Vol.67: e24240293, 2024
https://doi.org/10.1590/1678-4324-2024240293
ISSN 1678-4324 Online Edition

BABT
Brazilian Archives of Biology and Technology

Article - Agriculture, Agribusiness and Biotechnology

Standardization of the Cultivation of *Rhizopus arrhizus* Using Agroindustrial Residues: High Production of Amylases in Pineapple Peel

Paulo Henrique Silva Lopes[1]
https://orcid.org/0000-0003-1461-0208

Thiago Machado Pasin[2*]
https://orcid.org/0000-0003-3100-1674

Vivian Machado Benassi[1]
https://orcid.org/0000-0002-6030-0473

David Lee Nelson[1]
https://orcid.org/0000-0001-7435-3675

Tássio Brito de Oliveira[3]
https://orcid.org/0000-0002-4666-7930

Maria de Lourdes Teixeira de Moraes Polizeli[4]
https://orcid.org/0000-0002-5026-6363

[1]Universidade Federal dos Vales do Jequitinhonha e Mucuri, Programa de Pós-graduação em Biocombustíveis, Diamantina, Minas Gerais, Brasil; [2]University of Texas at San Antonio, Department of Chemistry, San Antonio, Texas, USA; [3]Universidade Federal da Paraíba, Centro de Ciências Exatas e da Natureza, Departamento de Sistemática e Ecologia, João Pessoa, Paraíba, Brasil; [4]Universidade de São Paulo, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Departamento de Biologia, Ribeirão Preto, São Paulo, Brasil.

Editor-in-Chief: Bill Jorge Costa
Associate Editor: Ana Cláudia Barana

Received: 29-Mar-2024; Accepted: 07-Jun-2024.

*Correspondence: thiagopasin@hotmail.com; (T.M.P.).

HIGHLIGHTS

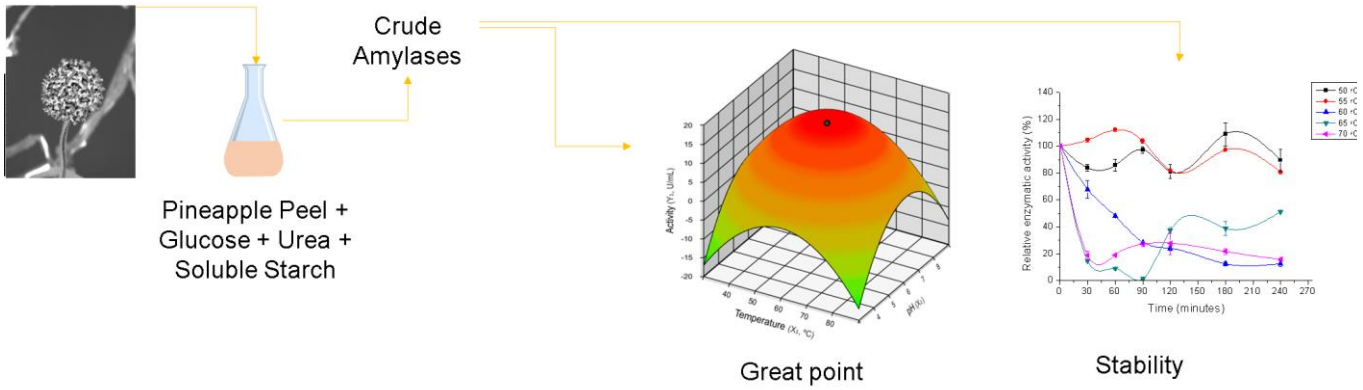
- Maximum production of amylases with residual carbon sources.
- Amylase production with less expensive nitrogen sources.
- Amylolytic activity is independent of the inoculum diameter.
- Crude amylases are stable for over two hours.

Abstract: Amylases, crucial in various industries such as food, textile, and biofuels, require optimized production and biochemical characterization. Thus, this study aimed to produce amylases from *Rhizopus* sp. I 1.2.1, characterize the cultivation conditions and the synthesized enzyme. The fungus was molecularly identified with 99.06% homology in the LSU gene, 98.08% identity in the ITS region, and placed phylogenetically closer to *Rhizopus arrhizus* CBS 112,07. The LSU gene and ITS region sequences were deposited in GenBank. The CP medium was optimal for amylase production by *Rhizopus arrhizus*, with peak activity on the 6th day. Supplementation with urea significantly increased amylolytic activity by 110-fold. CP salts outperformed other salts in enzyme production, achieving a maximum amylase activity of 15.5 U/ml. The pH 6.0 was optimal for amylase production, with higher specific activity at pH 5.0. Pumpkin peel and pineapple peel were the best carbon sources for amylase production with an amylolytic activity of 18 and 16 U/mL, respectively. The amylase production showed a significant increase when pineapple peel was used in the presence of glucose 0.5% showing an activity of 22.4 U/mL, representing a 1.5- and 1.6-fold increase

over the control. The optimal reaction of the amylase was observed at pH 6.0 and 60 °C. The enzyme remained stable for 240 minutes at 50-55 °C and at pH 4.0-7.0. Amylase was inhibited by glucose concentration and certain salts, but EDTA and K_2SO_4 increased activity by 25%. These results suggest industrial potential based on residual carbon source use, cultivation conditions, and crude enzyme stability.

Keywords: Agroindustrial residues; Amylases; filamentous fungus; *Rhizopus arrhizus*; stability.

GRAPHICAL ABSTRACT



INTRODUCTION

Starch is a polysaccharide stored in the form of semi-crystalline granules and can be found in rhizomes, tubers, seeds, and fruits. The chemical composition of the polysaccharide is based on amylopectin and amylose, which have high molecular weight [1, 2]. The molecular structure of starch constituents needs to undergo the action of stereospecific enzymes to aid in the release of fermentable sugars. Amylases are the enzymes that act as hydrolases on starch, breaking the α-1,4 and α-1,6-glycosidic bonds at branching points [1, 3, 4]. Filamentous fungi can produce amylases in the presence of starch-based substrates [5]. They are composed of hyphal filaments and secrete compounds and biomolecules to ensure the full development of their structure [6, 7]. The traditional process of starch debranching and hydrolysis occurs by adding amylolytic enzymes to liquefaction and saccharification processes. The liquefaction step is conducted at a temperature of 95 °C, reducing the viscosity of the polysaccharide [8]. Thus, this process represents a high energy expenditure for the industry. Therefore, studies with enzymes need to be ongoing, seeking new ways to optimize processes and characterize microbial enzymes with the potential to play a significant role in industrial processes involving starch hydrolysis [9, 10]. The objective of this work was to obtain amylases with high catalytic efficiency from a fungal isolate from decomposing fruit peels in the soil, named *Rhizopus arrhizus* I 1.2.1, which will aid in the hydrolysis of starch into fermentable sugars, as well as to biochemically characterize the crude amylases obtained and measure other types of enzymes produced by the fungus in the presence of residues.

MATERIAL AND METHODS

The work was conducted at the Laboratory of Mycology, Enzymology, and Product Development (LMEDP) of the Institute of Science and Technology, Federal University of the Valleys of Jequitinhonha and Mucuri (UFVJM), Diamantina campus.

