-terminal domain corresponds to the *C*-domain of α-amylases (El-Fallal et al., 2012; Janeček et al., 2014; Paul et al., 2021). The additional *N*-terminal domain, commonly found in the GH13_21 and GH13_20 subfamilies, folds into a distinct β-sandwich domain, which is classified as a carbohydrate-binding module 34 (CBM34) involved in protein stabilization, dimerization, and substrate specificity (Janeček et al., 2019). A structural model of the *K. pneumoniae* maltodextrin glucosidase (GenBank VGM24776.1) obtained from the AlphaFold data bank (A0A0H3GJ07.1.A) superposed well with the *K. variicola* CTI-79 maltodextrin glucosidase obtained structure model (Figure 2B).

The literature pointed out that the *E. coli* MalZ maltodextrin glucosidase, used as a template in the structure modeling analysis, is a dimer with two-fold rotational symmetry (Ahn et al., 2022). In addition, each molecule's *N*-terminal domain is near the adjacent molecule's catalytic domain with a head-to-tail arrangement (Ahn et al., 2022). Therefore, by inference, the *K. variicola* CTI-79 maltodextrin glucosidase is also a dimer.

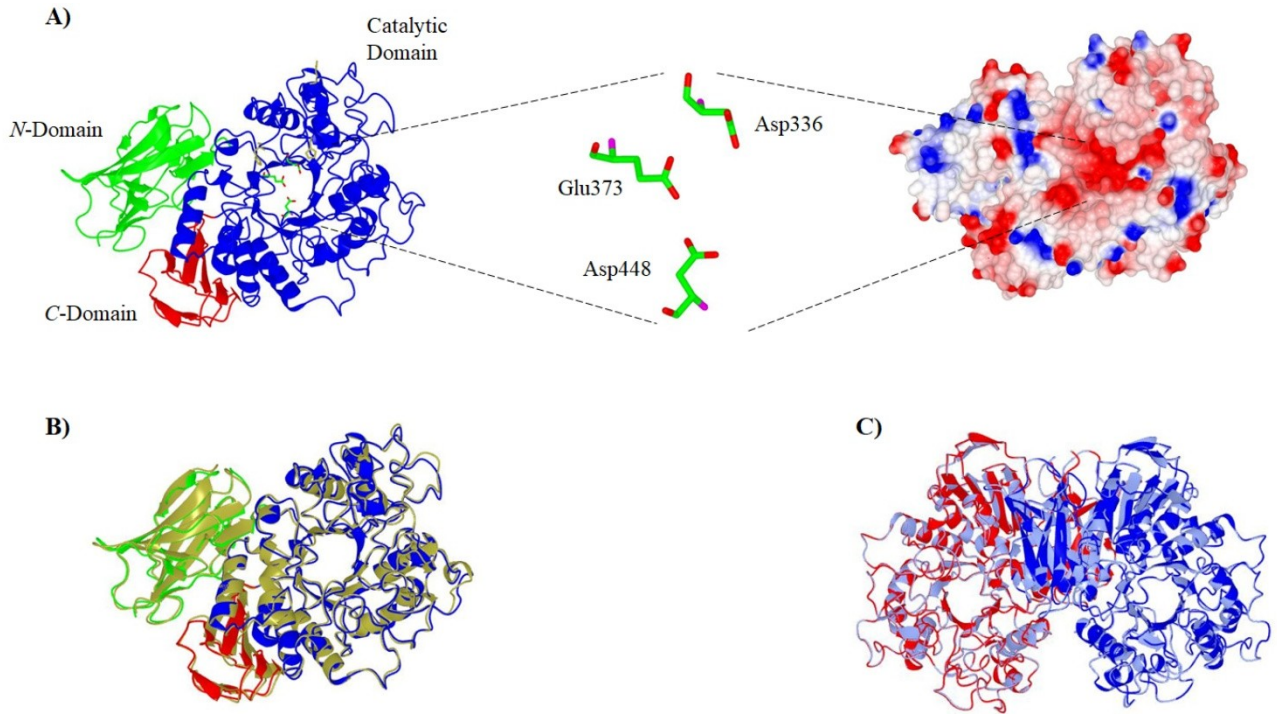

Figure 2. The homology structural model of the *K. variicola* CTI-79 maltodextrin glucosidase. A) The catalytic domain (in blue), constituted by $(\beta/\alpha)_8$-barrel, can be seen in the center. The *N*-domain is in green. The *C*-terminal domain is constituted of six antiparallel β-strands. The catalytic residues (enlarged) are shown in green, red, and magenta, numbered in the *K. variicola* CTI-79 maltodextrin glucosidase. The calcium-ligand residues in domain B [N251, D279, and H339] are shown in gold. The protein is also shown with its surface electrostatic potential (red–negative regions; blue–positive regions). B) Superposition of the *K. variicola* CTI-79 maltodextrin glucosidase structure (green, blue, and red) with the *K. pneumoniae* maltodextrin glucosidase structure (gold) obtained from AlphaFold Data Bank (https://alphafold.ebi.ac.uk/) (GenBank: VGM24776.1; AF PDB A0A486DID6 6WNU). C) The *K. variicola* CTI-79 maltodextrin glucosidase dimeric structure, as modeled in the SwissModel platform (blue and red), superposed with the *E. coli* maltodextrin glucosidase dimeric (PDB 7VT9) structure used as a model (ice blue).

