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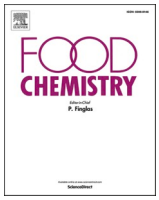

Production and application of inulinase by new isolates of *Aspergillus welwitschiae* from fermented peach-palm waste for the production of fructooligosaccharides

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ABSTRACT

Microbial inulinase enzymes have a number of applications in biotechnology. In this study, new strains of *Aspergillus welwitschiae* were investigated as producers of inulinases and their *endo*- and *exo*-inulases were characterized *in silico* and their protein modeling was performed. The inulinase production by *A. welwitschiae* employing the Dohelert method to assess the interaction between temperature and cultivation humidity, resulted in a fourfold increase in activity. The optimal temperature and humidity were 25–27 °C and 75–82 %, respectively. The enzyme exhibited optimal activity at a pH of 3.5 and at 60 °C. The hydrolysis of the sisal root yielded 272 mg/g of reducing sugars and oligosaccharides. Our *in silico* experiments predicted 10 well-validated structures of *endo*- (5) and *exo*-inulinases (5) from *A. welwitschiae*. The notable activity of these inulinases on inulin highlights their potential for the treatment of agro-industrial residues with the objective of producing high-value added products.

1. Introduction

Inulinases are enzymes produced by plants, filamentous fungi, yeasts, and bacteria, which have several biotechnological applications due to their mechanism of inulin hydrolysis, a fructose polymer present in some plants. They are used in the production of fructooligosaccharides (FOS), food preparations, pharmaceutical and beverage industries, ethanol, sorbitol, citric and lactic acids, and other chemicals, as well as in energy production and bioplastics (Dotsenko et al., 2023). They can be presented as *exo*-inulinases (fructan β-fructosidase) and *endo*-inulinases (β-2-1-D-fructan fructan hydrolase) and can act alone or synergistically hydrolyzing inulin-producing fructans such as fructooligosaccharides (FOS) (Holyavka et al., 2018; Hoshida et al., 2018; Saleh, Abd El-Galil, Sakr, Taie, & Mostafa, 2020).

Several studies have demonstrated the production of inulinase by different microorganisms, including fungi such as *Aspergillus sp.* (Guerrero-Urrutia, Volke-Sepulveda, Figueroa-Martinez, & Favela-Torres, 2021), *Fusarium sp.* (Kamble, Suryawanshi, Jadhav, & Attar, 2019), *Rhizopus oligosporus* (Mohamed, Salah, Moharam, Foda, & Fahmy, 2015), among others. However, there are few reports using *Aspergillus welwitschiae* (Dotsenko et al., 2023; Saleh et al., 2020; Stojanović et al., 2022). This filamentous fungus, belonging to the Nigri section of the genus *Aspergillus*, is the closest phylogenetic taxon to *Aspergillus niger*, a species used in various activities in the biotechnology industry (Quintanilha-Peixoto et al., 2019).

Commercial inulin is present in some plants (artichoke, asparagus root extracts, dahlia tuber, and dandelion root extract) and are considered the best inducer source for inulinase production (Holyavka et al.,

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2018; Mao et al., 2019; Singh, Singh, Hassan, & Kennedy, 2020). However, studies using substrates derived from agro-industrial residues for inulinase production by fermentation, such as carrot pulp (Singh, Chauhan, & Kennedy, 2017), wheat bran (Chen, Chen, Chen, Xu, & Jin, 2011; Trivedi, Divecha, & Shah, 2012), and sugarcane bagasse (Silva et al., 2013), have been conducted, but no studies reporting the production of inulinase from fermentation of peach palm, sisal, and cocoa husk residues were found.

Enzyme production costs are strongly associated with investment capital, which accounts for about 50 %, with one-third of this represented by raw materials. Therefore, it is interesting to look for ways to reduce these costs, such as the use of agro-industrial residues (Prado-Acebo, Cubero-Cardoso, Lu-Chau, & Eibes, 2024). The use of agro-industrial residues may result in a higher return on investment, as well as being considered an environmentally friendly alternative for these materials, since it is estimated that annually 5 billion tons of residues from agricultural activities are generated globally (Yafetto, 2022).

