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EXPRESSÃO RECOMBINANTE, PURIFICAÇÃO E CARACTERIZAÇÃO DE UMA α-AMILASE de *Massilia timonae*

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BRUNA YUKI TAGOMORI

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Dissertação apresentada ao Programa de Pós-graduação em Ciências Biológicas (Área de Concentração - Biologia Celular e Molecular) da Universidade Estadual de Maringá, para obtenção do grau de Mestre em Ciências Biológicas.

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“C'est le temps que tu as perdu pour ta
rose qui fait ta rose si importante.”

Antoine de Saint-Exupéry

APRESENTAÇÃO

Esta dissertação é composta de um artigo científico que descreve a clonagem de um gene que codifica uma α-amilase da bactéria *Massilia timonae*. Este gene foi expresso em *Eschericha coli* e a enzima recombinante obtida foi purificada e caracterizada. Em consonância com as regras do Programa de Pós-graduação em Ciências Biológicas, esta dissertação foi redigida como um artigo científico que será enviado para análise quanto à publicação para a revista 3 Biotech.

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SUMÁRIO

LISTA DE TABELAS

LISTA DE FIGURAS

LISTA DE ABREVIAÇÕES

°C – Grau Celsius

uL – Microlitro(s)

µmol – Micromol(s)

AN – GenBank Número de Acesso

bp – Base pairs.

CAI – Codon Adaptation Index

DNA – Ácido desoxirribonucleico

DNS – Ácido dinitrosalicílico

dNTP – trifosfato de desóxirribonucleosídeo

EDTA – Ácido etilenodiamino tetra-acético

g – Grama(s)

g/L – Grama(s) por litro

h – Hora(s)

kDa – KiloDalton(s)

K_M – Constante de Michaelis-Menten

L – Litro(s)

M – Molar

M.M. – Marcador Molecular

m/v – Massa por volume

mg – Miligrama(s)

mg/L – Miligrama(s) por litro

min – Minuto(s)

mL – Mililitro(s)

mM – Milimolar.

ng – Nanograma(s)

nm – Nanômetro(s)

OD – Densidade ótica

PAGE – Eletroforese em gel de poliacrilamida

PCR – Reação em cadeia da polimerase

pH – Potencial de hidrogênio

PMSF – Fluoreto de fenilmetanosulfonila

pmol - picomol

rpm – Rotações por minuto

SDS – Dodecil sulfato de sódio

SDS-PAGE – Dodecil sulfato de sódio – Eletroforese em gel de poliacrilamida

U – Unidade Internacional

UV – Ultravioleta

v/v – Volume/volume

V_{max}– Velocidade Máxima

w/v – Weight per volume

RESUMO GERAL

Introdução: A α-amilase é uma hidrolase com grande demanda no mercado industrial de enzimas. Esta enzima é uma endoamilase, que libera dextrina e oligossacarídeos de vários comprimentos. As α-amilases possuem três domínios, chamados A, B e C, no qual o primeiro possui uma estrutura tridimensional conservada $(\beta/\alpha)_8$ e contém os resíduos catalíticos chave altamente conservados. O aumento crescente da demanda industrial de α-amilases requer enzimas com diferentes características. O estudo com enzimas recombinantes é particularmente interessante, pois pode contribuir na descoberta de novas e melhores enzimas. Anteriormente em nosso laboratório, uma cepa de *Massilia timonae* apresentando atividade amilolítica foi isolada de grãos de milho apresentando sinais de podridão. **Objetivos:** Clonar o gene que codifica uma α-amilase de *M. timonae*, expressar a proteína em *Escherichia coli*, purificar e caracterizar a enzima recombinante expressa. **Material e métodos:** Com base na sequência do gene putativo de α-amilase encontrado no genoma sequenciado e anotado de *M. timonae* e alinhamento com outras sequências similares, iniciadores foram desenhados para amplificar este gene por PCR a partir do DNA genômico de *M. timonae.* O gene amplificado foi primeiramente inserido no vetor pCR2.1®, o qual foi sequenciado e a sequência de aminoácidos foi obtida utilizando-se ferramentas de tradução. Estudos filogenéticos e modelagem por homologia foram conduzidos. O gene foi posteriormente subclonado no vetor de expressão pTrcHis2B. O vetor de expressão construído foi utilizado na transformação em *E. coli* Rosetta™ (DE3). Após o crescimento bacteriano em meio Luria Bertani, as células foram coletadas por centrifugação, ressuspensas em tampão de lise e sonicadas. A amostra obtida foi centrifugada e a enzima foi purificada a partir do sobrenadante usando cromatografia de afinidade. A atividade da enzima recombinante purificada foi medida usando o método de descoloração do complexo amido-iodo. Para avaliar a pureza da enzima e a massa molecular, uma análise eletroforética foi conduzida. Uma avaliação da atividade usando zimograma também foi realizada. Os parâmetros cinéticos K_M e $V_{máx}$ também foram encontrados. A especificidade do substrato foi avaliada utilizando amido, amilopectina, maltodextrina, β-ciclodextrina e 4-nitrofenil α-D-glicopiranosídeo. Os produtos de hidrólise do amido foram avaliados por cromatografia descendente em papel e coloração com o método de nitrato de prata amoniacal. Os parâmetros pH e temperatura ótimos, estabilidade térmica e estabilidade em diferentes pHs foram avaliados. **Resultados e discussão:** A proteína expressa tinha uma massa molecular de 46,7 kDa. A estrutura modelada apresentou um monômero composto de três domínios, no qual o domínio A possui uma estrutura em barril $(\beta/\alpha)_8$ com os resíduos catalíticos conservados. A estrutura possui um pequeno domínio B irregular, com um pequeno domínio C composto de cinco fitas β antiparalelas. A análise filogenética mostrou que a α-amilase de *M. timonae* é relacionada com sequências de α-amilases de bactérias e plantas. Uma maior especificidade foi encontrada para o amido, seguido da amilopectina e maltodextrina. Os valores encontrados para K_M e $V_{máx}$ foram 0,79 mg/mL e 0,04 mg/mL.min. Os açúcares glicose e maltose foram obtidos da hidrólise do amido pela enzima. O íon cálcio é requerido para a atividade enzimática, enquanto que EDTA, molibdênio, cobalto e mercúrio mostraram fortes efeitos inibitórios. A enzima foi quase totalmente ativa na presença de SDS. A enzima exibiu temperatura e pH ótimos de 60 °C e 6,0, respectivamente. A T_m foi de 73,65 °C. **Conclusão:** Este trabalho reporta um sistema alternativo para a expressão recombinante de α-amilase em *E. coli* e descreve uma nova α-amilase bacteriana termofílica, termoestável e resistente a detergente aniônico, com uma alta identidade na sequência de aminoácidos com α-amilases de outras bactérias e de plantas.

Palavras-chave: Alfa-amilase, Hidrolase, *Massilia timonae*, Expressão recombinante.

GENERAL ABSTRACT

Introduction: The α-amylase is one hydrolase with great demand in the enzyme's industrial market. This enzyme is an endoamylase, which releases dextrin and oligosaccharides of various lengths. In general, α-amylases have three domains, named A, B, and C, where the first has a conserved $(\beta/\alpha)_8$ three-dimensional structure and contains the highly conserved critical catalytic residues. The crescent increase in the industrial demand for α-amylases requires enzymes with different characteristics. The study with recombinant enzymes is particularly interesting because it can contribute to the discovery of new and improved enzymes. Previously in our laboratory, a strain of *Massilia timonae* showing amylolytic activity was isolated from maize grains presenting rotten symptoms. **Objectives:** To clone the gene encoding for an α-amylase from *M. timonae*, express the recombinant protein in *Escherichia coli*, purify, and characterize the expressed enzyme. **Material and methods:** Based on the putative α-amylase gene sequence found in the sequenced and annotated genome of *M. timonae*, and alignment with other similar sequences, primers were designed to amplify this gene by PCR from the genomic DNA of *M. timonae*. The gene amplified was first inserted into the pCR2.1® vector, which was sequenced, and the amino acid sequence was obtained using translation tools. Phylogenetic studies and modeling by homology were conducted. The gene was further subcloned into the pTrcHis2B expression vector. The constructed expression vector was used in the transformation into the *E. coli* Rosetta™ (DE3). After bacterial growth in Luria Bertani medium, the cells were harvested by centrifugation, resuspended in a lysis buffer, and sonicated. The sample obtained was centrifuged, and the enzyme was purified from the supernatant using affinity chromatography. The purified recombinant enzyme activity was measured using the starch-iodine complex distaining method. An electrophoretic analysis was performed to evaluate enzyme purity and molecular mass. An activity evaluation using a zymogram was also performed. The substrate specificity was evaluated using starch, amylopectin, maltodextrin, β-cyclodextrin, and 4-nitrophenyl α-D-glucopyranoside. The kinetics parameters K_M and V_{max} were also found. The starch hydrolysis products were evaluated by descending paper chromatography and staining with the ammoniacal silver nitrate method. The parameters optima pH and temperature, thermal stability, and stability in different pHs were evaluated. **Results and discussion:** The expressed protein had a molecular mass of 46.7 kDa. The modeled structure showed to be a monomer composed of three domains, in which domain A had the $(\beta/\alpha)_8$-barrel structure with the conserved catalytic residues. The structure had a small irregular domain B, and a small domain C composed of five antiparallel β-strands. The phylogenetic analysis showed that *M. timonae* α-amylase is related to other bacterial and plant sequences. The K_M and V_{max} values were 0.79 mg/mL and 0.04 mg/mL.min. Higher specificity was found towards starch, followed by amylopectin, and maltodextrin. Glucose and maltose were obtained from starch hydrolysis by the enzyme. The calcium ion is required for the enzyme activity, while EDTA, molybdenum, cobalt, and mercury showed strong inhibitory effects. The enzyme was almost entirely active in SDS presence. The enzyme exhibited optimal temperature and pH of 60 °C and 6.0, respectively. The T_m was of 73.65 °C. **Conclusion:** This work reported an alternative *E. coli* system for α-amylase recombinant expression and described a thermophilic, thermostable, and anionic detergent-resistant novel bacterial α-amylase, with a high identity amino acid sequence to α-amylases from other bacteria as well as with plants.

Keywords: Alpha-amylase, Hydrolase, *Massilia timonae*, Recombinant expression.