The *K. variicola* CTI-79 maltodextrin glucosidase dimeric model, obtained in the Swiss Model platform with 100% of coverage and 90% of confidence using the *E. coli* maltodextrin glucosidase dimeric (PDB 7VT9) structure used as a model, was validated in the Procheck program with 86.3% of amino acid residues in most favored regions in the Ramachandran plot. This dimeric structural model of the *K. variicola* CTI-79 maltodextrin glucosidase superposed well with the dimeric structure of the *E. coli* MalZ (PDB 7VT9) dimer (Figure 2C).

The connection between the *K. variicola* CTI-79 maltodextrin glucosidase and other amylase's amino acid sequences was assessed in a phylogenetic tree (Figure 3). Proteins with identity superior to 31.53% with the *K. variicola* CTI-79 maltodextrin glucosidase were from the GH13_21 subfamily (Figure 3). Protein sequences from the subfamily GH13_20 shared less than 26.03% of identity with the *K. variicola* CTI-79 maltodextrin glucosidase. The *K. variicola* CTI-79 maltodextrin glucosidase GH13_21 clade clustered with the GH13_20 subfamily clade (Figure 3). Even though these proteins are clustered together, the amino acid residue's identity is low, probably because they have different activities. Proteins from the GH13_30 and GH_26, which have low sequence identity with the *K. variicola* CTI-79 maltodextrin glucosidase, formed two separate clades. These results are consistent with the GH13 family phylogeny (Stam et al., 2006).

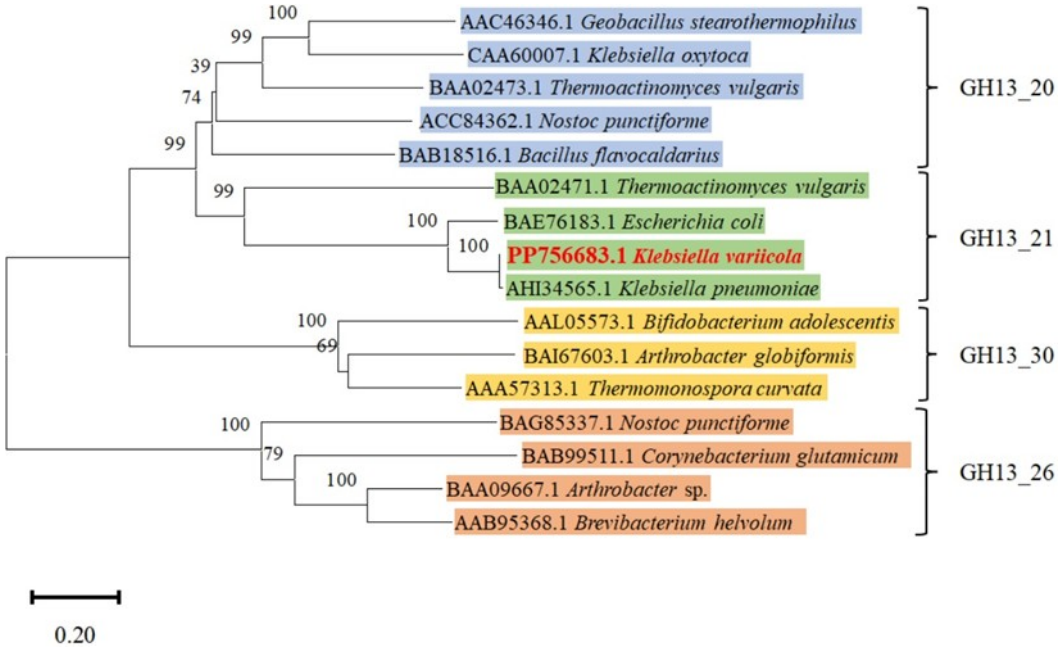

Figure 3. The phylogenetic analysis. The bootstrap test results (%) are shown next to the branches (1000 replicates). The branch lengths correspond to the units of the evolutionary distances used to infer the phylogenetic tree. This analysis involved 16 amino acid sequences. The evolutionary distances are in units of the number of amino acid substitutions per site. The ambiguous positions were removed (pairwise deletion option). There was a total of 994 positions in the final dataset. The *K. variicola* CTI-79 maltodextrin glucosidase is indicated in bold red. The GH13 subfamilies are indicated in different colors.