Microorganism, Inoculum, and Obtaining the Enzymatic Extract

The selected strain is registered in the National System for Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under the number A64AD93 and name I 1.2.1. The fungal inoculum previously developed on solid Quaker® oatmeal medium [11] was transferred onto a 250 mL Erlenmeyer flask containing 50 mL of sterile CP medium [12] using a sterilized 1'' diameter stainless steel tube. After an incubation period in a Tecnal TE-371 BOD Germination Chamber for 6 days at 35 °C without physical agitation, the amylolytic activity, protein quantification, and the apparent specific activity of the filtered extracellular crude extract were determined using a vacuum filtration funnel and Unifil® filter paper.

Total Protein and Amylase Assay

The protein quantification was estimated using the Bradford method [13], with albumin (100 µg/mL) in the standard curve. The enzymatic assay for amylases was performed at 55 °C using the colorimetric method with 3',5'-dinitrosalicylic acid (DNS) [14], using a 1% (w/v) soluble starch substrate in 100 mM sodium acetate buffer, pH 5.5. The standard curve was prepared with glucose (1.0 mg/mL) in 100 mM sodium acetate buffer, pH 5.5. Protein and amylase readings were taken on a Ray Leígh UV-2601 spectrophotometer at 595 nm and 540 nm, respectively.

Molecular Identification and Phylogenetic Analysis and Scanning Electron Microscopy

The molecular identification first involved the physical lysis of the mycelium to extract genomic DNA. For mycelium maceration, TES lysis buffer (Tris 100 mM; EDTA 10 mM; SDS 2%) and a pestle were used, followed by incubation at 65 °C for 15 min. 140 µL of 5 M sodium chloride (NaCl) was added and incubated on ice for 30 min. Then, 600 µL of chloroform: isoamyl alcohol mixture (24:1) was added and centrifuged at 10,000*xg* for 10 min at 4 °C. The supernatant was collected, and isopropanol and 3M sodium acetate (pH 5.2) were added in respective proportions of 300 µL and 50 µL. The mixture was centrifuged at 10,000*xg* for 10 min at 4 °C, and the supernatant was discarded. The precipitated material was washed again with 600 µL of 70% ethanol and centrifuged. Then, the supernatant was discarded, and the precipitated pellet was diluted in 50 µL of TE buffer (Tris 10 mM; EDTA 1 mM) with 5 µL of RNAse (10 mg/mL).

The DNA of the fungus I 1.2.1 was extracted and the Internal Transcribed Spacer (ITS) region was amplified with primers ITS4 and ITS5 [15], and the LSU gene was amplified with primers NL1 and NL4 [16]. The PCR amplification reactions were performed using the PCR Master Mix kit (Promega®), following the manufacturer's instructions. The amplification products were visualized using 1% agarose gel electrophoresis; the gel was stained with Nancy (Sigma-Aldrich®) and visualized under an ultraviolet (UV) transilluminator. For the purification of the amplification reaction, the Wizard® SV Gel and PCR Clean-up System kit (Promega®) was used, and the DNA was quantified using NanoDrop® (Thermo Scientific®). For sequencing, the BigDye® Terminator Cycle Sequencing kit (Life Technologies®) was used, following the manufacturer's protocol, and sequenced on an ABI 3500XL sequencer (Life Technologies®).

BioEdit v. 7.0.5.3 was used to check the quality of the forward and reverse sequences generated and to assemble them into a consensus sequence [17]. The contigs were compared with homologous sequences available in public databases such as NCBI-GenBank (www.ncbi.nlm.nih.gov) using the BLASTn tool. The ClustalW tool was used as a second quality control for the sequences by aligning them with homologous sequences from culture collections [18]. For taxonomic affiliation confirmation, phylogenetic analyses were performed with the obtained sequences. The alignment of the sequences was based on homologous sequences of phylogenetically related species using the ClustalW tool, followed by manual refinement. The Neighbor-Joining method was used to construct the phylogeny, calculating the evolutionary distance based on the Kimura 2-parameter model. Finally, the tree support was determined based on bootstrap analysis with 1,000 pseudo-repetitions. The tree was inferred using the MEGA v. 5.0 tool [19, 20].

Scanning Electron Microscopy (SEM) of the fungus *Rhizopus arrhizus* (formerly *R. oryzae*) I 1.2.1 was performed at the X-Ray Diffraction, AFM and SEM Laboratory, Department of Chemistry, UFVJM. The fungus was fixed on a sample holder (stub) using double-sided carbon conductive tape (Tedpella®), and the surface morphology was examined using a Hitachi TM3000 scanning electron microscope, generating images at an acceleration voltage of 15 kV, with a magnification of 1kx.

Fermentation Process Parameters and Optimization

The fungus was inoculated in Adams [21], SR [22], CP [12], and Khanna [23] media to evaluate their influence on amylase production, using Dinâmica® soluble starch as the carbon source. Inoculation was done in CP medium [12] with an initial pH of 6, using equal proportions of different nitrogen source variations (Himedia® peptone, Isofar® yeast extract, and Neon® urea).

Different salts were tested, evaluating amylase production in CP medium [12] with an initial pH of 6. The salts tested were SR [22], CP [12], Khanna [23], and MV - Modified Vogel [24]. The MV salt solution did not contain sodium citrate heptahydrate, chloroform, monohydrated citric acid, $Fe(NH_4)_2(SO_4)_2.6H_2O$, $MnSO_4.H_2O$, and $Na_2MoO_4.H_2O$.

The influence of initial pH was tested by varying the pH of the culture medium to 4.5, 5.0, 5.5, 6.0, and 6.5. The inoculum on disk was tested by varying the diameter to 0.5'', 1.0'', 1.5'', 2.0'', as well as 1.5'' and 2.0'' disks split in half, using sterile stainless-steel tubes. Different carbon sources at 1.5% (w/v) were tested to

assess their influence on amylase production in CP medium [12]. The influence of adding 0.5% (w/v) of Isofar® anhydrous glucose to different carbon sources at a concentration of 1.5% (w/v) was also analyzed.

Characterization of Crude Amylases and Hydrolysis of Different Substrates

The fungus was cultivated under optimal conditions for amylase production, and after obtaining the crude enzymatic extract, the biochemical characterization of the free amylases in the extract was performed.

The optimal temperature and pH of the crude amylases were determined using Central Composite Rotatable Design (CCRD), with three central points, one genuine repetition, α equal to 5%, and a desired R^2 of 95%, varying the substrate pH and reaction temperature and evaluating the amylolytic activity (U/mL) as the response variable. Substrates at different pH containing 1% (w/v) soluble starch (Cinética®) were prepared with 100 mM sodium citrate buffer (pH 3.2, 4.0, and 6.0) and 100 mM glycine buffer (pH 8.0 and 8.8). The reaction temperature in a water bath was evaluated from 40 to 80 °C. The enzymatic assay was performed according to the CCRD experimental matrix obtained from the variable levels table (Table 1). The CCRD was performed using commercially acquired Protimiza Experimental Design software.