RECOMBINANT EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF AN α-AMYLASE FROM *Massilia timonae*

Tagomori, B.Y.,[1] Barbosa-Tessmann, I.P.[2]

ABSTRACT

This work reports an α-amylase characterization from the bacteria *Massilia timonae*. A gene encoding this protein was expressed from the pTRCHis2B plasmid in *Escherichia coli* Rosetta™ (DE3). The purified protein had 46.7 kDa, and its modeled structure showed a monomer composed of three domains. The domain A had the characteristic $(\beta/\alpha)_8$ barrel structure and contained the active site with highly conserved catalytic amino acid residues. The B domain was small, and the domain C was similar to those found in the barley α-amylase. Phylogenetic analysis demonstrated a high sequence identity of the studied protein with other bacterial as well as plant α-amylases. The purified enzyme presented high starch specificity. The K_M for starch and V_{max} values were 0.79 mg/mL and 0.04 mg/mL.min, respectively. The calcium ion showed to be essential for the purified enzyme's activity, while EDTA, molybdenum, cobalt, and mercury were strong inhibitors. The enzyme was almost fully active in SDS presence. The enzyme's optimal pH and temperature were 6.0 and 60 °C, respectively, and its denaturation T_m was 73.65 °C. In conclusion, this work reported an alternative *E. coli* system for α-amylase recombinant expression and described a thermophilic, thermostable, and anionic detergent-resistant novel bacterial α-amylase, with a high amino acid sequence identity with bacteria and plants α-amylases.

Keywords: Alpha-amylase, Hydrolase, *Massilia timonae*, Recombinant expression.

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1 INTRODUCTION

The global market for industrial enzymes is expected to reach nearly US$6.2 billion by 2020 (Singh et al., 2016). Amylases represent 25% of this enzyme market (Gurung et al., 2013; Singh et al., 2016). The industrial starch enzymatic hydrolysis is environmentally benign and eco-friendly because it produces little residues (Tomasik and Horton, 2012; Singh et al., 2016; Läufer, 2017).

The primary application is in the starch hydrolysis and detergent industry, which require enzymes with activity at extreme conditions of pH and temperature (Prakash and Jaiswal, 2010; Gurung et al., 2013; Niyonzima and More, 2014). Amylases are also widely used in the food industry, in baking, brewing, starch liquefaction, and production of high-glucose and -fructose syrups (Raveendran et al., 2018). Besides, there are applications of amylases in pharmaceutical, textile, paper, and analytical industries (Gupta et al., 2003; Souza and Magalhães, 2010; Gurung et al., 2013; Raveendran et al., 2018). Another application of amylases is in biofuel production, to obtain fermentable sugars from starch (Läufer, 2017). The increasing demand in various industries requires enzymes with suitable characteristics for each application, and thus, the search from different sources and the microbial amylases improvement are of great value (Mehta and Satyanarayana, 2016; Gopinath et al., 2017; Sindhu et al., 2017; Chapman et al., 2018).

There are a few types of amylases classified according to their action pattern on the starch (Sindhu et al., 2017). β-amylases and glucoamylases are exoamylases. Filamentous fungi constitute the primary source of glucoamylases, while β-amylases are generally of plant origin (Pandey et al., 2000). α-Amylases are endo-acting enzymes that hydrolyze internal α-1,4-glycosidic linkages of glucose polymers, such as starch (Gupta et al., 2003; van der Maarel et al., 2002). This enzyme's action releases oligosaccharides of various lengths and

dextrins (El-Fallal et al., 2012; Gupta et al., 2003; van der Maarel et al., 2002; Sindhu et al., 2017).

The industrial amylases are produced mainly by microorganisms (Sun et al., 2010). Bacteria of genus *Bacillus* and fungi of genera *Aspergillus*, *Penicillium*, and *Rhizopus* are the primary sources of amylases (Sun et al., 2010; Gurung et al., 2013). Microbial enzymes are preferred due to their stability and higher catalytic activity (Gurung et al., 2013). Besides, microorganisms grow abundantly on low-cost substrates, and their production is not affected by seasonal fluctuations (Gurung et al., 2013; Singh et al., 2016). α-Amylases may be derived from bacteria, archaea, and eukaryote (Gupta et al., 2003; Mehta and Satyanarayana, 2016).

α-Amylases are classified in the glycosyl hydrolase family 13 (GH13), according to the amino acid sequence, and their structure is typically organized in three domains, named A, B, and C (El-Fallal et al., 2012; Prakash and Jaiswal, 2010). The *N*-terminal A domain's structure is highly conserved and composed of a barrel of eight β-strands encircled by eight α-helices. This domain has the three highly conserved catalytic residues in its *C*-terminal β-strands. The B domain, of variable length, protrudes between the third β-strand and the third α-helix of the A domain and has the substrate and calcium-binding sites. The active site is formed between these first two domains (van der Maarel et al., 2002; El-Fallal et al., 2012). The C domain is organized in antiparallel β-strands (El-Fallal et al., 2012).

In a previous study in our laboratory (Santos et al., 2019), a strain of *Massilia timonae*, isolated from maize grains with rotten symptoms presented amylolytic activity in a solid medium. *M. timonae* is a Gram-negative, aerobic, rod-shaped, non-fermenting, mesophilic bacterium from the phylum Proteobacteria and order Burkholderiales (La Scola et al., 1998; Lindquist et al., 2003). The *Massilia* genus species were first isolated from clinical samples and subsequently isolated from many environmental samples (La Scola, et al., 1998; Lindquist et al., 2003; Nagy et al., 2005; Gallego et al., 2006; Fahlgren et al., 2011). Species

of the *Massilia* genus are reported to be able to grow using starch as a carbon source and to produce polyhydroxyalkanoates, suggesting their ability to produce amylases (Cerrone et al., 2011). A search in the *M. timonae* CCUG 45783 sequenced genome was conducted, and five amylase genes were found. A gene coding for a cyclodextrinase was already cloned, and the recombinant enzyme was characterized (Santos and Barbosa-Tessmann, 2019). In this study, an α-amylase gene was cloned from this bacterium, expressed in *Escherichia coli*, and the recombinant enzyme was purified and characterized.

2 MATERIALS AND METHODS

2.1 Bacterial strains

E. coli TOP10™ was used for molecular cloning, while *E. coli* Rosetta™ (DE3) and *E. coli* Bl21 Star™ (DE3) were used as expression hosts. The *M. timonae* CTI-57 strain was previously obtained from the screening of hydrolytic enzymes producing bacteria isolated from maize grains presenting rotten symptoms collected from the soil as harvest leftovers, during August 2015, at the Irrigation Technical Center (CTI), at the main campus of the Universidade Estadual de Maringá, Paraná, Brazil (Santos et al., 2019). This isolate was being maintained in Nutrient slant agar culture at 4 °C and in Nutrient medium (6 g/L meat peptone; 0.5 g/L $MgSO_4$; 0.5 g/L KCl; pH 7.0) with 50% glycerol at −20 °C. However, this strain was of difficult maintenance, and it died during this work execution.

2.2 Genomic DNA extraction

Fresh culture of bacterial colony grown at 37 °C on nutrient agar (6 g/L meat peptone; 0.5 g/L $MgSO_4$; 0.5 g/L KCl; pH 7.0; 20 g/L agar) was used to inoculate 5 mL of liquid

Luria-Bertani (LB) medium (10 g/L tryptone; 5 g/L yeast extract; 10 g/L NaCl) and incubated for 24 h at 37 °C under agitation of 100 rpm. 1.5 mL of this culture was transferred, and the cells were harvested by centrifugation (12,000*g*, 1 min). The pellet was washed two times with 500 µL of TE buffer (10 mM Tris; 1 mM Na_2EDTA; pH 8.0) and finally resuspended in 200 µL of TE buffer, boiled for 10 min and centrifuged for 1 min (10,000*g*). The supernatant of genomic DNA obtained was stored at −20 °C.

2.3 Cloning of the α-amylase gene and construction of the expression plasmids

Previously, in a study conducted in our laboratory (Santos and Barbosa-Tessmann, 2019), five amylolytic enzymes were identified in the sequenced and annotated genome of *M. timonae* CCUG 45783, a species from the Human Microbiome Project (GenBank AN: EKU82996.1, EKU83004.1, EKU82989.1, EKU82293.1, EKU83291.1). Primer pairs were designed targeted for the five genes, and if a signal peptide is present, the mature forms of the proteins, i.e., without the signal peptide, were considered. When these primers were used in PCR reactions, one pair did amplify a cyclodextrinase gene (GenBank EKU82989.1), which was cloned and expressed in *E. coli* (Santos and Barbosa-Tessmann, 2019). In this present work, several homologous proteins of an α-amylase (GenBank EKU83291.1) from *M. timonae* genomes were retrieved from GenBank, aligned, and primers were designed targeting the entire coding region, without the initial region encoding the signal peptide. To later subclone the gene into the pTrcHis2B expression plasmid, the following primer pair was designed. The forward primer (5´-TTGGATCCAGCSACCCCGCCGGCCCAGTCC) contained a restriction site for the enzyme *BamH*I (underlined) and had no initial ATG, as the expression vector pTrcHis2B has it. An extra A (double underlined) was added to keep up the correct reading frame. The reverse primer (5´-TTTCTAGACGTTTGATCCAGACCGCG TAAT) contained a restriction site for the enzyme *Xba*I (underlined). The stop codon was

deleted, while the bases CG (double underlined) were added to keep up the reading frame so the recombinant protein could have poly-histidine Tag for further purification. To later subclone the gene to the pET21a expression plasmid, the following primer pair was designed. The forward primer (5´-TTCATATGGCSACCCCGCCGGCCCAGTCC) had a restriction site for the enzyme *Nde*I (underlined). The *Nde*I restriction sequence gave the initial ATG. The reverse primer (5´-TTAAGCTTTTTGATCCAGACCGCGTAAT) had a restriction site for the enzyme *Hind*III (underlined). The stop codon was deleted so that the recombinant protein could have poly-histidine Tag for further purification.

The PCR reaction was performed with 1 U of Accu *Taq*™ DNA Polymerase (Sigma Aldrich, USA), enzyme buffer [50 mM Tris-HCl, 15 mM ammonium sulfate (pH 9.3, adjusted with NH_4OH), 2.5 mM $MgCl_2$, 1% Tween 20], 0.2 mM of each dNTP, 25 pmol of each primer, and 2 µL of the obtained genomic DNA supernatant, in a final volume of 25 µL. The used conditions consisted of an initial incubation of 10 min at 94 °C, followed by 25 cycles of 1.5 min at 94 °C, 1.5 min at 60 °C, and 2 min at 68 °C. After that, the reactions were maintained for 10 min at 68 °C for a final extension of products.