Recombinant enzyme expression and purification

The *K. variicola* CTI-79 maltodextrin glucosidase recombinant pure protein yield, expressed in 50 mL of LB medium *E. coli* culture, was 1.0 mg (20 μg mL^{-1} of culture medium) under the glucose oxidase assay. The *E. coli* expression system is frequently used for α-amylase recombinant expression (Movahedpour et al., 2022). The obtained yield for the *K. variicola* CTI-79 maltodextrin glucosidase was low compared to the yield obtained from an affinity-chromatography purified α-amylase from *Pseudoalteromonas* sp., a GH13_36 subfamily protein, whose yield was 385.1 $\mu g\ mL^{-1}$ and specific activity 337.9 U mg^{-1} in 100 mL culture (Wang et al., 2018). However, the yield for the *K. variicola* CTI-79 maltodextrin glucosidase was similar to the one obtained for the *M. timonae* CTI-57 cyclodextrinase from the GH13_46 subfamily, which rendered a yield of 1.58 mg of protein (30 μg mL^{-1} of culture medium) (Santos & Barbosa-Tessmann, 2019).

The *K. variicola* CTI-79 recombinant maltodextrin glucosidase expressed from the pET21a(+) plasmid had 20 additional amino acids in the carboxylic region, including the His-tag (GNSSSVDYLAAALEHHHHHH). Thus, the in silico predicted mass of the expressed protein is 70.7 kDa. The purified protein experimentally estimated molecular mass of 70.0 kDa (Figure 4) is very close to the predicted value. Although variable, most amylases have a molecular mass of around 40–60 kDa (Paul et al., 2021; Sindhu et al., 2017). In agreement,

the *Thermoactinomyces vulgaris* pullulanase TVAI, a protein from the GH13_21 subfamily, has a similar molecular mass of 71 kDa (Ohtaki et al., 2003), and the *E. coli* MalZ maltodextrin glucosidase is reported to have a similar molecular mass of 65 kDa (Tapio et al., 1991).

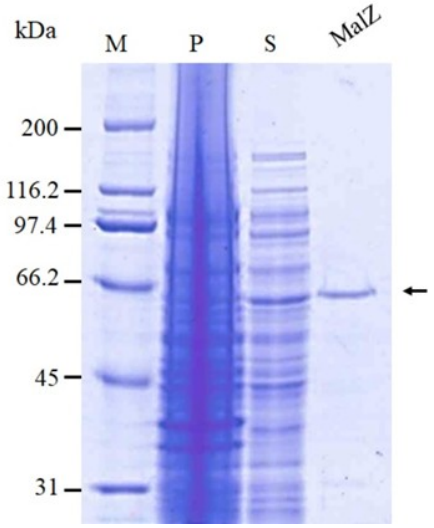

Figure 4. Electrophoretic analyses. A) SDS-PAGE gel. M - Broad range molecular marker (BioRad, USA); P – pellet; S – supernatant; MalZ – purified *K. variicola* CTI-79 maltodextrin glucosidase. Proteins were expressed in *E. coli* BL21 Star™ (DE3) transformed with the pET21a(+)-*malZ* plasmid and cultured in LB/ampicillin medium with 0.5 mM IPTG induction. The arrow indicates the purified enzyme. The gel was stained with Coomassie Brilliant Blue.

The recombinant enzyme characterization

The maltoheptaose saturation curve followed the Michaelis-Menten kinetics (Figure 5A). The *K. variicola* CTI-79 recombinant maltodextrin glucosidase calculated K_M for maltoheptaose was 32.7 mM, and V_{max} was 2.58 mmol/min of glucose released. Similarly, the reported K_M value of the *E. coli* maltodextrin glucosidase for maltotriose is 25 mM, but for maltoheptaose is 3.4 mM (Tapio et al., 1991). Unfortunately, data in the literature for maltodextrin glucosidase K_M and V_{max} are restricted to the *E. coli* enzyme, which is the only characterized enzyme.