Table 1. DCCR Levels pH vs. Temperature

[1]**Variables**	**Levels**				
	-1,41	-1	0	+1	+1,41
Temperature	32	40	60	80	88
pH	3.2	4.0	6.0	8.0	8.8

[1]External control was performed with 1% (w/v) soluble starch substrate in 100 mM sodium acetate buffer, pH 5.5, and at a reaction temperature of 55 °C.

The stability of the crude amylases was analyzed using 1% (w/v) soluble starch substrate (Cinética®) in 100 mM sodium citrate buffer, pH 6.0, in the temperature range of 50 to 70 °C, at intervals of 5 °C, for 30, 60, 90, 120, 180, and 240 minutes for each incubation temperature in the aforementioned water bath.

pH stability was evaluated by incubating the crude enzymatic extract in 100 mM sodium citrate buffer for pH 3.0 to 6.5 and in 100 mM glycine buffer for pH 7.0 to 8.0, for 30, 60, 90, 120, 180, and 240 minutes for each pH of incubation of the crude extract.

This procedure aimed to assess the inhibition of amylolytic activity in the presence of different concentrations of glucose in the reaction. Different aliquots of the 400 mM anhydrous glucose stock solution (Vetec®) were added so that the final volume of the enzymatic assay reaction had glucose concentrations of 5, 10, 20, 50, 100, 150, and 200 mM.

This procedure aimed to determine the different enzymes produced and released by the fungus under study, which act together with the amylases in the hydrolysis of complex substrates. The enzymes assayed and their substrates were: (1) amylases – 1% soluble starch (Cinética®) in 100 mM sodium citrate buffer, pH 6.0; (2) xylanases – 1% xylan (Uniscience®) in 200 mM sodium phosphate buffer, pH 5.5; (3) endoglucanases (CMCase) – 1% sodium carboxymethyl cellulose (CMC) medium viscosity (Sigma Aldrich®) in 100 mM sodium citrate buffer, pH 5.5; (4) invertase – 1% sucrose (Vetec®) in 100 mM sodium citrate buffer, pH 6.0; (5) avicelase – 1% avicel (Fluca®) in 100 mM sodium citrate buffer, pH 5.0; (6) endoglucanases (CMCase) – 1% low viscosity sodium carboxymethyl cellulose (CMC) (Sigma Aldrich®) in 100 mM sodium citrate buffer, pH 5.5; and (7) endoglucanase and exoglucanase (FPase) – J Prolab® filter paper (one unit, 6 cm X 1 cm, 205 µm thick, and pores of 14 µm) in sodium citrate buffer, pH 6.0. All enzymes were assayed under the optimal conditions of the crude amylases for 15 minutes, except for FPase, which was assayed for 30 minutes [25-27].

This procedure aimed to evaluate the induction or inhibition of amylolytic activity in the presence of different salts, including ethylenediaminetetraacetic acid (EDTA). For this, stock solutions of each salt at 400 mM were prepared, and aliquots were incubated with the crude enzymatic extract so that the final concentration of the salt in the enzymatic assay reaction was 5 mM.

Repeatability and Statistical Analysis

The experiments were conducted in quadruplicate, and errors and standard deviations were obtained. Statistical analyses were performed using the free software Sisvar 5.8 - Build 92. Data were evaluated

using analysis of variance (ANOVA), and when significant differences were found, means were compared using the Scott-Knott test, with α set at 0.05.

RESULTS

Molecular Identification and Phylogenetic Analysis of I 1.2.1

The molecular and phylogenetic analysis showed that the LSU gene exhibited 99.06% homology, and the ITS region showed 98.08% identity, both converging to the species *Rhizopus arrhizus* (formerly *Rhizopus oryzae*), based on the alignment performed [28]. The dendrogram of I 1.2.1 reveals that the fungus is in the same phylogenetic branch as *Rhizopus arrhizus* CBS 112,07 (NR 103595), with a bootstrap value of 78% (Figure 1A). The nucleotide sequences obtained for the LSU gene and the ITS region were deposited in the GenBank database under the accession numbers OP903020 and OP903016, respectively. Figure 1B shows part of the long, tubular, non-septate sporangiophore, with the sporangium enclosing a considerable number of ellipsoidal or subglobose sporangiospores.

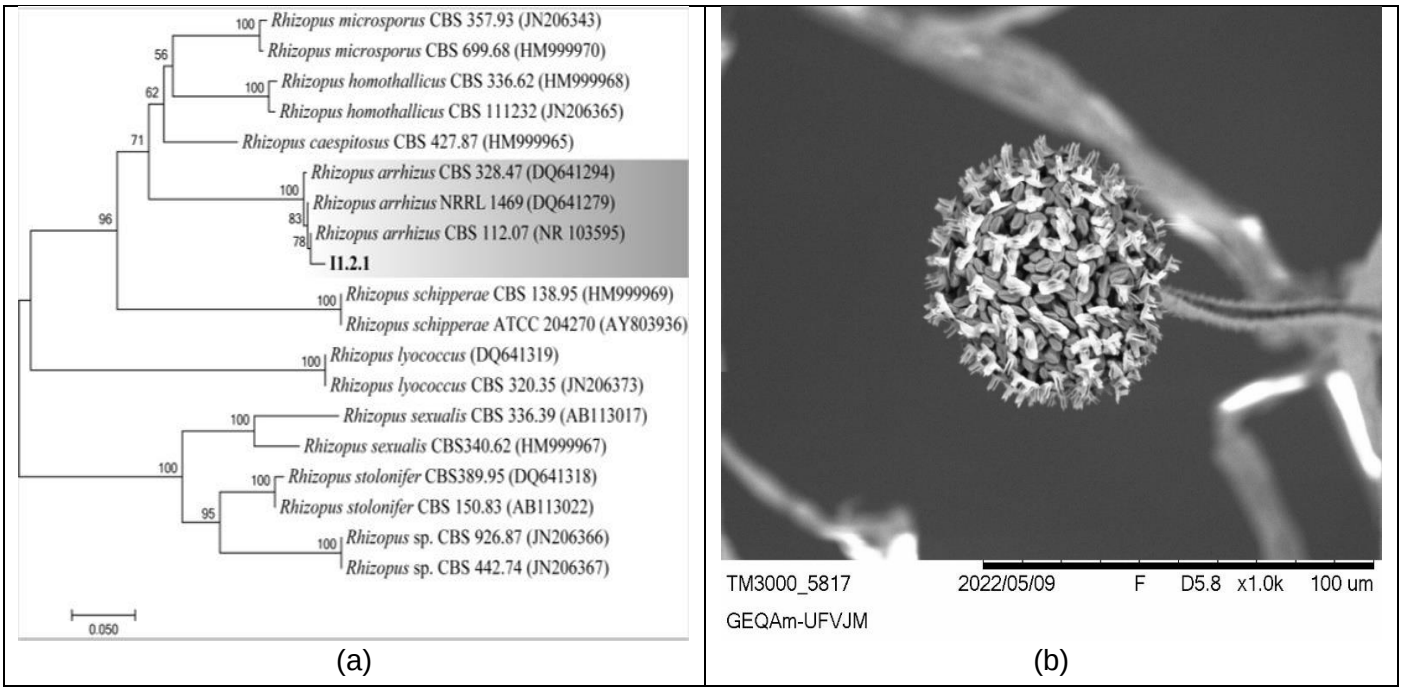


Figure 1. The microorganism under study: (a) Dendrogram for I 1.2.1 based on the ITS region of the extracted genomic DNA; (b) SEM of the fungus *Rhizopus arrhizus* (formerly *R. oryzae*) I 1.2.1, with a magnification of 1kx, showing the sporangiospores, sporangium, and part of the sporangiophore.