The PCR obtained products were cloned into the pCR2.1® plasmid from the TOPO TA Cloning kit (Thermo Fisher Scientific, USA) or pGEM®-T plasmid from the pGEM®-T Vector Systems (Promega, USA), after the user guide instructions. The recombinant plasmids were transformed in *E. coli* TOP10™ (Chung et al., 1989) and recovered by alkaline lysis (Sambrook and Russel, 2001). Then, the truncated genes were transferred from the pCR2.1® or pGEM®-T vectors to the pTrcHis2B and pET21a(+) expression vectors, using digestion and ligation technology (Sambrook and Russel, 2001). The recombinant plasmids were transformed in *E. coli* TOP10™ strain (Chung et al., 1989), recovered by alkaline lysis, and analyzed by restriction digestion (Sambrook and Russel, 2001).

The cloned gene in the pCR2.1® vector containing the *BamH*I and *Xba*I restriction sites in the 5´ and 3´, respectively, was sequenced at the CEGH at the University of São Paulo, using internal primers designed for the α-amylase gene from *M. timonae* CCUG 45783 and primers targeting the plasmid regions flanking the insert. The contig was generated using the BioEdit program (Hall, 1989).

2.4 *In silico* analysis

Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) was used for sequences alignment and to check the level of identity between our protein sequence and others found in GenBank and UniProt databases. A phylogenetic tree was constructed using the neighbor-joining method (Saitou, 1987) in the MEGA 7.0 program (Kumar et al., 2016). The confidence limits of the branching were assessed using Bootstrap analyses with 1000 heuristic replicates (Felsenstein, 1985). Values higher than 70% in this test were considered reliable phylogenetic grouping among proteins.

A structural model for the protein of *M. timonae* from our sequenced gene was obtained with the Modeller v9.16 program (Webb and Sali, 2016) using as a template the *Hordeum vulgare* (barley) α-amylase (pdb 1AMY) (Kadziola et al., 1994) and the *Pseudomonas stutzeri* maltotetraose-forming exo-amylase structure (pdb 2AMG) (Morishita et al., 1997). These templates were selected by a search using the GenTHREADER prediction method inside the PSIPRED server (http://bioinf.cs.ucl.ac.uk/psipred/) and a search in the PDB database. The best models among 250 generated in each run were selected based on the Modeller DOPE Score. The stereo-chemical quality of the obtained models was evaluated using Procheck, and the percentage of secondary structure was obtained using VADAR version 1.8 (http://vadar.wishartlab.com/). The DiANNA 1.1 webserver (http://clavius.bc.edu/~clotelab/DiANNA/) was used for disulfide bond prediction. The PISA

server (https://www.ebi.ac.uk/pdbe/pisa/) was used to verify macromolecular interfaces and probable multimeric structures. The modeled PDB structure was visualized and colored using the CCP4MG program. A search for structural domains was performed in the Conserved Protein Domain Database (CDD) from NCBI (https://www.ncbi.nlm.nih.gov) and Pfam from EMBL-EBI (https://pfam.xfam.org/).

The secretion signal peptide presence and the molecular weight and pI were obtained using the SignalP-5.0 Server (http://www.cbs.dtu.dk/services/SignalP/) and Compute pI/Mw tool from ExPASy (https://www.expasy.org/), respectively. A search in the Carbohydrate-Active enZYmes Database (CAZY; http://www.cazy.org/) was conducted to find the protein's family. The RNA folder Web Server program (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) was used to predict the mRNA 5´secondary structure with its minimal free energy and the Rare Codon Analyser program (https://www.biologicscorp.com/tools/RareCodonAnalyzer) was used to predict mRNA Codon Adaptation Index (CAI) value.

2.5 Expression analysis and purification of the recombinant α-amylase

For the expression analysis, *E. coli* Rosetta™ (DE3) was transformed using 50 ng of the pTrcHis2B-*amy1* or pET21a-*amy1* vectors, and *E. coli* Bl21 Star™ (DE3) was transformed using 50 ng of the pET21a-*amy1* vector, as described in Chung (1989). The obtained bacteria were transferred to 10 mL of LB medium containing 50 µg/mL ampicillin and incubated overnight at 37 °C with agitation (100 rpm). An aliquots of 100 µL (1% inoculum) of each culture was transferred to tubes containing 10 mL of LB medium with 50 µg/mL ampicillin. An aliquot of 100 µL of the *E. coli* Bl21 Star™ (DE3) transformed with the pET21a-*amy1* vector was also transferred to a tube containing 10 mL of the auto-induction ZYM-5052 medium (Studier, 2005) containing 50 µg/mL ampicillin and prepared

slightly modified as described in Santos and Barbos-Tessmann (2019). All tubes were and incubated at 37 °C with agitation (100 rpm). After 4 h of incubation, when the optical density (OD) reached values of 0.6-0.8 at 620 nm, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to the LB medium cultures. All cultures were then incubated at 20 °C, for 18 hours, under agitation (100 rpm). The cells were harvested by centrifugation (2,000*g*, for 10 min, at 4 °C). The obtained bacterial pellet was resuspended in 10 mL of lysis buffer (50 mM Tris pH 7.5, 50 mM NaCl, 2 mM $CaCl_2$, 1 mM PMSF) and the cells were disrupted through sonication (30 cycles of 10 s – 5 s on and 5 s off – with an amplitude of 30 %). The samples were centrifuged at 10,000*g* for 5 min. The enzyme expression was tested in the obtained supernatant using the enzyme assays and SDS-PAGE analysis.

For the enzyme purification, *E. coli* Rosetta™ (DE3) was transformed using 50 ng of the pTrcHis2B-*amy1*, as described in Chung (1989). The obtained bacteria were transferred to 10 mL of LB medium containing 50 µg/mL ampicillin and incubated overnight at 37 °C with agitation (100 rpm). An aliquot of 500 µL of the obtained culture was transferred to a 250 mL Erlenmeyer containing 50 mL of LB medium with 50 µg/mL ampicillin and incubated at 37 °C with agitation (100 rpm). IPTG was added to a final concentration of 1 mM when the OD reached 0.6-0.8 at 620 nm, and the culture was incubated at 20 °C, for 18 hours, under agitation (100 rpm). After cell collection and lysis as described above, the supernatant was applied in a HisTrap™ HP column (GE Healthcare Life Sciences, USA) loaded with Ni^{2+} and equilibrated with 50 mM Tris buffer, pH 7.5, containing 50 mM NaCl and 20 mM imidazole. After washing the column a few times with this equilibrium buffer, the recombinant protein was eluted with a 50 mM up to 150 mM imidazole gradient in the elution buffer (50 mM Tris buffer, pH 7.5, 50 mM NaCl). Fractions of 1.5 mL each were collected during the elution. Aliquots of 60 µL of each elution fraction were used for the enzyme assay by the starch-

iodine complex distaining method (Palanivelu, 2001). The fractions which contained the enzyme were pooled and dialyzed against 50 mM Tris buffer, pH 7.5. For storage of the sample, glycerol and $CaCl_2$ was added up to the 50% (v/v) and 2 mM, respectively, unless otherwise specified. The samples were kept at -20 °C until use.

2.6 Enzyme assays

The starch-iodine complex distaining method (Palanivelu, 2001) was used to measure the amylase activity. A sample of 500 µL was added to 1.5 mL of 0.1 % (w/v) soluble starch in 100 mM Tris buffer, pH 6.0. The reaction was incubated for 10 min at 60 °C and then stopped with 500 µL of 0.1 M HCl. For the remaining starch evaluation, 500 µL of iodine solution [KI 2.0% (w/v); I_2 0.2% (w/v)] was added, followed by absorbance measurement at 690 nm in a spectrophotometer. The enzyme unit was defined as the amount of enzyme that causes a 20% reduction in the absorbance per min and mL, under the assay conditions.

Alternatively, amylase activity was evaluated using the dinitrosalicylic acid (DNS) method for reducing sugars release (Miller, 1959). For this purpose, a sample of 500 µL was added to 1.5 mL of 0.5% (w/v) substrate in 50 mM Tris buffer, pH 6.0. The reaction was incubated for 10 min at 60 °C and then stopped with 500 µL of the DNS solution [1% (w/v) DNS; 0.4 M NaOH; 1 M potassium sodium tartrate]. The samples were boiled for 5 min and had 2.5 mL of deionized water added. The absorbance was measured in a spectrophotometer at 540 nm. A calibration curve was drawn using values obtained using different concentrations of glucose (0.5 to 4 µmol). The enzyme unit was defined as the amount of enzyme which releases 1 µmol of reducing sugar/min under the assay conditions.

2.7 SDS-PAGE and zymogram

Protein concentration was evaluated with the method of Bradford (1976) using albumin as standard. An aliquot (7.2 μg) of the purified protein was precipitated with trichloroacetic acid, and the obtained pellet was resuspended with 20 μL of the sample elution buffer [20% (v/v) glycerol, 1% (w/v) SDS, 0.03 mg/mL bromophenol blue, 125 mM Tris, pH 6.8, 0.72 M β-mercaptoethanol]. Aliquots (10 μg) of the cell homogenate and the supernatant obtained after centrifugation was treated as well. The samples were boiled for 10 min 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. The broad range molecular marker (BioRad, USA) was loaded together to estimate the protein size. Tris-glycine buffer system was used for running. The gel was stained using Coomassie brilliant blue R-250.

For the zymogram analysis, a 7.5% PAGE gel was run as described above, except that the samples were not TCA precipitated or boiled, and SDS and β-mercaptoethanol were not added to the gel or samples. Aliquots of 7 μL of the homogenate (6.5 μg) and purified enzyme (0.16 μg; 0.4 U) were applied into the gel. After the run, the gel was incubated in 50 mL of 50 mM Tris buffer, pH 6.0, containing 2 mM $CaCl_2$, and 0.5% soluble starch (w/v), for 1 hour at 4 ºC, allowing the substrate to impregnate on the gel, and for 2 h, at 40 °C, under 60 rpm of agitation. For the revealing, the gel was stained with the same iodine solution used in the enzyme assay and then photographed.

2.8 Substrate specificity and kinetic parameters

For evaluation of enzyme's substrate specificity, reactions were conducted using maltodextrin (dextrose equivalent 4.0–7.0, Sigma-Aldrich 419672, USA), amylopectin from maize (Sigma-Aldrich 10120, Germany), and β-cyclodextrin (Sigma-Aldrich W402826,

Germany), with the DNS method, conducted for 10 min, using 0.5% (w/v) of each substrate and 2.4 μg of the purified enzyme with 2 mM $CaCl_2$.