The recombinant *K. variicola* CTI-79 maltodextrin glucosidase is most active at 30°C (Figure 5C). This enzyme is intracellular, and its optimal temperature may reflect the microorganism's habitat (Pandey et al., 2000). The literature does not provide data on maltodextrin glucosidases' optimal temperature. However, bacterial α-amylases are reported to be active at a broad optimum temperature (Gupta et al., 2003; Pandey et al., 2000; Sindhu et al., 2017; Sivaramakrishnan et al., 2006).

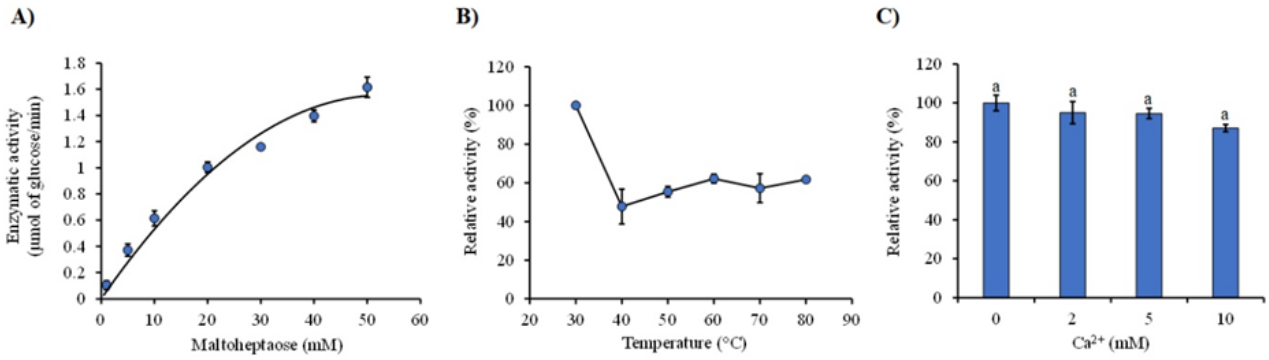

Figure 5. *K. variicola* CTI-79 recombinant maltodextrin glucosidase characterization. A) Determination of K_M and V_{max} using maltoheptaose as substrate. The saturation curve varying starch concentration from 1 to 50 µM. The initial velocity was measured in A and B as µmol of glucose released per min. B) Optimum temperature. C) Effect of calcium on the purified recombinant enzyme's activity. The average with the same letter indicates no statistical difference at 0.01% significance. The data represent the average and SD of three experimental sets.

Most α-amylases are metalloenzymes and require Ca^{2+} for structural and catalytic activity (Gupta et al., 2003; Paul et al., 2021; Sivaramakrishnan et al., 2006). However, the purified *K. variicola* CTI-79 maltodextrin

glucosidase was active without calcium (Figure 5D), indicating that this ion is unnecessary for its catalytic activity. The *K. variicola* CTI-79 maltodextrin glucosidase has shown three conserved amino acids (N251 at CSR I, D279 at CSR V, and H339 at CSR II) in the high-affinity calcium-binding site (Figure 1) (Boel et al., 1990; Buisson et al., 1987; Cihan et al., 2018). In the *K. variicola* CTI-79 maltodextrin glucosidase obtained structural model (Figure 2A), two of those amino acids (N251 and H339) are located next to the active site. Nevertheless, the third one (D279) is far from the active site. Calcium-independent amylases are essential because their purification is less expensive and simpler (Paul et al., 2021).

TLC analysis of enzyme products

In the *K. variicola* CTI-79 maltodextrin glucosidase maltoheptaose hydrolysis product analysis, after 18 h incubation at 37°C, glucose (G1) and other degradation products, probably maltose and maltotriose, can be seen (Figure 6). It is reported that the *E. coli* MalZ maltodextrin glucosidase has the same pattern of product release from maltoheptaose (Tapio et al., 1991). Therefore, the *K. variicola* CTI-79 maltodextrin glucosidase can be considered a glucose-forming amylase.

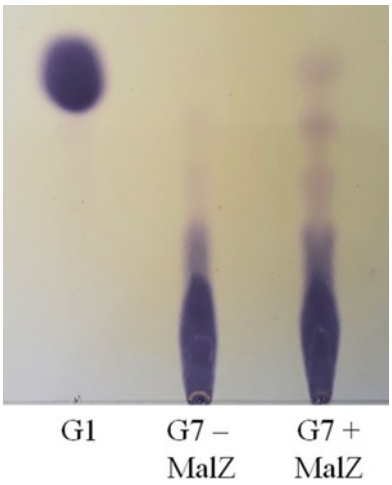

Figure 6. Maltoheptaose hydrolysis products by the *K. variicola* CTI-79 recombinant maltodextrin glucosidase. A TLC plate with the hydrolysis products from maltoheptaose incubated (or not) with the recombinant maltodextrin glucosidase at 37°C for 18 h in a rotatory mixer (10 rpm). Standards: G1 - glucose. MalZ - *K. variicola* CTI-79 recombinant maltodextrin glucosidase. G7 – Maltoheptaose.