Peach-palm (*Bactris gasipaes* Kunth.) processing data indicate that approximately 84 % of the total weight of the palm is wasted. The peach palm strain has in its composition 34.2 % cellulose; 21.3 % hemicellulose; 19.5 % lignin (de Cássia Spacki et al., 2022). These residues can be used as a carbon source for enzyme production, as well as contributing to reducing environmental pollution, and lowering the final cost of the produced enzymes, adding greater value to this residue (Melo Neto, Carvalho, Costa, Uetanabaro, & Melo, 2021).

In Brazil, activities for obtaining cocoa beans generate around 42 thousand tons of residues, and to obtain a ton of cocoa almond, 80–120 kg of the seed husk is generated after processing (Casteliano et al., 2022). Sisal (*Agave sisalana)* is also an important residue, as it is widely distributed in the Northeast region of Brazil, where it is exploited only as a source of hard fiber, with Brazil being the world's largest exporter. Furthermore, some studies have demonstrated the potential of *A. sisalana* in various new biotechnological applications as a source of secondary metabolites and new products of interest for the pharmaceutical and food industries (Apolinário et al., 2017).

Recent studies on the production and characterization of inulinases have revealed significant advances in understanding the molecular mechanisms and optimizing the production of these enzymes, with potential applications in the synthesis of fructooligosaccharides (FOS) and other products of industrial interest. For instance, N-glycosylation, for example, has been identified as a crucial factor affecting the catalytic parameters and active site dynamics of *exo*-inulinase from *Aspergillus awamori*, thereby undercoring the impact of post-translational modifications on enzymatic efficiency (Dotsenko, Denisenko, Zorov, Rozhkova, & Shashkov, 2025). Moreover, inulinase cocktails derived from *Aspergillus welwitschiae*, cultivated in media comprising agromaterials that stimulate FOS production, have demonstrated potential for the generation of this bioproduct, emphasizing the feasibility of utilizing agro-industrial substrates for biotechnological applications (Stojanović et al., 2022). Another study employing coffee residues and extracts as substrates for the production of inulinase, β-mannanase, and oligosaccharides corroborates the significance of agro-industrial waste in enzymatic bioconversion (Basmak & Turhan, 2024). The investigation of the interaction between inulin and inulinases from *Botrytis cinerea* has yielded significant insights into oligosaccharide formation, offering a new perspective on enzyme engineering (Versluys, Porras-Domínguez, Voet, Struyf, & Van den Ende, 2024). Ultimately, the sequential optimization of solid-state fermentation parameters for inulinase production by *Kluyveromyces marxianus* using brewery waste exemplifies the potential of biotechnology in industrial processes, particularly in the sustainable production of enzyme from waste materials (Yupanqui-Mendoza, Vaz de Arruda, & Castelo da Silva, 2022).

The objective of this study was to analyze the new strains of *Aspergillus welwitschiae* as producers of inulinases and to characterize and predict their 3D structures for both *endo*- and *exo*-inulases using computational approaches. *In silico* experiments are relatively faster in comparison to wet lab techniques, and they could complement the understanding about how these fungal inulinases catalyze inulin, as well as they can foster new studies in bioengineering and innovation for using this type of enzyme with different applications. Additionally, the objective was to ascertain the optimal fermentation conditions for the production of inulinases by the fungus *A. welwitschiae* using low-cost substrates and to evaluate the potential of novel sources of agro-industrial residues for the production of oligosaccharides and reducing sugars from enzymatic hydrolysis.

2. Materials and methods

2.1. Chemical reagents

Inulin was obtained from Megazyme, sucrose from Vetec, and Potato Dextrose Agar (PDA) from Acumedia Neogen. All other chemical reagents used in this work were of analytical grade.

2.2. Obtaining and maintaining microorganisms

The fungi *A. welwitschiae* CMB663 (CCI, under the GenBank accession code GCA_023718355.1) and *A. welwitschiae* CCMB674 (CSDI, accession code GCA_009761105.1) were provided by the culture collection of the Microbiology Laboratory at the Federal University of Minas Gerais - UFMG. The microorganisms were cultivated on PDA at 30 °C for up to 6 days and stored at 4 °C until use.