The enzyme action using the synthetic substrate 4-nitrophenyl α-D-glucopyranoside was also tested using the method described by Akinloye et al. (2012), with modifications as follows. An aliquot of 80 μL (1.9 μg) purified enzyme was added to 1920 μL of 100 mM Tris buffer, pH 6.0, containing 2 mM $CaCl_2$ and 20 mM of 4-nitrophenyl α-D-glucopyranoside (Sigma N7006, Switzerland). The reaction was incubated for 30 min at 60 °C. Afterward, an aliquot of 500 μL of the reaction was transferred to a clean tube, which had added 5 mL of 0.1 M sodium carbonate. The absorbance was then estimated in a spectrophotometer at 405 nm.

The initial velocities of enzyme activity were determined with the enzymatic assay (starch-iodine complex distaining method), with 2 mM $CaCl_2$, with a fixed amount of enzyme (0.48 μg; 4.4 U), and varying the starch concentration from 0.2 to 2.0 mg/mL. Residual starch concentration after enzyme action was calculated using a calibration curve using starch as a standard. The initial velocities were expressed as mg of starch consumed/min. K_M and V_{max} values were obtained by non-linear regression using GraphPad Prism® Version 6.01. The k_{cat} was calculated using the obtained V_{max} value in seconds divided by the moles of enzyme used in the reaction. The obtained constants were used to calculate k_{cat}/K_M.

2.9 Analysis of the enzyme reaction products

An aliquot of 250 μL (1.1 U) of the purified enzyme was added to 750 μL of 1.0% (w/v) starch in 100 mM Tris buffer containing 2 mM $CaCl_2$, pH 6.0, and incubated for 16 h at 40 °C under 65 rpm of agitation. Twenty μL of this hydrolysis solution was applied on a Whatman paper chromatogram n°. 1, along with 20 μL of the following standards at 100 mM: D(+)anhydrous glucose, D(+)maltose monohydrate, maltotetraose, and maltoheptaose. Twenty μL of the substrate solution of 1.0% (w/v) starch in 100 mM Tris buffer containing 2

mM $CaCl_2$ was also applied in the chromatographic paper. The descending chromatography was conducted having butanol/pyridine/acetic acid/water (12:6:1:4) as the mobile phase. The chromatogram was developed with the ammoniacal silver nitrate method (Trevelyan et al., 1950). For that, the chromatogram was first sprayed with a saturated solution of $AgNO_3$ in acetone (1.5 g of $AgNO_3$ dissolved in 0.5 mL of distilled water, added to 12 mL of 60% (v/v) acetone), then sprayed with a solution which consisted of 0.5 mL of 10 M NaOH added to 12 mL de ethanol. The chromatogram was then, exposed to water vapor until the clear visualization of dark spots, and dipped in a 10% (w/v) sodium thiosulfate bath. Finally, the chromatogram was dried and photographed.

2.10 Effect of potential activators and inhibitors, pH, and temperature in the purified enzyme

All the assays were carried out in 100 mM Tris buffer, pH 6.0, containing 2 mM $CaCl_2$, except in the $CaCl_2$ effect evaluation on the enzyme activity, where no calcium was added. The activity was examined as a percentage of the activity at standard conditions unless stated otherwise. Putative activators and inhibitors were investigated by adding metal ions or detergents in the enzyme assay up to a final concentration of 5 or 10 mM for solids and 0.5 or 1% (v/v) for liquids. All reactions were incubated at 60 °C for 10 min.

Optimum pH was determined by performing the enzyme assay in 100 mM Tris buffer adjusted to different pH values – 5.0, 6.0, 7.0, and 8.0. A 24 h pH stability time course was conducted by incubating the enzyme at these same pH values at room temperature. Aliquots were collected and used in the enzyme assay, under the standard conditions.

Optimum temperature determination was carried out by varying the enzyme assay temperature from 30 °C to 80 °C with increments of 10 °C. Thermal stability was evaluated by incubating the enzyme at these same temperatures, during 30 min, before the enzyme assay

in the standard conditions. The results were evaluated relative to the best value. The temperature in which the enzyme loses 50% of its activity (T_m) after incubation was calculated with the second-order polynomial regression equation obtained curve. A thermal stability 24 h time course was carried out at 60 and 70 °C.

2.11 Statistical analysis

When indicated, averages and standard deviations (SD) were submitted to ANOVA and compared using the Tukey test, in the SASM–Agri program (Canteri et al., 2001). Identical letters show no difference among averages (α = 0.01).

3 RESULTS AND DISCUSSION

3.1 Sequence analysis of the *M. timonae* AMY1 protein

A partial α-amylase gene from *M. timonae* CTI-57 genomic DNA, without the signal peptide and the stop codon, was amplified, cloned into the pCR2.1® plasmid, and sequenced. This gene, named *amy1*, was the first α-amylase gene cloned from *M. timonae*. The partial amplified gene had 1,203 bp, encoding for 401 amino acids protein. The obtained gene sequence was deposited in GenBank under the accession number MN990470.

The predicted molecular mass and isoelectric point of the protein encoded by the *amy1* gene, without the signal peptide, were 43.6 kDa and 6.25, respectively. The protein sequence alignment (Fig. 1) showed that the *M. timonae* CTI-57 AMY1 protein shared 90.52% of identity with the α-amylase from *M. timonae* CCUG 45783 (GenBank EKU83291.1), 43.12% with the barley α-amylase (*Hordeum vulgare*, GenBank P04063.3), and 29.06% with the exo-amylase (GenBank P13507.2) from *P. stutzeri* (Morishita et al., 1997). The three invariably

conserved catalytic residues in all α-amylases (Asp179, Glu204, and Asp281, given positions in our protein) were identified (Fig. 1). The α-amylases comprise a large variety of enzymes, known to have highly variable sequences among them. However, seven short sequences are conserved (Janeček et al., 2014; Janeček and Gabriško, 2016), which are present in the *M. timonae* CTI-57 AMY1 protein (Fig. 1).

A search for domains in the CDD and Pfam platforms indicated that the *M. timonae* CTI-57 AMY1 protein has an alpha-amylase catalytic domain (Family: Alpha-amylase, PF00128, and Clan CL0058 – Tim barrel glycosyl hydrolase superfamily) with an e-value of $1.3 \times e^{-09}$, and an Alpha-amylase *C*-terminal beta-sheet domain (Family: Alpha-amyl_C2, PF07821, and Clan CL0369 – Glycosyl hydrolase domain superfamily) with an e-value of $1.8 \times e^{-19}$. The catalytic and *C*-terminal beta-sheet domains correspond to the domains A and C of the α-amylase family, respectively. Those platforms could not recognize the B domain, which is smaller in α-amylases. Proteins containing the A domain are classified into the Glycosyl hydrolase family 13 (GH13), according to CAZY classification. The alignment and domain analysis results together confirm that the studied protein is an α-amylase.

The modeled structure for the *M. timonae* CTI-57 AMY1 α-amylase, using the *H. vulgare* (PDB 1AMY, Kadziola et al., 1994) as a template, is shown in Fig. 2. To produce a model using as template a bacterial protein instead of a plant protein, the *P. stutzeri* exo-amylase structure (PDB 2AMG) (Morishita et al., 1997) was used as a template, although it has less sequence identity with the *M. timonae* CTI-57 AMY1 α-amylase (not shown). The obtained model shows that the *M. timonae* CTI-57 AMY1 protein has three domains: an *N*-terminal catalytic domain A, composed of $(\beta/\alpha)_8$-barrel, an irregular domain B, protruding between the third β-strand and the third α-helix of the domain A, and a small *C*-terminal domain C, constituted of five antiparallel β-strands (Fig. 2A). An analysis of the obtained model in the PISA server evidenced a secondary structure composed of α-helix (31%), β-

conformation (22%), coil (46%), and turns (17%) (Fig. 2B). The active site is located between the first two domains, and the critical catalytic residues are shown in Fig. 2C. The active site has a predominantly negative environment in the cleft (Fig. 2D), probably due to the catalytic residues which are negatively charged. Near this site, there is a Ca^{2+} binding site, where three of the corresponding Ca^{2+} binding residues present in the model were also identified in the *M. timonae* CTI-57 AMY1 sequence (Fig. 2C). The binding of this Ca^{2+} ion stabilizes the loop of domain B and is conserved in α-amylases (Morishita et al., 1997). The other two putative Ca^{2+} binding sites present in the template sequence (Kadziola et al., 1994) were not identified in the *M. timonae* CTI-57 AMY1 amino acid sequence. The obtained structure showed the predicted disulfide bond between the residues Cys201-Cys232, which is shown in Fig. 2A. The PISA results pointed out that our protein is a monomer, as this form has the lowest, and therefore, more stable Gibbs free energy.

A phylogenetic tree was built based on the amino acids sequence similarities of proteins found in the NCBI database to verify the evolutionary relationship between the *M. timonae* and other bacterial α-amylases. A search for bacterial protein sequences with more than 50% of identity with the *M. timonae* AMY1 sequence resulted mostly in proteins of other *Massilia* species and a few proteins from other bacterial genera, such as from *Herbaspirilum*, *Empedobacter*, and *Pseudomonas* (Fig. 3). Two other prominent clades of bacterial amylases were also formed, one that grouped with the *M. timonae* CTI-57 AMY1 protein and another that was a bit more distant (Fig. 3). Plant (barley and rice) α-amylases, which share 40% of identity with the *M. timonae* CTI-57 protein, commercially available α-amylases sequences from species of the *Bacillus* genus, which share less than 30% of identity with the *M. timonae* CTI-57 AMY1 protein, as well as the *P. stutzeri* exo-amylase protein sequence were also included in the phylogenetic analysis. The plant proteins were closer to the *M. timonae* CTI-57 AMY1 protein than the bacterial proteins from *Bacillus* spp. or *P. stutzeri*. According to

Janeček and Gabriško (2016), there is a close relatedness between the plant, archaeal, and Flavobacteria α-amylases from the GH13 family. The bacterial analyzed proteins in our phylogenetic analysis belonged to the phyla Proteobacteria, Firmicutes, and Bacteroidetes. The *Empedobacter* α-amylase clustered together with the *M. timonae* CTI-57 AMY1 protein. The *Empedobacter* genus belongs to the family Flavobacteriaceae from the Bacterioidetes Phylum.