The industrial starch market is estimated to be valued between USD 67.1 and 112 billion in 2024 (Adewale et al., 2022). These industries prefer enzymatic hydrolysis because it is less environmentally harmful and yields higher (Läufer, 2017; Singh et al., 2016). Enzymes from microbial sources are preferred due to their stability, low-cost production, and no seasonal fluctuations (Gurung et al., 2013; Singh et al., 2016). Because each industry branch requires different enzymes, searching for new microbial sources and varied enzymes is a plus (Chapman et al., 2018; Gopinath et al., 2017; Mehta & Satyanarayana, 2016; Paul et al., 2021).

Industrially, two enzymatic procedures are performed after starch gelatinization. Initially, liquefaction is conducted by α-amylases that reduce the solution viscosity and produce maltodextrins (El-Fallal et al., 2012; Van der Maarel et al., 2002). The second process is saccharification, performed by glucoamylases and other amylases that release maltose and glucose from the maltodextrins obtained in the liquefaction process (Van der Maarel et al., 2002). Considering its maltodextrin specificity and glucose production, the *K. variicola* CTI-79 maltodextrin glucosidase could be used for starch saccharification.

Conclusion

A new maltodextrin glucosidase gene was cloned from *K. variicola* CTI-79. The cloned gene's coded protein showed high identity with the CAZy GH13_21 subfamily bacterial maltodextrin glucosidases. A recombinant *K. variicola* CTI-79 maltodextrin glucosidase was expressed in *E. coli*. After purification, the recombinant protein was characterized. The results aid in better understanding maltodextrin metabolism in a *K. pneumoniae* species complex isolate. In addition, the expressed and purified enzyme characteristics indicate that it could be applied in industrial starch saccharification.

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References

Adewale, P., Yancheshmeh, M. S., & Lam, E. (2022). Starch modification for non-food, industrial applications: Market intelligence and critical review. *Carbohydrate Polymers, 291*, 119590. https://doi.org/10.1016/j.carbpol.2022.119590

Ahn, W. C., An, Y., Song, K. M., Park, K. H., Lee, S., Oh, B. H., Park, J. T., & Woo, E. J. (2022). Dimeric architecture of maltodextrin glucosidase (MalZ) provides insights into the substrate recognition and hydrolysis mechanism. *Biochemical and Biophysical Research Communications, 586*, 49–54. https://doi.org/10.1016/j.bbrc.2021.11.070

Benassi, V. M., Pasin, T. M., Facchini, F. D. A., Jorge, J. A., & Polizeli, M. de L. T. de M. (2014). A novel glucoamylase activated by manganese and calcium produced in submerged fermentation by *Aspergillus phoenicis*. *Journal of Basic Microbiology, 54*(5), 333–339. https://doi.org/10.1002/jobm.201200515

Binder, F., Huber, O., & Böck, A. (1986). Cyclodextrin-glycosyltransferase from *Klebsiella pneumoniae* M5a1: cloning, nucleotide sequence and expression. *Gene, 47*(2–3), 269–277. https://doi.org/10.1016/0378-1119(86)90070-3

Bloch, M. A., & Raibaud, O. (1986). Comparison of the malA regions of *Escherichia coli* and *Klebsiella pneumoniae*. *Journal of Bacteriology, 168*(3), 1220–1227. https://doi.org/10.1128/jb.168.3.1220-1227.1986

Boel, E., Brady, L., Brzozowski, A. M., Derewenda, Z., Dodson, G. G., Jensen, V. J., Petersen, S. B., Swift, H., Thim, L., & Woldike, H. F. (1990). Calcium binding in α-amylases: an X-ray diffraction study at 2.1-Å resolution of two enzymes from *Aspergillus*. *Biochemistry, 29*(26), 6244–6249. https://doi.org/10.1021/bi00478a019

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*(1–2), 248–254. https://doi.org/10.1006/abio.1976.9999

Buisson, G., Duée, E., Haser, R., & Payan, F. (1987). Three-dimensional structure of porcine pancreatic α-amylase at 2.9 Å resolution. Role of calcium in structure and activity. *The EMBO Journal, 6*(13), 3909–3916. https://doi.org/10.1002/j.1460-2075.1987.tb02731.x