2.3. In silico characterization of endo and exo-inulinases from A. welwitschiae

The genomes of isolates CCMB663 and CCMB 674 of *A. welwitschiae* were sequenced and annotated as seen in Quintanilha-Peixoto et al. (2019). Briefly, isolate CCMB 663 (under the GenBank accession code GCA_023718355.1) and CCMB 674 (accession code GCA_009761105.1) were annotated with the MAKER2 workflow (Holt & Yandell, 2011), using a set of protein sequences of *A. welwitschiae* and *Aspergillus niger* as input for *ab initio* gene predictors SNAP (Korf, 2004), GeneMark (Lukashin, 1998), and Augustus (Stanke, Steinkamp, Waack, & Morgenstern, 2004).

Endo- and *exo*-inulinases from these two isolates were identified through similarity analysis with BLASTp against 11 curated inulinases sequences from UniProt (Q96TU3.1, A2R0E0.1, Q76HP6.1, E1ABX2.1, O74641.1, A5ABL2.1, O94220.1, O74642.1, P28999.1, A8W7I5.3, and A5DHM6.2), and then latter confirmed through the presence of the typical inulinase protein domain Glycoside hydrolase, family 32 (IPR001362) on InterProScan (Blum et al., 2020).

2.4. Molecular modeling of the endo and exo-inulinases from A. welwitschiae CMB663 (CCI) e A. welwitschiae CCMB674 (CSDI)

The corresponding endo and exo-inulinases amino acid sequences were initially submitted to BLASTp (https://blast.ncbi.nlm.nih.gov/) [PMID: 2231712] for performing an alignment with all protein sequences deposited in the Protein Data Bank (PDB) (https://www.rcsb.org/) to find homologous templates to be used in the 3D modeling process. Endo and exo-inulinase sequences were then submitted to the Swiss-Model webserver (https://swissmodel.expasy.org/) [PMID: 29788355] for protein homology modeling, following the steps: (I) confirming previously selected 3D templates from BLASTp against PDB, (II) Automated rigid-body protein modeling based on the sequence-template structural alignment and (III) Protein assessment evaluation. The best models were selected according to QMEANDisco quality with values between 0.6 and 1, with the last one representing the best quality close to crystallographic models, as well as GMQE (Global Model Quality Estimation) that is another important parameter in SWISS-MODEL, used to predict the overall quality of a protein model even

before its construction. Values of GMQE close to 1 represent models with higher quality. Furthermore, we checked the built models for possible structure clashes using the Pymol Molecular Graphics System, Version 3.0 (Schrödinger, 2023).

2.5. Culture media

The root and trunk of sisal (*Agave sisalana*) and the agro-industrial wastes cocoa husk (*Theobroma cacao*) and the cocoa bean peel were obtained from local producers, and the agro-industrial peach-palm (*Bactris gasipaes* Kunth.) waste was obtained from the company Inaceres, Uruçuca, Bahia, Brazil. The residues were dried in an oven (SOLAB) at 40 °C for 5 days, and ground in a Wiley type mill to a particle size of 2 mm. Soy flour was purchased from a local market in the municipality of Itabuna, Bahia. The root and trunk of sisal and the cocoa husk and peach-palm waste were used as carbon sources and the cocoa bean peel and soy flour as nitrogen sources.