3.2 Recombinant enzyme expression and purification

There was no expression of the *M. timonae* CTI-57 AMY1 protein from the pET21a-*amy1* plasmid using *E. coli* Bl21 Star™ (DE3) or Rosetta™ (DE3) grown in the LB medium or ZYM-5052 medium. No insoluble protein was produced, as confirmed with an SDS-PAGE analysis of the homogenized and supernatant samples of the transformed and cultured bacteria lysates (not shown). The truncated *M. timonae* CTI-57 *amy1* mRNA has a codon adaptation index (CAI) value of 0.81, which is lower than the considered ideal value of 1.0, indicating that it could indicate a higher amount of rare codons. However, this may not be the only reason this gene was not expressed from the pET21a plasmid since its expression also did not occur in the Rosetta™ (DE3) strain, which has the tRNAs for the rare codons in *E. coli*.

There was a *M. timonae* CTI-57 AMY1 α-amylase expression from the pTrcHis2B plasmid using the *E. coli* Rosetta™ (DE3) grown in LB medium. The vector pTrcHis2B has a mini cistron, which provides a sufficient translational restart and, therefore, is useful for eukaryotic protein expression in *E. coli*. The better translation initiation provided by the pTrcHis2B plasmid combined with the rare codons tRNAs presence in Rosetta™ (DE3) *E. coli* may have been responsible for the improved α-amylase expression. Indeed, an analysis of the predicted structure of the *M. timonae* CTI-57 *amy1* mRNA 5´ region initial 255

nucleotides sequence (without the signal sequence) identified minimum free energy of -94.50 kcal/mol, which demands a ribosomal effort to translate it.

The recombinant protein yield from the pTrcHis2B, from in 50 mL LB medium, after column and dialysis, was 76.2 μg (1.52 μg/mL of culture medium) with a specific activity of 558.5 U/mg, under the starch-iodine complex distaining assay. The DNS method was not used for measuring the activity, because the reducing sugars production by the purified enzyme was deficient, probably for the enzyme's endo-activity. This yield is lower than the found one for the *B. subtilis* WB800 α-amylase of 3.35 mg in 50 mL of culture medium, after two-step purification in gel filtration and ion-exchange chromatography (Chen et al., 2015) and for the *Halothermothrix orenii* α-amylase of 0.9 mg in 50 mL of culture, after Ni-affinity chromatography (Mijts and Patel, 2002).

The expressed recombinant protein from the pTrcHis2B had three additional amino acids at the *N*-terminal end (Met-Asp-Pro) and 23 amino acids at the *C*-terminal end, including the His-tag (RLEQYLISEEDLQSAVDHHHHHH). The predicted expressed protein molecular mass was 46.8 kDa, and the isoelectric point was 5.98. The SDS-PAGE analysis confirmed the predicted molecular mass of approximately 47.0 kDa (Fig. 4A), as calculated graphically (Fig 4B). Although being variable, most amylases have a molecular mass around 40-60 kDa (Gupta et al., 2003; Sindhu et al., 2017). In agreement with our results, the recombinant *B. amyloliquefaciens* α-amylase is reported to have 58.4 kDa (Chen et al., 2015) and the *Laceyella* sp. recombinant α-amylase is reported to have 51.5 kDa (El-Sayed et al., 2019). In the zymogram analysis (Fig. 4C), a clear hydrolysis halo can be seen in the same position in the homogenized lane as well in the purified enzyme lane, indicating that the produced α-amylase was purified. An identical PAGE gel was performed and revealed with the silver staining method (Blum et al., 1987), but the *M. timonae* CTI-57 AMY1 protein could not be detected (Fig. 4D).

3.3 Substrate specificity and kinetic parameters

The *M. timonae* CTI-57 AMY1 showed the highest activity against starch, followed by amylopectin, and maltodextrin in the DNS test (Fig. 5A). There was no activity against β-cyclodextrin in the same test (Fig. 5A) and no activity against with 4-nitrophenyl α-D-glucopyranoside when the release of *p*-nitrophenol was monitored. In agreement, the *T. fonticaldi* strain HB23 α-amylase was also more active against potato starch and less active against amylopectin and maltodextrin (Allala et al., 2019). The substrate preference of α-amylases for starch has been reviewed before (Gupta et al., 2003). The purified recombinant *M. timonae* CTI-57 AMY1 has this same preference as it was more active against larger in size and α-1,4 linkages more abundant substrates, such as starch and amylopectin than against smaller and partially digested substrate, such as the maltodextrin. The lack of activity against the synthetic substrate 4-nitrophenyl α-D-glucopyranoside, which has a single glucose unit, also corroborates the fact that this enzyme prefers larger substrates, such as starch, and a glucose unit is probably too small for the substrate-binding cleft.

By measuring the initial velocities of starch consumption, it was possible to see that the enzyme followed the Michaelis-Menten kinetics (Fig. 5B). The purified recombinant *M. timonae* CTI-57 AMY1 α-amylase presented the following kinetics parameters: K_M for starch of 0.79 mg/mL, V_{max} of 0.04 mg/min, k_{cat} of 652,777,778 Mol/mL.s, and k_{cat}/K_M of 8.2×10^5 Ms^{-1}. The α-amylases K_M values for bacterial α-amylases depends on the microorganism and are reported to be in the range of 0.3 to 14 mg/mL (Gupta et al., 2003). The *M. timonae* CTI-57 AMY1 α-amylase K_M for starch is among the lower ones, and this indicates that this enzyme needs less substrate to reach half of the V_{max}. The k_{cat}/K_M of the purified enzyme indicates its high catalytic efficiency, which was also seen in the zymogram analysis, where a small amount of the enzyme was able to degrade the starch present in the gel (Fig. 4C). Truthfully, members of the α-amylase family are considered to belong to the most efficient

known enzymes (van der Maarel et al., 2002). V_{max} and k_{cat}/K_M values for bacterial α-amylases are rare and use different units, depending on the method used to measure the enzyme activity, which makes difficult the comparison among data.

3.4 Enzyme products

In the starch hydrolysis product analysis by the *M. timonae* CTI-57 AMY1 α-amylase, it is possible to see a small production of glucose (G1) and maltose (G2) after 16 h of hydrolysis at 40 °C (Fig. 6). This small production of glucose and maltose reinforces the endo-acting mechanism of the purified α-amylase. The enzyme probably also produces dextrin and longer oligosaccharides that cannot be seen in the reducing sugar staining of the chromatogram. According to our results, glucose and maltose were also detected on the paper chromatography of hydrolysis products of the *Micrococcus luteus* α-amylase (Ilori et al., 1997). Besides, several other bacterial α-amylases were also reported to produce glucose and maltose from starch hydrolysis (Gupta et al., 2003).

3.5 Effect of potential activators and inhibitors, pH, and temperature in the purified enzyme

The purified *M. timonae* CTI-57 AMY1 α-amylase was inactive in the Ca^{2+} ion absence (Table 1), which indicates that this cation is required for the enzyme's activity. The enzyme was practically fully active with 2 mM Ca^{2+}, and higher concentrations of Ca^{2+} did not cause a significant increase in the enzyme activity (Table 1). This calcium activation model suggests that there is a limit value in which all the enzymes molecules are already supplied with the required amount of Ca^{2+}, and further increases do not improve the enzyme activity. Most α-amylases are metalloenzymes and require Ca^{2+} for structural and catalytic activity (Gupta et al., 2003; Sivaramakrishnan et al., 2006). Calcium-independent α-amylases

have also been reported, such as the *Laceyella* sp. DS3 α-amylase, which was activated by Ca^{2+} but was also active in this cation absence (El-Sayed et al., 2019). Therefore, there is a calcium variation dependency among α-amylases from different microorganisms.

The chelating agent EDTA caused a potent inhibition of the purified *M. timonae* CTI-57 AMY1 enzyme (Table 1), and this corroborates the result obtained by the Ca^{2+} ion on the enzyme's activity. Although there are reports of amylases which are not affected by the EDTA presence, such as the *T. fonticaldi* strain HB23 α-amylase (Allala et al., 2019), most α-amylases are metalloenzymes, and addition of EDTA causes inhibition of its activity, as reported for the *B. subtilis* DR8806 recombinant α-amylase (Emtenani et al., 2015). The calcium ion removal seems to cause reversible denaturation of α-amylases, due to loss of tridimensional structure (Prakash and Jaiswal, 2010).

The anionic detergent SDS did not inhibit the enzyme (Table 1). Other amylases have been reported to be resistant to SDS inhibition, as the *Bacillus halodurans* and *Bacillus* sp. α-amylases (Murakami et al., 2007; Kim et al., 2012). The enzyme's activity with neutral detergents addition, such as Tween 80 and Triton X-100, could not be determined because those reagents were not compatible with the starch-iodine distaining method. The purified enzyme activity with addition of an anionic detergent indicates its possible use in the detergent industry, considering that anionic surfactants are the oldest and most widely used synthetic detergents and may be found in many personal-care products and household-care products (Kosswig, 2005).

The monovalent cations Na^{+} and Mg^{2+} presence in the reaction assay at 10 mM caused a slight inhibition of the enzyme (Table 1). The divalent cations Mn^{2+}, Co^{2+}, Hg^{2+}, and Mo^{2+}, were potent inhibitors of the purified enzyme. Many metal cations, especially heavy metal ions, are reported to inhibit α-amylases (Gupta et al., 2003). In agreement, the *Laceyella* sp. DS3 α-amylase was reported to be inactivated by $HgCl_2$ and $MgSO_4$, and the *Tepidimonas*

fonticaldi strain HB23 α-amylase was utterly inhibited by Hg^{2+} and Mn^{2+} (El-Sayed et al., 2019; Allala et al., 2019). However, in opposition to our results, this later enzyme was activated by Mg^{2+} (Allala et al., 2019). Therefore, the effect of metal ions on α-amylase activity varies between the sources organisms.

The optimum pH for the *M. timonae* CTI-57 AMY1 was 6.0 (Fig. 7A). Most bacterial α-amylase has optimum pH on a slightly acidic to a neutral range of pH, mainly at 5.0 to 6.5 (Pandey et al., 2000; Gupta et al., 2003). This profile seems to repeat in recombinant enzymes, such as the *Laceyella* sp. DS3 and the *B. subtilis* WB800 recombinant α-amylases, which have optimum activity at pH 6.0 (El-Sayed et al., 2019; Chen et al., 2015). This optimum acidic pH is essential for the starch industry, considering that the natural pH of the starch slurry is generally around 4.5 (Sivaramakrishnan et al., 2006). The optimum acidic pH is attributed to the ionization state of the critical catalytic residues in the active site of α-amylases, the nucleophile aspartate, which should stay negatively charged, and the catalyst glutamic acid residue, which should have a proton to donate (Nielsen et al., 2001).