Parameters and Optimization of the Fermentation Process

The optimization of amylase production was evaluated based on the expressed amylolytic activity, although the values of total protein and specific apparent activity were also determined.

The Scott-Knott test revealed that among the analyzed media, the CP medium was statistically the most favorable for the production of amylases by I 1.2.1. Regarding the days of cultivation, the 6th day corresponded to the highest amylolytic activity for the medium, with an activity of 0.160 U/mL, although it is statistically equal to the fifth day (Figure 2).

Supplementation with urea contributed to the highest amylolytic activity (17.738 U/mL) and specific apparent activity, according to the "a" index of the statistical test. The combination of urea with other nitrogen sources contributed to the highest protein values, as in (E+U) with 3.595 mg of protein/mL (Table 2).

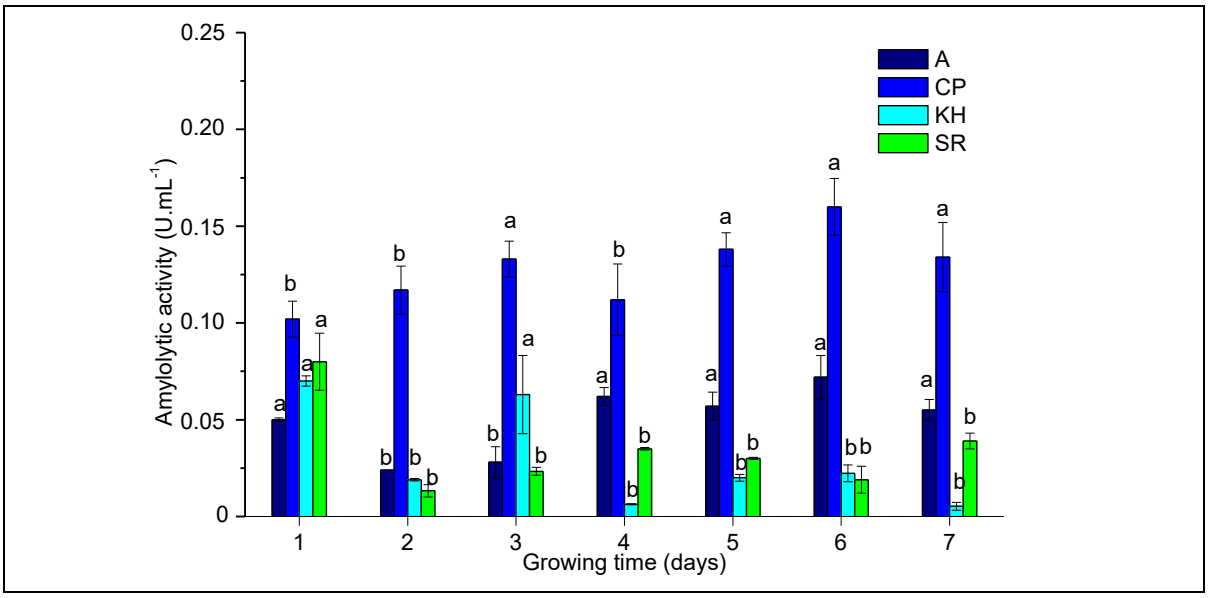


Figure 2. Amylolytic activity as a function of medium and days of cultivation. The statistical test used was the Scott-Knott test, with α set at 0.05. The results are expressed along with their respective errors. The index representing the highest mean is "a", and equal indices represent statistically equal means. A - Adams medium, KH - Khanna medium. The graph was evaluated first in relation to the media and then in relation to the days of the most significant medium of cultivation.

Table 2. Production of amylases, proteins, and specific apparent activity as a function of different nitrogen source supplements.

Nitrogen Source[1]	Activity (U.mL^{-1})	Protein (mg.mL^{-1})	Specific Activity (U.mg^{-1})
E (control)	0.185 ± 0.019 ^{c}	0.289 ± 0.027 ^{c}	0.645 ± 0.096 ^{c}
P	4.319 ± 0.564 ^{b}	0.334 ± 0.013 ^{c}	12.922 ± 1.347 ^{b}
U	17.738 ± 3.268 ^{a}	0.178 ± 0.017 ^{c}	99.251 ± 12.437 ^{a}
P+U	0.743 ± 0.146 ^{c}	2.974 ± 0.720 ^{b}	0.258 ± 0.067 ^{c}
Y+U	1.181 ± 0.164 ^{c}	3.595 ± 0.230 ^{a}	0.329 ± 0.049 ^{c}
Y+P	4.216 ± 0.474 ^{b}	0.319 ± 0.042 ^{c}	13.341 ± 2.091 ^{b}
Y+P+U	0.970 ± 0.103 ^{c}	3.489 ± 0.286 ^{a}	0.277 ± 0.008 ^{c}

[1]Y - yeast extract, P - peptone, U - urea. Results obtained by the Scott-Knott test, α = 0.05. Results expressed with their standard deviations. Index of the highest mean is “a”.

The CP salts are more efficient for the production of amylolytic enzymes, as well as a higher specific apparent activity than with the other salts analyzed, although they are not efficient for protein production. Additionally, the data reveal that with CP medium salts, the fungus produced activity 42, 11, 15, and 7 times greater than with Wesson - W, Khanna - K, SR, and MV salts, respectively. This result is important because it means that fewer reagents are needed in the production process of amylases by *Rhizopus arrhizus* I 1.2.1 (Table 3).

The initial pH of the CP medium most favorable for amylase production is around 6.0, as observed by the "a" index. Protein production at pH 4.5 was higher than at pH 6.5; however, the production of the biomolecule does not depend exclusively on pH since the values were not decreasing. Although the highest amylolytic activity is at pH 6.0, the specific apparent activity was higher at pH 5.0 (Table 4).

Table 3. Result for the different salt supplements.

Salts	Activity (U.mL^{-1})	Protein (mg.mL^{-1})	Specific Activity (U.mg^{-1})
CP (control)	15.527 ± 0.945 ^{a}	0.467 ± 0.008 ^{d}	33.291 ± 2.270 ^{a}
SR	1.053 ± 0.071 ^{b}	8.185 ± 0.943 ^{a}	0.130 ± 0.020 ^{b}
Khanna	1.395 ± 0.095 ^{b}	8.883 ± 0.657 ^{a}	0.157 ± 0.001 ^{b}
Wesson	0.366 ± 0.019 ^{b}	1.438 ± 0.224 ^{c}	0.257 ± 0.025 ^{b}
Modified Vogel	2.076 ± 0.648 ^{b}	3.654 ± 0.192 ^{b}	0.573 ± 0.196 ^{b}

Analysis by the Scott-Knott test, α = 0.05. Results expressed with standard deviations. The index of the highest mean is "a".