Canteri, M. G., Althaus, R. A., Virgens Filho, J. S., Giglioti, É. A., & Godoy, C. V. (2001). SASM-AGRI - System for analyses and separation of averages in agriculture experiments using the methods of Scott-Knott, Tukey and Duncan. *Brazilian Journal of Agrocomputation, 1*(2), 18–24. https://ainfo.cnptia.embrapa.br/digital/bitstream/item/175651/1/SASM-AGRI.pdf

Chapman, J., Ismail, A., & Dinu, C. (2018). Industrial applications of enzymes: Recent advances, techniques, and outlooks. *Catalysts, 8*(6), 238. https://doi.org/10.3390/catal8060238

Chung, C. T., Niemela, S. L., & Miller, R. H. (1989). One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. *Proceedings of the National Academy of Sciences, 86*(7), 2172–2175. https://doi.org/10.1073/pnas.86.7.2172

Cihan, A. C., Yildiz, E. D., Sahin, E., & Mutlu, O. (2018). Introduction of novel thermostable α-amylases from genus *Anoxybacillus* and proposing to group the *Bacillaceae* related α-amylases under five individual GH13 subfamilies. *World Journal of Microbiology and Biotechnology, 34*(7), 95. https://doi.org/10.1007/s11274-018-2478-8

D'Enfert, C., Chapon, C., & Pugsley, A. P. (1987). Export and secretion of the lipoprotein pullulanase by *Klebsiella pneumoniae*. *Molecular Microbiology, 1*(3), 107–116. https://doi.org/10.1111/j.1365-2958.1987.tb00534.x

Dippel, R., & Boos, W. (2005). The maltodextrin system of *Escherichia coli*: metabolism and transport. *Journal of Bacteriology, 187*(24), 8322–8331. https://doi.org/10.1128/JB.187.24.8322-8331.2005

El-Fallal, A., Abou, M., El-Sayed, A., & Omar, N. (2012). Starch and microbial α-amylases: From concepts to biotechnological applications. In *Carbohydrates - Comprehensive Studies on Glycobiology and Glycotechnology* (Vol. 21, pp. 259–288). InTech. https://doi.org/10.5772/51571

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution, 39*(4), 783. https://doi.org/10.2307/2408678

Freundlieb, S., & Boos, W. (1986). Alpha-amylase of *Escherichia coli*, mapping and cloning of the structural gene, *malS*, and identification of its product as a periplasmic protein. *Journal of Biological Chemistry, 261*(6), 2946–2953. https://doi.org/10.1016/S0021-9258(17)35878-7

Gopinath, S. C. B., Anbu, P., Arshad, M. K. M., Lakshmipriya, T., Voon, C. H., Hashim, U., & Chinni, S. V. (2017). Biotechnological processes in microbial amylase production. *BioMed Research International, 2017*, Article ID 1272193. https://doi.org/10.1155/2017/1272193

Gupta, R., Gigras, P., Mohapatra, H., Goswami, V. K., & Chauhan, B. (2003). Microbial α-amylases: a biotechnological perspective. *Process Biochem, 38*(11), 1599–1616. https://doi.org/10.1016/S0032-9592(03)00053-0

Gurung, N., Ray, S., Bose, S., & Rai, V. (2013). A broader view: Microbial enzymes and their relevance in industries, medicine, and beyond. *BioMed Research International, 2013*, Article ID 329121. https://doi.org/10.1155/2013/329121

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series, 41*, 95–98. https://doi.org/10.14601/Phytopathol_Mediterr-14998u1.29

Hudson, C. M., Bent, Z. W., Meagher, R. J., & Williams, K. P. (2014). Resistance determinants and mobile genetic elements of an NDM-1-encoding *Klebsiella pneumoniae* strain. *PLoS ONE, 9*(6), e99209. https://doi.org/10.1371/journal.pone.0099209

Janeček, Š. (2002). How many conserved sequence regions are there in the α-amylase family? *Biologia, Bratislava, 57*(11), 29–41. http://imb.savba.sk/~janecek/Papers/PDF/Biologia_02_Suppl_11.pdf

Janeček, Š., & Gabriško, M. (2016). Remarkable evolutionary relatedness among the enzymes and proteins from the α-amylase family. *Cellular and Molecular Life Sciences, 73*(14), 2707–2725. https://doi.org/10.1007/s00018-016-2246-6