The pH stability assay showed that the *M. timonae* CTI-57 AMY1 remained 53%, 45%, 77%, and 42% active when incubated for 24 h in pH 5.0, 6.0, 7.0, and 8.0, respectively (Fig. 7B). This quite broad range of stability agrees with that α-amylases are stable over a range of pH, from 4 to 11 (Gupta et al., 2003). However, in pH 7.0, the stability was statistically significant ($\alpha = 0.01$) higher than in other pHs, including the optimum pH of 6.0. This stability pattern could be explained by the better preservation of the enzyme's tertiary structure in pH 7.0, which could be altered in more acidic or basic pHs, due to changes in the ionic state of the key residues. As all the enzymatic assays were conducted in the optimum pH (6.0), even after incubating in another pH, the catalytic residues' charges could be restored during the reaction.

The recombinant *M. timonae* CTI-57 AMY1 enzyme showed to be very active in a broader range of temperatures, ranging from 40 to 70 °C (Fig. 8A), but its optimum temperature in pH 6.0 was 60 °C, and in pH 7.5 the highest activity was obtained at 70 °C. It is reported in the literature that the species *M. timonae* seems to be more evolutionary related to environmental organisms, which optimum growth temperature is 28-30 °C (La Scola et al., 1998). Nonetheless, the purified enzyme showed an optimum temperature higher than what was expected, as the enzyme's optimum conditions should be like that of the microorganism's habitat condition (Pandey et al., 2000). Regarding other recombinant α-amylases, the recombinant *Laceyella* sp. DS3 α-amylase was reported to have an optimum temperature of 55 °C (El-Sayed et al., 2019). Moreover, bacterial α-amylases are reported to be active in a broad range of temperature, but several have high optima temperatures, what classifies them as thermophilic enzymes (Gupta et al., 2003; Sivaramakrishnan et al., 2006). The broader range of temperature in which the *M. timonae* CT57 AMY1 α-amylase could work and its resistance to the anionic detergent SDS could be useful in the detergent industry, considering the modern trend among consumers of using colder temperatures for doing the laundry or dishwashing (El-Fallal et al., 2012).

In the thermal stability assay, the enzyme remained very active after 30 min of incubation, in temperatures ranging from 30 to 70 °C, but lost its activity when incubated at 80 °C, having a T_m of 73.65 °C, calculated from the second-order polynomial curve (Fig. 8B). In an activity time course on incubation at different temperatures, the recombinant *M. timonae* CTI-57 AMY1 enzyme retained 86.1% and 44.3% of its activity after incubation at 60 °C and 70 °C, respectively, for 24 h (Fig. 8C). Prakash and Jaiswal (2010) consider enzymes acting in a temperature equal or above 60 °C as thermostable, as most enzymes lose activity at temperatures of 50-60 °C. Considering the *M. timonae* CTI-57 AMY1 α-amylase T_m of 73.65 °C and stability when incubated at 60 °C for 24 h, it could be considered a thermostable

enzyme, which is comparable to α-amylases of some bacteria from the *Bacillus* and *Pyrococcus* genera (Pandey et al., 2000; Sivaramakrishnan et al., 2006; Prakash and Jaiswal, 2010; Mehta and Satyanarayana, 2016).

The starch industry requires α-amylases to be active at high temperatures to economize the process, and there has been a need and continual search for more thermophilic and thermostable α-amylase (Sivaramakrishnan et al., 2006). In the industrial starch hydrolysis, two processes are conducted. The first one is the liquefaction, which is carried out by amylases that allows a rapid reduction in the solution viscosity and which is performed at pH 6.0 and high temperatures (80-110 °C) (van der Maarel et al., 2002; El-Fallal et al., 2012). The second process corresponds to the partially hydrolyzed starch saccharification, which uses enzymes that can release maltose and D-glucose and is carried out at pH 4.5 and lower temperatures (60-70 ºC) (van der Maarel et al., 2002). α-Amylases are divided into two categories according to the degree of hydrolysis. Liquefying α-amylases, which do not produce free sugars and cause a more rapid reduction in the starch paste viscosity, and saccharifying α-amylases, which produce free sugars and reduce the starch viscosity less rapidly (El-Fallal et al., 2012; Tomasik and Horton, 2012). Considering the *M. timonae* CTI-57 AMY1 α-amylase characteristics, such as optimum pH of 6.0, T_m of 73.65, starch specificity, and no release of reducing sugars after 30 min of hydrolysis in the DNS reaction, it could be considered as a liquefying enzyme.

4 CONCLUSIONS

In this study, a gene from a novel α-amylase from *M. timonae* CTI-57 was cloned. The gene was successfully expressed using the pTrcHis2B plasmid and the *E. coli* Rosetta™ (DE3) strain. The protein sequence had a high identity with proteins from other *Massilia* species, as well as with proteins from other bacteria and plants. The *M. timonae* CTI-57 AMY1 α-amylase was not inhibited by SDS and could have an application in the detergent industry. The biochemical characteristics of the purified protein, such as activity in pH 6.0, thermal stability, starch specificity, and endo-acting mechanism, classify it as a liquefying starch enzyme.

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Table 1. Effect of activators and inhibitors on the purified α-amylase activity.

	Relative activity (%)*			
Substance	**0 mM**	**2 mM**	**5 mM**	**10 mM**
$CaCl_2$	0	100	110 ± 7.9	110 ± 4.3

	Relative activity (%)*	
Substance	**5 mM**	**10 mM**
Control (2 mM $CaCl_2$)	100 ± 3.8^{a}	100 ± 4.9^{a}
NaCl	103 ± 6.1^{a}	80.9 ± 8.0^{b}
KCl	94.3 ± 8.0^{a}	82.5 ± 3.2^{ab}
NH_4Cl	89.8 ± 3.4^{a}	82.6 ± 4.8^{ab}
$MgSO_4 \cdot 7H_2O$	57.6 ± 6.1^{b}	75.8 ± 6.7^{b}
$CoCl_2 \cdot 6H_2O$	58.7 ± 7.2^{b}	49.7 ± 5.1^{c}
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	19.7 ± 3.8^{de}	25.8 ± 4.8^{d}
$HgCl_2$	44.3 ± 5.7^{bc}	9.9 ± 4.0^{de}
$MnSO_4 \cdot H_2O$	39.7 ± 4.9^{cd}	53.8 ± 2.9^{c}
$CuSO_4 \cdot 5H_2O$	0^{f}	0^{f}
EDTA	11.3 ± 2.5^{e}	4.1 ± 4.1^{e}
SDS	104.3 ± 3.5^{a}	82.5 ± 2.4^{ab}

*The data corresponds to the average and standard deviation of four repetitions. Equal letters indicates no statistical differences at 1% ($\alpha = 0.01$) of significance.

Fig. 1. Sequence alignment. Clustal Omega sequence alignment of the *M. timonae* CTI-57 AMY1 (GenBank MN990470), *M. timonae* CCUG 45783 (GenBank EKU82989.1), and *H. vulgare* (GenBank P04063.3) α-amylases. The secretion sequences are in small letters and brown color. 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 and Gabriško, 2016) are highlighted in bright yellow and boxed. The amino acid residues that bind to calcium are in bold magenta and have a diamond at the top.

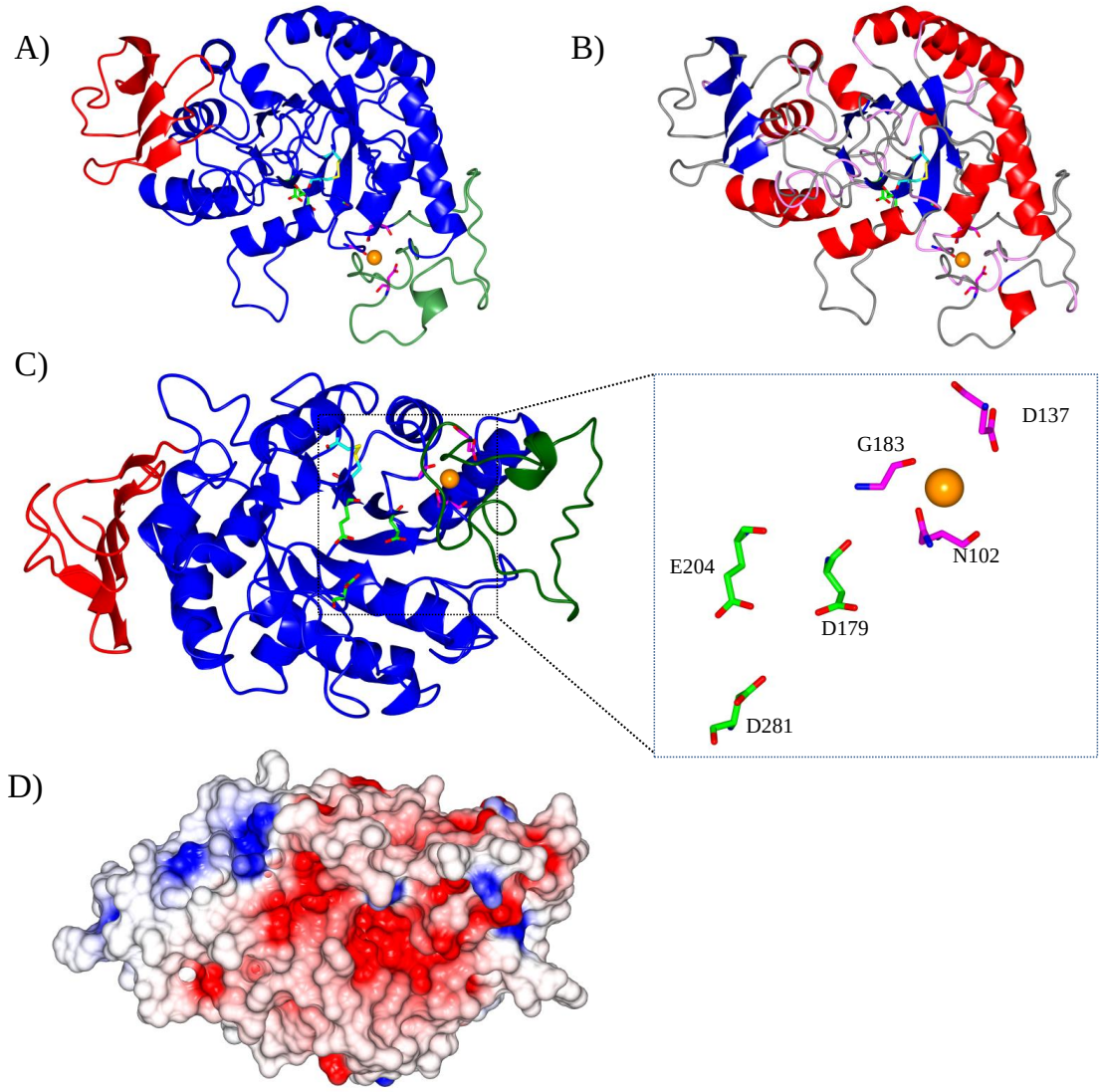

Fig. 2. The homology structural model of the *M. timonae* CTI-57 AMY1 α-amylase. A) Domains A, B, and C are shown in blue, green, and red, respectively. The disulfide bond formed between Cys201-Cys232 is shown in yellow with the Cys residues in cyan. The Ca^{2+} ion is displayed in orange. B) The α-helices, β-strands, turns, and coils are shown in red, blue, pink, and gray, respectively. The A domain, constituted by $(\beta/\alpha)_8$-barrel, can be seen in the center. The domain B protrudes between β3-sheet and α3-helix of domain A. The C-terminal domain is constituted of five antiparallel β-strands. C) The catalytic residues are shown in green and red. The Ca^{2+} coordinating residues are shown in magenta and red. D) Electrostatic potential (red – negative regions; blue – positive regions) in the protein surface positioned as in C).