Table 4. Production of amylases, proteins, and specific activity as a function of initial pH.

Initial pH	Activity (U.mL^{-1})	Protein (mg.mL^{-1})	Specific Activity (U.mg^{-1})
6.0 (control)	14.374 ± 0.154 ^{a}	0.623 ± 0.047 ^{b}	23.009 ± 1.550 ^{c}
4.5	13.003 ± 0.825 ^{a}	1.074 ± 0.063 ^{a}	12.141 ± 1.096 ^{c}
5.0	13.695 ± 2.352 ^{a}	0.201 ± 0.019 ^{c}	68.394 ± 11.379 ^{a}
5.5	11.799 ± 0.200 ^{b}	0.843 ± 0.283 ^{b}	14.961 ± 4.379 ^{c}
6.5	10.172 ± 1.111 ^{b}	0.211 ± 0.044 ^{c}	49.075 ± 6.989 ^{b}

Analysis by the Scott-Knott test, α = 0.05. Results expressed with standard deviations. The index of the highest mean is "a".

The results for the variation in inoculum diameter, with means accompanied by the index "a," are statistically equal and more significant. Although the 0.5'' diameter provided less activity than the 1.0'', it is convenient to work with a smaller inoculum diameter, as there is less consumption of solid culture medium to achieve equivalent enzymatic activities. However, the smaller diameter negatively interferes with protein production, and consequently, higher specific apparent activity (Table 5).

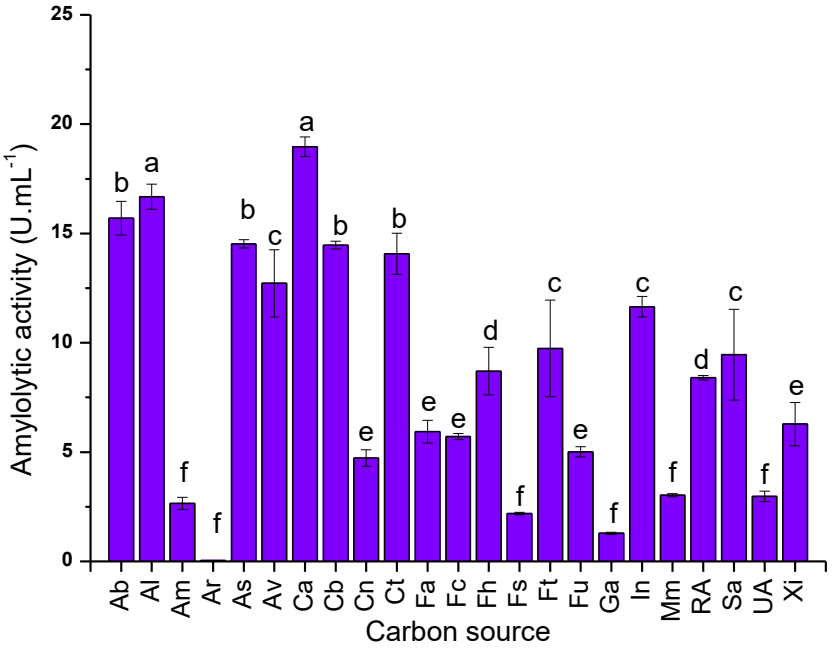
Table 5. Result for the different inoculum diameters.

Inoculum diameters[1]	Activity (U.mL^{-1})	Protein (mg.mL^{-1})	Specific Activity (U.mg^{-1})
1.0'' (control)	14.989 ± 0.915 ^{a}	0.800 ± 0.037 ^{d}	18.763 ± 1.440 ^{b}
0.5''	14.515 ± 0.810 ^{a}	0.433 ± 0.106 ^{d}	34.719 ± 7.623 ^{a}
1.5''	12.286 ± 0.847 ^{b}	9.707 ± 0.820 ^{b}	1.268 ± 0.052 ^{c}
1.5'' halved	14.412 ± 1.132 ^{a}	5.115 ± 0.577 ^{c}	2.825 ± 0.119 ^{c}
2.0''	13.938 ± 0.256 ^{a}	9.752 ± 0.518 ^{b}	1.431 ± 0.067 ^{c}
2.0'' halved	13.925 ± 1.170 ^{a}	15.780 ± 2.167 ^{a}	0.893 ± 0.140 ^{c}

[1] Halved corresponds to the inoculum being divided into two halves. Analysis by the Scott-Knott test, α = 0.05. Results expressed with standard deviations. The index of the highest mean is "a".

The pumpkin peel was the carbon source that showed the highest amylolytic activity, 1.3 times higher than the control (soluble starch), and one of the sources with the highest specific activity (Figure 3). The highest protein values were identified for yam peel, pineapple peel, oat flakes, oat bran, fine wheat bran, chia flour, potato peel, and banana peel, with values between 25.898 and 33.246 mg/mL. Protein and specific activity results were not discussed, as the inducing source in amylase production was selected based on enzyme activity.

The most promising results for amylase production in the presence of glucose were with sweet potato peel (21.733 U/mL) and pineapple peel (22.484 U/mL), representing a 1.5- and 1.6-fold increase over the control (soluble starch without glucose). Regarding specific activity, soluble starch in the presence of glucose provided the highest activity, at 151.466 U/mg. For protein production, the most suitable sources were pumpkin residue and potato peel (Table 6). Therefore, to reduce the lag phase time of the fungus under study and maximize microbial amylase production using residual carbon source, pineapple peel supplemented with anhydrous glucose was chosen.



Where it reads:
Ab - pineapple peel,
Al - avicel,
Am - corn starch,
Ar - white rice flour,
As - soluble starch,
Av - oat flakes,
Ca - pumpkin peel,
Cb - potato peel,
Cn - banana peel,
Ct - sweet potato peel,
Fa - flaxseed flour,
Fc - chia flour,
Fh - oat bran,
Fs - corn cob flour,
Ft - fine wheat bran,
Fu - cornmeal,
Ga - galactose,
In - yam peel,
Mm - ground corn,
RA - Pumpkin residue (peel, seed, fiber),
Sa - sucrose,
UA - Purple grape peel,
Xi - Xylose.

Figure 3. Screening of amylolytic production *by Rhizopus arrhizus* using different agroindustrial residues as carbon sources. The results obtained were analyzed using the Scott-Knott test, with α = 0.05. Results are expressed with errors. The index with the highest average is "a". ** Residual carbon sources were dehydrated at 60 °C for 24 hours and then ground in an analytical mill to obtain a fine particle size. Other carbon sources were commercially obtained. Pumpkin residue corresponds to seeds, peels, and fibers from pumpkin. Soluble starch (c) is the experiment's control.

Table 6. Influence of glucose on amylase production.