Janeček, Š., Mareček, F., MacGregor, E. A., & Svensson, B. (2019). Starch-binding domains as CBM families–history, occurrence, structure, function and evolution. *Biotechnology Advances, 37*(8), 107451. https://doi.org/10.1016/j.biotechadv.2019.107451

Janeček, Š., & Svensson, B. (2022). How many α-amylase GH families are there in the CAZy database? *Amylase, 6*(1), 1–10. https://doi.org/10.1515/amylase-2022-0001

Janeček, Š., Svensson, B., & MacGregor, E. A. (2014). α-Amylase: an enzyme specificity found in various families of glycoside hydrolases. *Cellular and Molecular Life Sciences, 71*(7), 1149–1170. https://doi.org/10.1007/s00018-013-1388-z

Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N., & Sternberg, M. J. E. (2015). The Phyre2 web portal for protein modeling, prediction and analysis. *Nature Protocols, 10*(6), 845–858. https://doi.org/10.1038/nprot.2015.053

Laemmli. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680–685. https://doi.org/10.1038/227680a0

Läufer, A. (2017). Starch biorefinery enzymes. *Advances in Biochemical Engineering/Biotechnology, 166*, 137–152. https://doi.org/10.1007/10_2016_60

Lin, L., Wei, C., Chen, M., Wang, H., Li, Y., Li, Y., Yang, L., & An, Q. (2015). Complete genome sequence of endophytic nitrogen-fixing *Klebsiella variicola* strain DX120E. *Standards in Genomic Sciences, 10*(1), 22. https://doi.org/10.1186/s40793-015-0004-2

Lombard, V., Golaconda Ramulu, H., Drula, E., Coutinho, P. M., & Henrissat, B. (2014). The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Research, 42*(D1), D490–D495. https://doi.org/10.1093/nar/gkt1178

McNicholas, S., Potterton, E., Wilson, K. S., & Noble, M. E. M. (2011). Presenting your structures: the CCP4mg molecular-graphics software. *Acta Crystallographica Section D Biological Crystallography, 67*(4), 386–394. https://doi.org/10.1107/S0907444911007281

Mehta, D., & Satyanarayana, T. (2016). Bacterial and archaeal α-amylases: Diversity and amelioration of the desirable characteristics for industrial applications. *Frontiers in Microbiology, 7*, 1129. https://doi.org/10.3389/fmicb.2016.01129

Momma, M. (2000). Cloning and sequencing of the maltohexaose-producing amylase gene of *Klebsiella pneumoniae*. *Bioscience, Biotechnology, and Biochemistry, 64*(2), 428–431. https://doi.org/10.1271/bbb.64.428

Movahedpour, A., Asadi, M., Khatami, S. H., Taheri-Anganeh, M., Adelipour, M., Shabaninejad, Z., Ahmadi, N., Irajie, C., & Mousavi, P. (2022). A brief overview on the application and sources of α-amylase and expression hosts properties in order to production of recombinant α-amylase. *Biotechnology and Applied Biochemistry, 69*(2), 650–659. https://doi.org/10.1002/bab.2140

Nakajima, R., Imanaka, T., & Aiba, S. (1986). Comparison of amino acid sequences of eleven different α-amylases. *Applied Microbiology and Biotechnology, 23*(5), 355–360. https://doi.org/10.1007/BF00257032

Ohtaki, A., Iguchi, A., Mizuno, M., Tonozuka, T., Sakano, Y., & Kamitori, S. (2003). Mutual conversion of substrate specificities of *Thermoactinomyces vulgaris* R-47 α-amylases TVAI and TVAII by site-directed mutagenesis. *Carbohydrate Research, 338*(15), 1553–1558. https://doi.org/10.1016/S0008-6215(03)00219-2

Palmer, T. N., Ryman, B. E., & Whelan, W. J. (1976). The action pattern of amylomaltase from *Escherichia coli*. *European Journal of Biochemistry, 69*(1), 105–115. https://doi.org/10.1111/j.1432-1033.1976.tb10863.x

Pandey, A., Nigam, P., Soccol, C. R., Soccol, V. T., Singh, D., & Mohan, R. (2000). Advances in microbial amylases. *Biotechnology and Applied Biochemistry, 31*(2), 135. https://doi.org/10.1042/BA19990073

Paul, J. S., Gupta, N., Beliya, E., Tiwari, S., & Jadhav, S. K. (2021). Aspects and recent trends in microbial α-amylase: A review. *Applied Biochemistry and Biotechnology, 193*(8), 2649–2698. https://doi.org/10.1007/s12010-021-03546-4

Puyet, A. (1993). Expression of bacterial genes involved in maltose metabolism. *World Journal of Microbiology and Biotechnology, 9*(4), 455–460. https://doi.org/10.1007/BF00328033

Rodríguez-Medina, N., Barrios-Camacho, H., Duran-Bedolla, J., & Garza-Ramos, U. (2019). *Klebsiella variicola*: an emerging pathogen in humans. *Emerging Microbes & Infections, 8*(1), 973–988. https://doi.org/10.1080/22221751.2019.1634981

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution, 4*(4), 406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Sambrook, J., & Russell, D. (2001). *Molecular cloning: a laboratory manual*, third edition. Spring Harbor Laboratory Press.