2.6. Solid-state fermentation (SSF) for Inulinase production

The initial fermentation was performed using 2.5 g of sisal root and trunk, peach-palm waste, and cocoa husk and 0.5 % glucose. The residues were moistened up to 80 % using Czapek salt medium (0.3 % $NaNO_3$, 0.1 % $KHPO_4$, 0.05 % $MgSO_4 \cdot 7H_2O$, 0.05 % KCl, and 0.001 % $FeSO_4 \cdot 7H_2O$; pH 6.0). After sterilization in an autoclave at 121 °C for 15 min, the medium was inoculated with 10^6 spores/mL of medium. Fermentation was conducted for 5 days, without agitation at 28 °C. To test the effect of different moistening agents on SSF, a test was conducted with four different moistening agents using peach-palm waste as substrate. The moistening agents tested were tap water (potable water), Czapek salts (as described above), MSS-1 (0.3 % $NaNO_3$, 0.1 % $KHPO_4$, 0.05 % $MgSO_4$ $7H_2O$, 0.05 % KCl, and 0.001 % $FeSO_4$ $7H_2O$; pH 5.0) and MSS-2 (0.3 % $(NH_4)2SO_4$, 0.3 % KH_2PO_4 and 0.6 % CH_3COONH_4; pH 6.0). After selecting the best moistening agent for enzyme production, experiments were conducted to verify the influence of glucose in SSF for enzymatic production. The experiment was carried out with peach-palm waste (2.5 g) with the addition of 0.5 % glucose compared to cultivation containing only 2.5 g of peach-palm waste. The cultures were moistened with MSS-1 solution at 80 % humidity. To identify the best time for the production of inulinase enzyme, fermentations were conducted over different incubation periods using peach-palm waste (2.5 g), 80 % of MSS-1 as a wetting agent, and the cultures were carried out over times of 3, 5, 8, 13, and 15 days. To test the influence of nitrogen source on enzyme production, fermentations were conducted using peach-palm waste (2.5 g) added with different nitrogen sources at 1 % (peptone, cocoa bean peel, and soy flour). The cultures were moistened with MSS-1 (80 %) and the fermentation was conducted at 28 °C for 5 days. All experiments were carried out in triplicate.

2.7. Obtaining crude enzymatic extract (CEE)

After fermentation, the crude enzymatic extract (CEE) was extracted with 25 mL of distilled water. The culture was thoroughly mixed with a glass rod and then placed on a rotary shaker (150 rpm) for 15 min, filtered through four-fold gauze, and centrifuged at 10,000*g* for 10 min. The supernatant was considered as the CEE and was analyzed for inulinase activity (Carvalho et al., 2018).

2.8. Enzyme assay and protein quantification

The enzymatic activity of inulinase was determined by the DNS method (Miller, 1959). The method involved 125 μL of a 1 % inulin solution prepared in 50 mM sodium acetate buffer pH 4.5, and 125 μL of the sample in test tubes incubated in a thermostated bath at 40 °C for 30 min. After the reaction time, 250 μL of DNS reagent was added, boiled for 5 min, and topped up with distilled water to a final volume of 2.5 mL. An enzymatic unit was defined as the ability to release 1 μmol of fructose from inulin per minute under assay conditions. Protein was estimated by the Bradford (1976) using bovine serum albumin as a standard and absorbance at 595 nm.

2.9. Optimization of Inulinase production

To obtain the best cultivation conditions for inulinase production, a statistical design using the Dohlert matrix with two variables was applied to observe the simultaneous, systematic, and efficient variation of important components on the fermentation process. Peach-palm waste was selected as the substrate for optimization studies and MSS-1 was used as the moistening agent. Two parameters, moisture (U) and temperature (T), were analyzed, and inulinase activity (Z) was the response variable. The independent temperature variable was studied at three levels (20 °C, 30 °C, and 40 °C) and moisture at five levels (50 %, 60 %, 70 %, 80 %, 90 %). The mathematical correlation between the two variables in the production of inulinase included a total of 9 experiments with three repetitions at a central point. The fermentation time was 5 days for all experimental runs.

The replicability of the method was verified through validation tests conducted at distinct experimental points, differing from those initially analyzed but within the same experimental domain. For *A. welwitschiae* CCI, validation was performed at 30 °C with 80 % moisture content, and at 27 °C with 82 % moisture content. For *A. welwitschiae* CSDI, the method was validated at 30 °C with 80 % moisture content, and at 26 °C with 70 % moisture content. These tests confirmed the robustness and consistency of the experimental approach.

2.10. Partial purification of Inulinase

The crude enzymatic extract was partially purified on peach-palm activated charcoal (Santos et al., 2020). 50 mg of activated charcoal was added to 5 mL of crude inulinase extract (5 U/mL). This mixture was incubated on a shaker at 120 rpm, 25 °C for 14 h (overnight) and after the incubation period the samples were centrifuged at 4000*g* for 10 min, the supernatant was removed and the supports were washed with distilled water to remove adsorbed compounds. The wash consisted of placing the activated charcoal in contact with distilled water for 10 min on a shaker at 120 rpm, followed by a centrifugation step as described above. After this procedure, the obtained samples were analyzed for total protein concentration (Bradford, 1976) and enzymatic activity (Miller, 1959).