Carbon Source[1]	Activity (U.mL^{-1})	Protein (mg.mL^{-1})	Specific Activity (U.mg^{-1})
Soluble starch w/o glucose	14.545 ± 0.027 ^{b}	0.162 ± 0.013 ^{e}	89.870 ± 7.361 ^{b}
Pumpkin peel w/o glucose	16.507 ± 0.772 ^{b}	2.333 ± 0.050 ^{d}	7.757 ± 0.155 ^{c}
Soluble starch	12.817 ± 2.147 ^{b}	0.090 ± 0.039 ^{e}	151.466 ± 42.537 ^{a}
English potato peel	19.985 ± 2.555 ^{a}	5.732 ± 0.018 ^{a}	3.486 ± 0.435 ^{c}
Pineapple peel	22.848 ± 0.788 ^{a}	4.271 ± 0.966 ^{c}	5.469 ± 1.053 ^{c}
Pumpkin peel	13.009 ± 2.147 ^{b}	2.970 ± 0.098 ^{d}	4.394 ± 0.868 ^{c}
Sweet potato peel	21.733 ± 3.940 ^{a}	4.264 ± 0.126 ^{b}	5.113 ± 1.076 ^{c}
Ground corn	10.665 ± 4.158 ^{b}	0.785 ± 0.032 ^{e}	13.487 ± 4.573 ^{c}
Pumpkin peels/seeds	10.319 ± 0.951 ^{b}	5.681 ± 0.108 ^{a}	1.815 ± 0.133 ^{c}

Results analyzed by the Scott-Knott test, α = 0.05. Results expressed with standard deviations. The index representing the highest average is "a". Controls: Soluble starch and Pumpkin peel w/o glucose.

Characterization of Raw Amylases and Enzyme Cocktail

The fungus was cultivated under optimal conditions for amylase production. After obtaining the crude enzyme extract, the biochemical characterization of the free amylases in the extract was performed.

The results of the DCCR (Central Composite Rotatable Design) showed that the pH (x_2), the square of the temperature (x_1^2), and the square of the pH (x_2^2) were statistically significant, as the p-value was less than 5%, as shown in Equation 1. With an R^2 of 97.06%, the model describes the experimental data well (Figure 4 A). Analyzing the ANOVA (Analysis of Variance) using the Fischer-Snedecor test (F-test), it was concluded that the model obtained with regression was statistically significant. This is because the R^2 of the model was relatively high; the F-regression/residuals was higher than F-tab1, and the F-lack of fit/pure error was lower than F-tab[2]. Additionally, the p-value of the regression was less than 5%, while that of the lack of fit was higher (Table 7). Thus, Figure 4 B shows the response surface in 2D, where it can be observed that the optimal point of action for the raw amylases was at 60.0 ± 0.4 °C and pH 6.0 ± 0.2, with amylolytic activity (Y_{max}) of 17.498 ± 0.750 U.mL^{-1}.

$$Y_1 = 17.450 - 6.88.x_1^2 + 1.23.x_2 - 7.63.x_2^2 \quad (1)$$

Table 7. ANOVA Table for the DCCR pH and temperature optima.

Variation source[1]	Sum of squares	Degrees of freedom	Mean square	Fcalc	p-value
Regression	473.9	3	158.0	95.0	<0.01
Residuals	11.6	7	1.7	-	-
Lack of fit	8.1	5	1.6	0.9	0.59044
Pure error	3.5	2	1.7	-	-
Total	485.5	10	-	-	-

[1]The symbol "-" indicates absence.

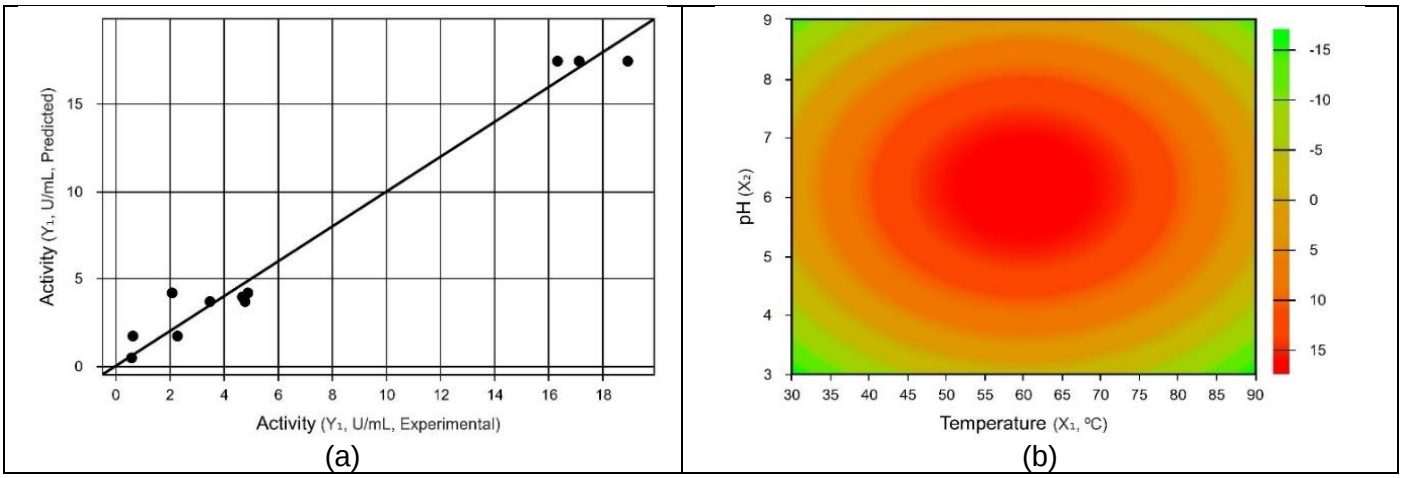


Figure 4. ANOVA Result for the DCCR: (a) Experimental versus predicted values by the quadratic model of activity as a function of temperature and pH; (b) Response surface in 2D for activity as a function of temperature and pH (colors indicate gradient, with more red indicating closer to the optimum point).

The validation of the optimum point (pH 6.0 and temperature of 60 ºC) was performed, and an experimental amylolytic activity (Y_{exp}) of 19.908 ± 1.781 U.mL^{-1} was obtained. Based on this, the difference between Y_{exp} and Y_{max} was calculated, and then divided by Y_{exp}, resulting in a variation of less than 10%, indicating that the model was validated.

Evaluating the stability of the crude amylases, it was observed that they remained stable for 240 minutes at 50 and 55 °C, being suitable for the hydrolysis of corn starch for a longer period of time (Figure 5A). Furthermore, they were able to maintain their stability above 50% at pH 4.0, 5.0, 5.5, and 7.0, at all analyzed time points (Figure 5B, C). While at other pH levels the enzymes were close to denaturation.

In 5 mM glucose, the activity decreased to 67% and with 10 mM to 30%. Therefore, in the presence of glucose, the amylases have a decreasing enzymatic activity. As the carbohydrate concentration increased, they were completely inhibited.

The fungus produced all tested enzymes, although the activity of the amylases was the most expressive and statistically the most significant, with an activity of 69.727 ± 3.271 U.mL^{-1}, which was also observed when evaluating the specific apparent activity. It is believed that due to the use of pineapple peel in its cultivation, a complex carbon source, different types of enzymes were produced to promote hydrolysis. In the crude extract, the total protein concentration was 2.801 ± 0.031 mg.mL^{-1}, and the specific apparent activity of the enzymes was obtained under the optimal conditions of the crude amylases (pH 6.0 and temperature of 60 °C). Therefore, it is assumed that the fungus mainly produces amylases under the established cultivation conditions (Figure 5D).