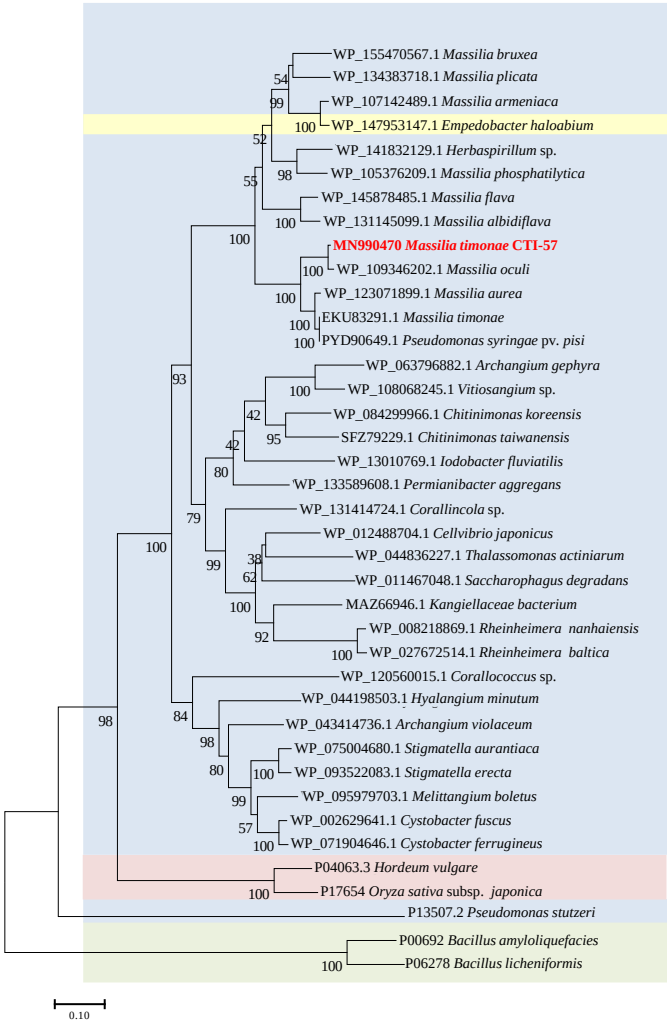

Figure 3. The phylogenetic analysis. The optimal tree with the sum of branch length = 6.47218210 is shown. The bootstrap test values are shown below the branches. The tree is drawn to scale, with branch lengths equal to evolutionary distances. The analysis involved 39 amino acid sequences. The ambiguous positions were removed and there were a total of 730 positions in the final dataset. The *M. timonae* CTI-57 protein is indicated in bold red. All sequences share more than 40% of identity, except for the *Bacillus* spp. and *P. stutzeri* sequences. Proteobacteria phylum species are squared in blue, Bacteroidetes phylum species in yellow, Firmicutes phylum species in green, and plant species in pink.

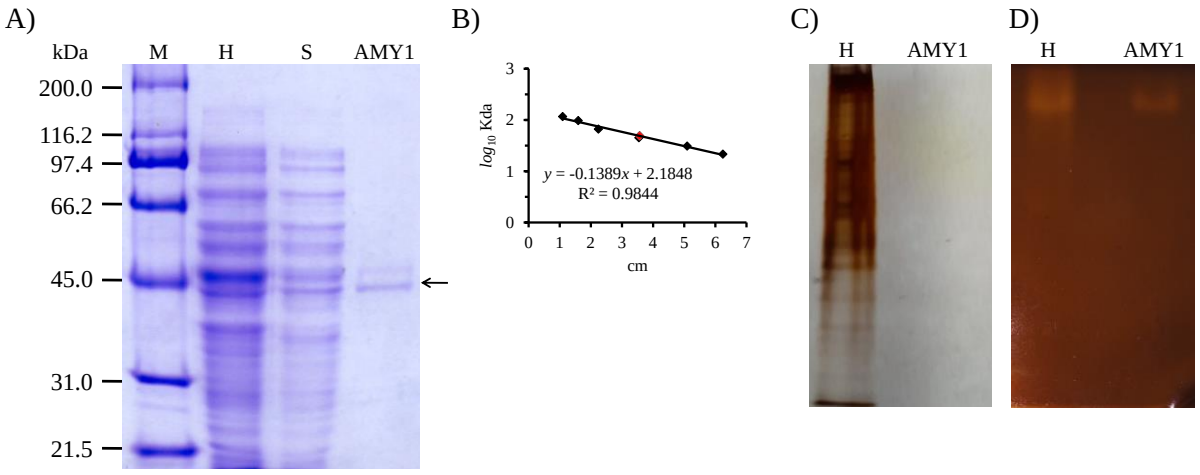

Figure 4. Electrophoretic analyses. A) SDS-PAGE gel. M - Broad range molecular marker (BioRad, USA); H – homogenized; S – supernatant; AMY1 – purified *M. timonae* CTI-57 α-amylase. The arrow indicates the purified enzyme. The gel was stained with Coomassie Blue. B) The graph used to calculate the enzyme's molecular weight. The red diamond shows the enzyme's data. C) The PAGE gel containing the homogenized (H) and purified enzyme (AMY1). The silver staining was used. D) The PAGE gel zymogram showing the activity of the native enzyme from the homogenized (H) and purified enzyme (AMY1) on soluble starch. The gel was stained with the iodine reagent and photographed. The bright halos indicate amylase activity.

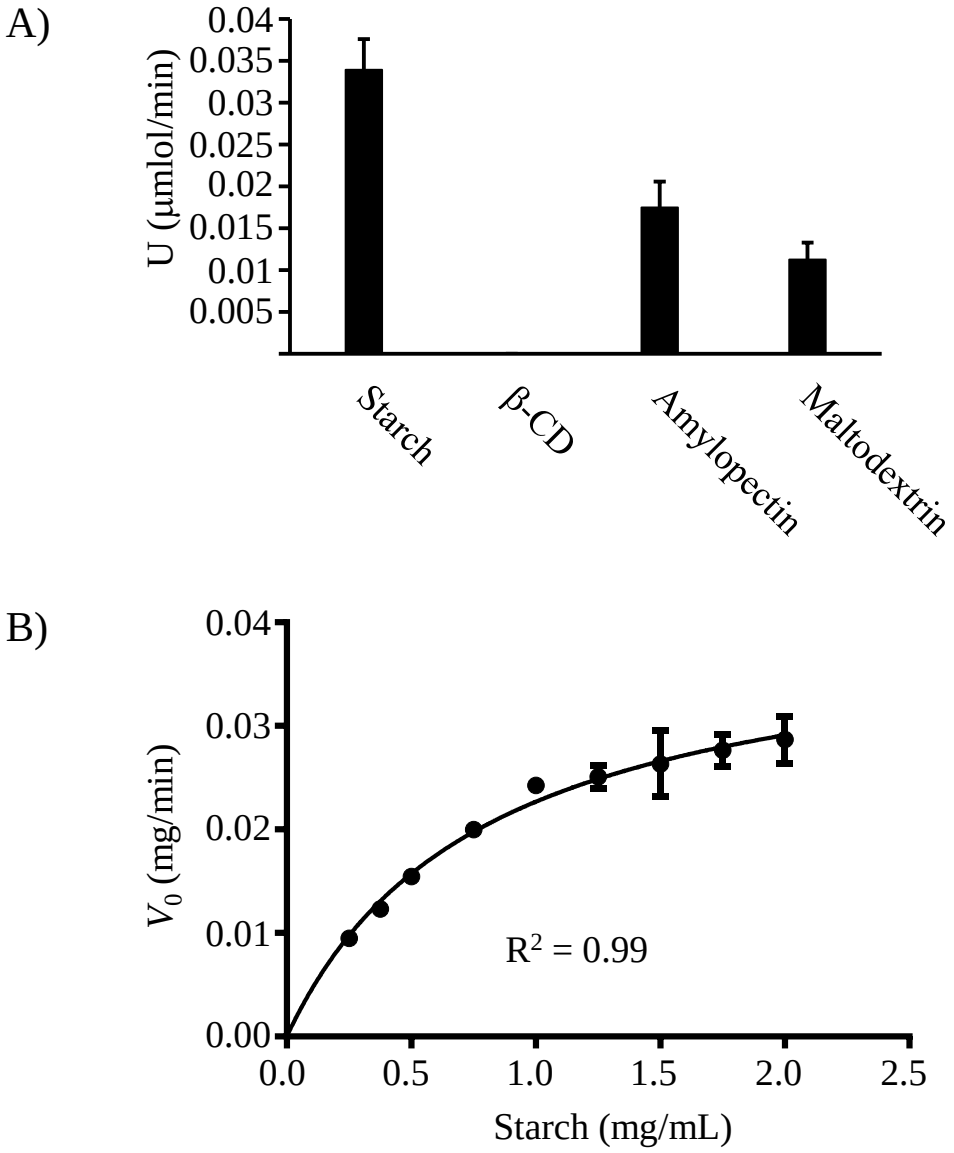

Figure 5. Substrate specificity and saturation curve of the purified recombinant *M. timonae* CTI-57 α-amylase AMY1. A) Substrate specificity. Starch, β-cyclodextrin (β-CD), amylopectin, and maltodextrin at 0.5% were used in the DNS reaction method. B) Saturation curve varying starch concentration from 0.2 to 2.0 mg/mL. The initial velocity was measured as mg of starch that is degraded per min. All data represent the average and SD of three experimental sets.