2.11. Optimal pH and temperature and stability

A Doehlert experimental design of two variables was conducted to verify the interaction between pH and temperature, aiming to find an optimal point for enzyme activity. The independent temperature variable was studied at three levels (50 °C, 60 °C, and 70 °C) and pH at five levels (2.0; 3.0; 4.0; 5.0; and 6.0) with a total of 9 experiments with three repetitions at a central point. Enzymatic activity was analyzed under standard assay conditions. To determine stability at different temperatures, the enzymatic extract was incubated for 30 min and 1 h at temperatures ranging from 30 to 60 °C. Enzymatic activity was measured under standard assay conditions and the residual activity was expressed as the percentage (%) of the initial activity.

Validation tests were conducted at pH 3.0 and 60 °C for both isolates, *A. welwitschiae* CCI and *A. welwitschiae* CSDI.

2.12. Influence of ions and other compounds on enzymatic activity

Different ion salts were added to the enzymatic assay ($CaCl_2$, $CoCl_2$, $CuSO_4$, $FeCl_3$, KCl_2, $MgCl_2$, $MnCl_2$, NaCl, and $ZnSO_4$) and the compounds β-mercaptoethanol and EDTA (ethylenediaminetetraacetic acid) at 1 mM and SDS (sodium dodecyl sulfate) at 0.1 % were also added. The

enzymatic activity was analyzed by the standard assay method and expressed as a percentage (%) compared to the assay without the addition of any ion or reagent (control). All assays were carried out in triplicate.

2.13. Application of *A. welwitschiae* CCI inulinase in the hydrolysis of commercial inulin and sisal root

After the enzymatic extract was semi-purified with peach-palm charcoal, a volume of 3 mL (50 U/g) of inulinase enzyme in 0.05 M sodium acetate buffer pH = 4.5 was added to dehydrated sisal root and commercial inulin. The conditions for sisal treatment were 3 % at 40 °C for 5 h, 3 % at 50 °C for 6 h and 4 % at 55 °C for 7 h. The conditions for commercial inulin were 3 % at 40 °C for 5 h, 3 % at 50 °C for 6 h and 4 % at 55 °C for 7 h. The control for each reaction was carried out using acetate buffers instead of the enzyme. After the hydrolysis process, the supernatant was obtained by centrifugation at 5000*g* at 4 °C for 3 min, and the released reducing sugars were quantified by the DNS method (Miller, 1959), using fructose as the standard. The reading was performed on a spectrophotometer at 540 nm. The reducing sugars produced were expressed in milligrams per gram of sisal hydrolysate (mg/g). The hydrolysis products were evaluated by thin-layer chromatography (TLC). The sample run on the TLC plate (0.20 mm thickness of silica gel 60, MN) was performed with a mixture of acetonitrile and water (8.5:1.5 *v*/v) as the solvent. Compounds were detected by spraying with a mixture of methanol and sulfuric acid (9:1 v/v) and subsequently heating the plate at 100 °C for 5 min (Jayapal et al., 2013). Starch, sucrose, and glucose (4 mg/mL) were used as reference standards.

3. Results and discussion

3.1. Molecular modeling of *A. welwitschiae* CCMB663 (CCI) CCMB674 (CSDI) inulinases based on their genomes

All the 3D structures modeled for both *A. welwitschiae* CCI and *A. welwitschiae* CSDI had their models validated by GMQE and QMEANDisco values. The Table 1 brings all details about templates and model assessment for each modeled protein.

All 3D models from *A. welwitschiae* CCMB663 (CCI) had their structures (Fig. 1A to E) validated by GMQE (between 0.90 and 0.98) and QMEANDisco (between 0.88 and 0.95) values in which all of them were positive. In addition, their template coverage ranged from 96 to 100 % and identity from 86 to 100 %.

A. welwitschiae CCMB674 (CSDI) (Fig. 2A to E) followed similar prediction validations with GMQE between 0.90 and 0.99, as well as QMEANDisco ranging from 0.88 to 0.95. Futhermore, *A. welwitschiae* CSDI presented templates with sequence alignment coverage between 96 and 100 %, and identity 86–99 %. These results indicate that all models reproduced well-validated crystallographic templates and could be used for further *in silico* predictions such as docking with their natural substrates and molecular dynamics simulations.