The enzymes were active in the presence of EDTA and the salts K_2SO_4, $MgSO_4.7H_2O$, and KI, providing an increase of more than 22% in enzymatic activity. The other salts inhibited a significant portion of the enzymatic activity, from 3 to 40%. Therefore, statistically, the salts that provided an increase in activity, such as K_2SO_4, were the most significant among those tested, indicating that the ions of these salts are optimal inducers of activity of the amylases produced under the best cultivation conditions of the fungus under study (Table 8).

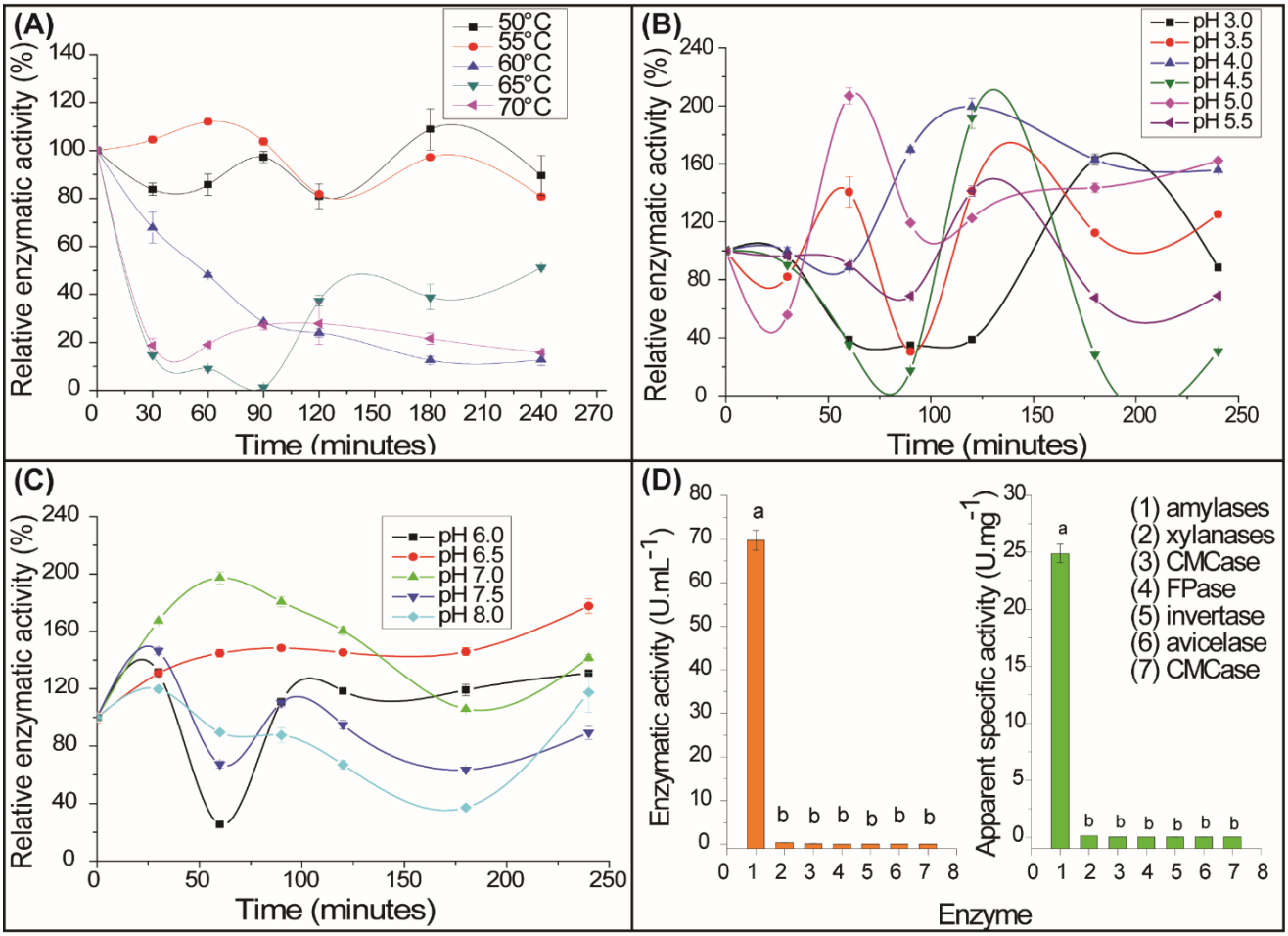


Figure 5. Relative activity: (a) Temperature and stability; (b) stability at pH 3.0 to 5.5; (c) stability at pH 6.0 to 8.0; (d) Enzymatic cocktail. The data is expressed together with the error bar.

Table 8. Chemical compounds incubated with the crude amylases.

Salts (5 mM)	Relative Activity (%)	Salts (5 mM)	Relative Activity (%)
Control	100 (± 2.01) [c]	$Ca(CH_3COO)_2.H_2O$	96.664 (± 4.61) [d]
K_2SO_4	126.459 (± 5.146) [a]	$Na_2B_4O_7.10H_2O$	96.74 (± 1.715) [d]
EDTA	125.095 (± 4.074) [a]	Na_2CO_3	93.404 (± 3.431) [d]
$BaCl_2$	121.683 (± 4.825) [a]	KH_2PO_4	88.931 (± 5.683) [e]
KI	122.517 (± 3.86) [a]	Na_3PO_4	87.87 (± 4.182) [e]
$MgSO_4.7H_2O$	123.275 (± 2.788) [a]	NH_4Cl	86.96 (± 2.895) [e]
Na_2SO_4	117.892 (± 4.182) [a]	$C_4H_{11}NO_3.HCl$	84.382 (± 4.825) [e]
$Ba(NO_3)_2$	112.813 (± 5.146) [b]	K_2HPO_4	85.368 (± 2.788) [e]
$Mg(NO_3)_2.6H_2O$	112.661 (± 2.788) [b]	$KMnO_4$	82.866 (± 4.825) [e]
$Na_2HPO_4.7H_2O$	110.462 (± 3.967) [b]	NaCl	83.7 (± 2.573) [e]
$NaNO_3$	109.553 (± 4.825) [b]	$ZnSO_4.7H_2O$	83.169 (± 2.466) [e]
$FeSO_4.7H_2O$	104.094 (± 7.827) [c]	$CaCl_2$	84.155 (± 1.072) [e]
NH_4CH_3COOH	110.614 (± 0.965) [b]	$CuSO_4$	76.725 (± 3.431) [f]
$Na_2S_2O_3.5H_2O$	106.293 (± 2.359) [c]	$NaHCO_3$	77.559 (± 2.466) [f]
KCl	105.838 (± 1.715) [c]	$Ca(H_2PO_4)_2.H_2O$	77.483 (± 2.359) [f]
$(NH_4)_2CO_3$	104.397 (± 1.823) [c]	$CaCO_3$	77.255 (± 2.037) [f]
NaH_2PO_4	100.531 (± 4.932) [c]	$NH_4H_2PO_4$	66.49 (± 0.107) [g]
$CH_3COONa.3H_2O$	96.816 (± 6.111) [d]	$Ca(NO_3)_2.4H_2O$	59.818 (± 5.254) [g]
$Na_3C_6H_5O_7$	96.285 (± 5.361) [d]		

The data were analyzed using the Scott-Knott means test, with a significance level of α = 0.05. No salts were added to the control.