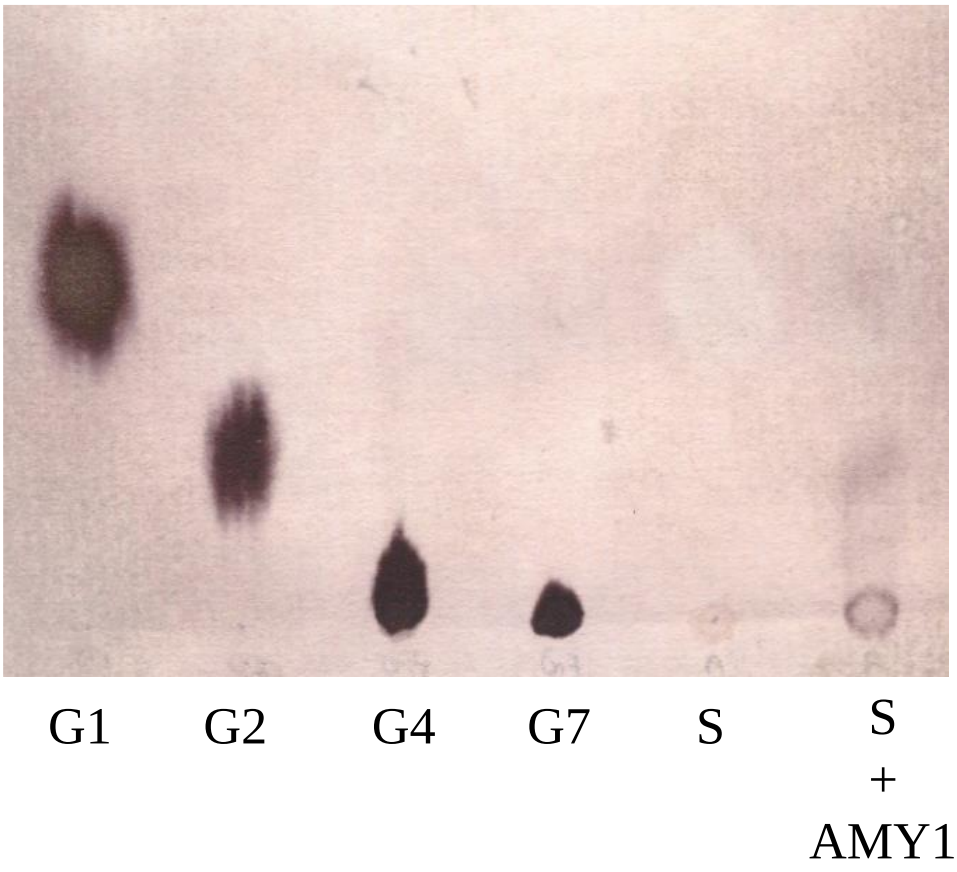

Figure 6. Starch Hydrolysis products by the *M. timonae* CTI-57 recombinant α-amylase AMY1. A descending chromatogram with the hydrolysis products from soluble starch incubated with the recombinant enzyme at 40 °C for 16 h under 65 rpm of agitation. Standards: G1 - glucose, G2 - maltose, G4 - maltotetraose, and G7-maltoheptaose. AMY1 - *M. timonae* CTI-57 recombinant α-amylase. S – soluble starch.

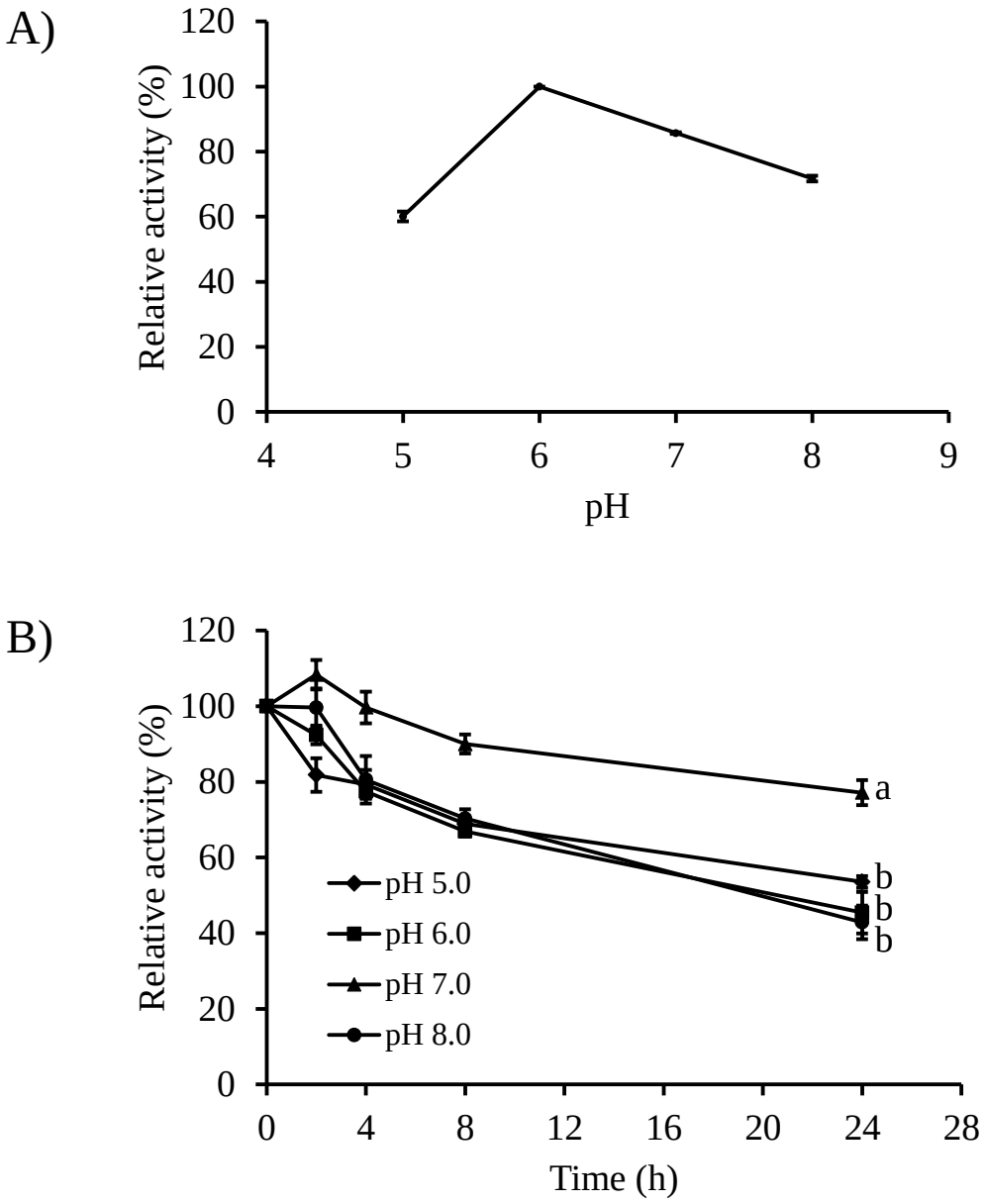

Figure 7. Effect of pH on the purified recombinant *M. timonae* CTI-57 α-amylase AMY1. A) Optimum pH. B) pH stability over time. Averages of the 24 h time point were submitted to ANOVA and compared using the Tukey test. Identical letters show no difference among averages (α = 0.01). All data represent the average and SD of three experimental sets.

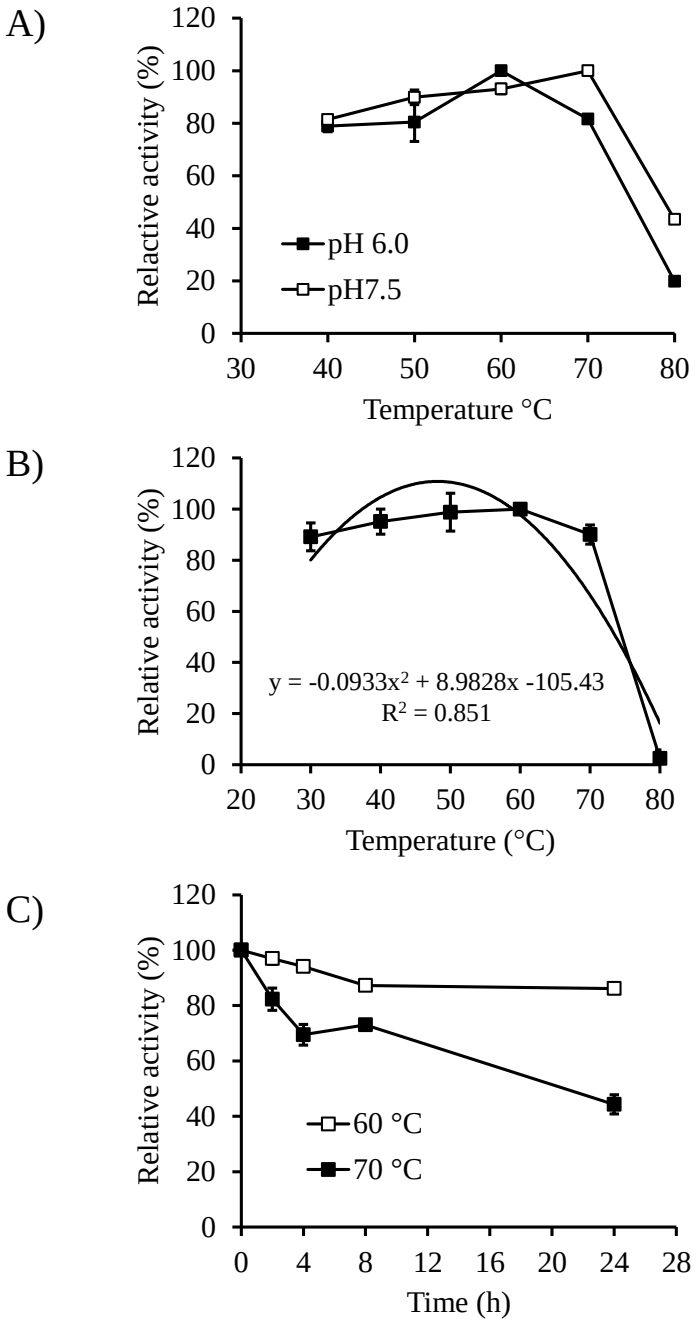

Figure 8. Effect of temperature on the purified recombinant *M. timonae* CTI-57 α-amylase AMY1. A) Optimum temperature. B) Thermal stability. C) Thermal stability over time. All data represent the average and SD of three experimental sets.