Santos, F. C. dos, & Barbosa-Tessmann, I. P. (2019). Recombinant expression, purification, and characterization of a cyclodextrinase from *Massilia timonae*. *Protein Expression and Purification, 154*, 74–84. https://doi.org/10.1016/j.pep.2018.08.013

Santos, F. C. dos, de Castro, F. F., Apolonio, T. M., Yoshida, L., Martim, D. B., Tessmann, D. J., & Barbosa-Tessmann, I. P. (2019). Isolation, diversity, and biotechnological potential of maize (*Zea mays* L.) grains bacteria. *Genetics and Molecular Research, 18*(3), gmr18320. https://doi.org/10.4238/gmr18320

Schwartz, M. (1987). The maltose regulon. In F. C. Neidhardt (Ed.), *Escherichia coli and Salmonella typhimurium: cellular and molecular biology* (Vol. 2, pp. 1482–1502). American Society of Microbiology.

Sindhu, R., Binod, P., Madhavan, A., Beevi, U. S., Mathew, A. K., Abraham, A., Pandey, A., & Kumar, V. (2017). Molecular improvements in microbial α-amylases for enhanced stability and catalytic efficiency. *Bioresource Technology, 245*, 1740–1748. https://doi.org/10.1016/j.biortech.2017.04.098

Singh, R., Kumar, M., Mittal, A., & Mehta, P. K. (2016). Microbial enzymes: Industrial progress in 21st century. *3 Biotech, 6*(2), 174. https://doi.org/10.1007/s13205-016-0485-8

Sivaramakrishnan, S., Gangadharan, D., Nampoothiri, K. M., Soccol, C. R., & Pandey, A. (2006). α-Amylases from microbial sources – An overview on recent developments. *Food Technology and Biotechnology, 44*(2), 173–184. https://hrcak.srce.hr/file/161876

Stam, M. R., Danchin, E. G. J., Rancurel, C., Coutinho, P. M., & Henrissat, B. (2006). Dividing the large glycoside hydrolase family 13 into subfamilies: towards improved functional annotations of α-amylase-related proteins. *Protein Engineering Design and Selection, 19*(12), 555–562. https://doi.org/10.1093/protein/gzl044

Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: Molecular evolutionary genetics analysis version 11. *Molecular Biology and Evolution, 38*(7), 3022–3027. https://doi.org/10.1093/molbev/msab120

Tapio, S., Yeh, F., Shuman, H. A., & Boos, W. (1991). The *malZ* gene of *Escherichia coli*, a member of the maltose regulon, encodes a maltodextrin glucosidase. *Journal of Biological Chemistry, 266*(29), 19450–19458. https://doi.org/10.1016/S0021-9258(18)55017-1

Trinder, P. (1969). Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen. *Journal of Clinical Pathology, 22*(2), 158–161. https://doi.org/10.1136/jcp.22.2.158

Van der Maarel, M. J. E. C., Van der Veen, B., Uitdehaag, J. C. M., Leemhuis, H., & Dijkhuizen, L. (2002). Properties and applications of starch-converting enzymes of the α-amylase family. *Journal of Biotechnology, 94*(2), 137–155. https://doi.org/10.1016/S0168-1656(01)00407-2

Wang, X., Kan, G., Ren, X., Yu, G., Shi, C., Xie, Q., Wen, H., & Betenbaugh, M. (2018). Molecular cloning and characterization of a novel α-amylase from Antarctic Sea ice bacterium *Pseudoalteromonas* sp. M175 and its primary application in detergent. *BioMed Research International, 2018*, Article ID 3258383. https://doi.org/10.1155/2018/3258383

Zuckerkandl, E., & Pauling, L. (1965). Evolutionary divergence and convergence in proteins. In H. J. Bryson, V., & Vogel (Eds.), *Evolving Genes and Proteins* (pp. 97–166). Elsevier. https://doi.org/10.1016/B978-1-4832-2734-4.50017-6