DISCUSSION

The ITS region is sufficient to identify species belonging to the genus *Rhizopus* sp. [29]. The structures observed by SEM have also been reported by other authors analyzing fungi of the genus *Rhizopus*, who describe long, tubular sporangiophores and sporangia, which are sacs enclosing spores with ellipsoidal shapes [30-31]. The sporangiophore is characteristic of fungi like *R. microsporus*, currently classified in the family Rhizopodaceae, such as *R. arrhizus* (*R. oryzae*) [28, 32].

The CP medium is well-suited for enzyme production as it is essential for the growth of filamentous fungi [33]. It has been noted in the literature that *R. oryzae* produced more active amylases in CP and Adams media, around 2.9 $U.mL^{-1}$, as yeast extract is essential for the microorganism [34]. The growth time is also an important factor, as there is an adaptation phase to the medium, as well as a growth and mass production phase of primary metabolites, such as enzymes [35-36].

Nitrogen compounds like urea are essential for the synthesis of amino acids in the biomass of *Rhizopus* fungi [37]. Peptone and urea were highly beneficial for the production of amylases by *R. oligosporus*, while yeast extract did not have the same effect [38]. Additionally, urea is a relatively cheaper organic nitrogen source than peptone and yeast extract, which encourages its use in culture media [37].

Microorganisms require salts in the growth and enzyme production process. However, solutions with high salt concentrations inhibit enzyme production. This phenomenon is observed in the cultivation of *Rhizopus* sp., which produced amylases with more significant activity in the presence of SR salts than in KH salts [34, 39]. KH_2PO_4 and $MgSO_4.7H_2O$ salts induce the production of more expressive amylolytic enzymes by filamentous fungi [39].

Extracellular pH is one of the factors that affect the growth and enzyme release of *Rhizopus oryzae* [40]. It has been reported that the filamentous fungus *Rhizopus* sp. AL131 produced amylases with more significant activity at an initial pH of 4.5 [39]. Meanwhile, *Rhizopus stolonifer* produced more active amylases at an initial pH of 6 to 7 [41].

The cultivation of *Rhizopus oligosporus* resulted in higher enzymatic activities with smaller inocula, while the protein concentration increased with the size of the inoculum [42]. A high number of spores causes overcrowding in the medium, leading to competition and rapid nutrient depletion, resulting in a reduction in enzyme production [40].

Pineapple peel is one of the by-products that is generated in greater proportion during pineapple processing and causes a great deal of environmental pollution when improperly disposed of. It has a great potential to be used as an inducing carbon source in amylase production due to their starch content [43-44]. This by-product has also many bioactive compounds that could be of interest to the food, pharmaceutical, and cosmetic industries. There is a current trend to develop environmentally friendly extraction processes to obtain good yields of high-added valued compounds [45]. To demonstrate the great potential of pineapple peels as a substrate for obtaining bioactive compounds, in this study we were able to produce high amounts of amylase for the first time using this residue during the fermentation of *Rhizopus arrhizus*. Also, Pumpkin and potato peels contain considerable carbohydrate fractions and are very interesting carbon sources for enzyme production [46-47]. In this study, we were also able to produce high concentrations of amylase using these two residues, which is the first report in the literature.

Glucose is a carbohydrate easily absorbed by fungi [39]. Other authors affirm its ability to improve amylase production through glucose supplementation in the fermentation of *Rhizopus stolonifer*, *Aspergillus oryzae*, and *Penicillium chrysogenum* [41].

The optimal temperature represents the point at which the substrate hydrolysis level is maximum, and it is also the condition in which the enzyme is in its ideal conformation. A temperature increase up to a certain point increases the speed of the atoms and consequently, the interaction, leading to the formation of products. However, there is a point beyond which enzymes begin to lose their conformation. Additionally, pH influences the interaction with the substrate, as enzymes may have ionizable active sites, protonating or deprotonating amino acid residues essential in catalysis [48-50].

Some authors report that the optimal point for amylases produced by *Rhizopus microsporus* var. *oligosporus* and *Rhizopus stolonifer* was around 37 °C and in the pH range between 5.0 and 6.0 [51-52]. The amylases produced by *Rhizopus* sp. AL131 under the best cultivation conditions had an optimum pH of 5.0 at 50 °C [39]. Regarding temperature stability, amylases were stable in the range of 40 to 60 °C, maintaining up to 57.6% of the activity after 60 minutes of incubation [53]. It is worth pointing out that in different temperatures and pH, it may happen conformational changes in the enzyme [54]. Because of that, effectors/inhibitors (ions) that activate or inhibit the enzyme may have favorable access to it, leading to a significant fluctuation in the activity, as observed.

The inhibition or activation of amylases by ions depends on their origin; therefore, some ions formed from their salts can act as enzyme cofactors or activity inhibitors during hydrolysis [55]. It has been reported that in the presence of 5 mM copper and iron (II) ions, amylase activity is strongly reduced [56]. Some studies report that EDTA, a chelating agent, has no positive effect on amylases [57]. Other authors have reported that amylases may not lose activity in the presence of EDTA, but they slightly decrease in the presence of calcium, sodium, and zinc ions [56, 58]. One reason for the reduction in activity in the presence of EDTA is that it covalently binds to essential metal ions in the active sites of enzymes [59]. Contrary to the cited studies, in this study, the results with EDTA were promising, indicating that the crude enzymes produced do not require metal ions to function.

The addition of glucose to the reaction was investigated, and the amylolytic activity decreased to 63% with increasing carbohydrate concentration [60]. Enzymes have domains whose function is to bind to the substrate and carry out hydrolysis at specific bonds; thus, glucose residues have the ability to strongly interact with the active sites of amylases through hydrogen interactions with amino acids, such as glutamic acid and aspartic acid [61-63].

In the hydrolysis of complex substrates, several enzymes are released, including various types of amylases, which act synergistically to hydrolyze the available starch and release fermentable sugars [25]. *Rhizopus* sp. AL131 produced amylases with activity around 20 $U.mL^{-1}$ [39]. The species *R. oryzae* UC2 was investigated in the production of cellulases and xylanases in the presence of agroindustrial residues, and analyzing the crude extract showed a considerable production of xylanase, CMCase, and FPase [26]. It was also found that avicelase was produced by *Aspergillus flavus* L-2007/2012 in the presence of cellulose residue [27]. Although there are no studies from the last two years that produced avicelases and invertases using the fungus under study, *R. arrhizus* I 1.2.1 has shown the ability to produce different types of enzymes, enabling future application in the hydrolysis of more complex substrates.

Funding: This research was funded by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.
Acknowledgments: The authors acknowledge the contributions of the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001, the Universidade Federal dos Vales do Jequitinhonha e Mucuri, CNPq, and the support from LMMA sponsored by FAPEMIG (CEX-112-10), sects/MG and RQ-MG (Fapemig: CEX-RED-00010-14).
Conflicts of Interest: The authors declare no conflict of interest.

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