3.2. Solid-state fermentation (SSF)

Four low-cost substrates were used, two agro-industrial residues (peach-palm waste and cocoa husk) and two natural substrates (sisal root and trunk) for SSF. For both *A. welwitschiae* isolates CCI and CSDI, only the use of peach-palm waste as substrate showed a significant difference in inulinase production with enzymatic activity of 10.58 U/g (CCI) and 12.23 U/g (CSDI) as seen in Fig. 3A.

The utilization of agro-industrial residues for inulinase production represents a promising strategy for the enhancement of by-product value, as well as a sustainable and cost-effective approach to biotechnology. The present study demonstrated that the use of peach-palm residue as a substrate for inulinase production yielded significant results in terms of enzymatic yield. In comparison to alternative substrates, such as coffee residues and extracts, the inulinase production in this study demonstrated comparable efficiency, thereby substantiating the feasibility of utilizing diverse agro-industrial residues for this purpose (Basmak & Turhan, 2024).

Moreover, inulinase cocktails derived from *Aspergillus welwitschiae* isolate FAW1, employing agromaterial inducers, demonstrated considerable FOS production yields, emphasizing the potential of diverse agro-industrial residues for bioconversion, resulting in the production of an inulinase in this study (1.1–5.6 U/mL). These results were found to be highly similar to those previously reported by Stojanović et al. (2022) who observed values of 1–1.2 U/mL. The inulinase production obtained through solid-state fermentation (SSF) using peach-palm waste in the present study was found to be 10.58–12.58 U/g. This activity was higher than that observed by *Penicillium amphipolaria* using sugarcane stalks (5.52 U/g), indicating that lignocellulosic substrates with high carbon content may be a highly promising source for large-scale enzyme production (Das, Bhat, & Selvaraj, 2020).

The physicochemical, kinetic, and thermodynamic studies on *Aspergillus welwitschiae* MN056175 inulinase with the extraction of prebiotic and antioxidant fructo-oligosaccharides from *Cynara scolymus* leaves also reported an inulinase production similar to that found in this study (3 U/mL) (Saleh et al., 2020). The use of peach-palm wastes may be aligned with this trend, offering a nutrient-rich substrate that is available in large quantities in regions where production is concentrated.

Considering the economic viability of using peach-palm waste, this option deserves special attention due to the abundance and low cost of this material, which is often discarded or underutilized. It is estimated that approximately 5420 tons of peach-palm waste are generated annually in Brazil (Carvalho et al., 2018; De Carvalho et al., 2023). Converting of this residue into a valuable substrate for inulinase production not only reduces production costs but also mitigates the environmental impact associated with waste accumulation. Compared to other residues, such as coffee extracts and carob, peach-palm residue offers an additional economic advantage because it is widely available in

Table 1
Modeled endo and *exo*-inulinases from both *A. welwitschiae* CCMB663 (CCI) and CCMB674 (CSDI).

Strain	Sequence	Type	Template	Cov. (%)	Identity (%)	GMQE	QMEANDisco
	CCMB663_NODE_2gene2	Endo	3RWK	100	100	0.98	0.95
	CCMB663_NODE_31gene3	Exo	1Y4W	96	92	0.96	0.95
CCMB663	CCMB663_NODE_53gene1		5XH8	96	97	0.90	0.88
	CCMB663_NODE_76-gene0		A0A317W028*	100	86	NA	0.91
	CCMB663_NODE_106-gene0		A2QLU1*	100	99	NA	0.92
	CCMB674_NODE_7-gene7		5XH8	96	97	0.90	0.88
	CCMB674_NODE_7-gene8		A2QLU1*	100	98	NA	0.92
CCMB674	CCMB674_NODE_12-gene6	Endo	3RWK	100	99	0.99	0.94
	CCMB674_NODE_19-gene3	Exo	1Y4W	96	92	0.96	0.95
	CCMB674_NODE_38-gene2		A0A317W028*	100	86	NA	0.91

* AlphaFold2 template.

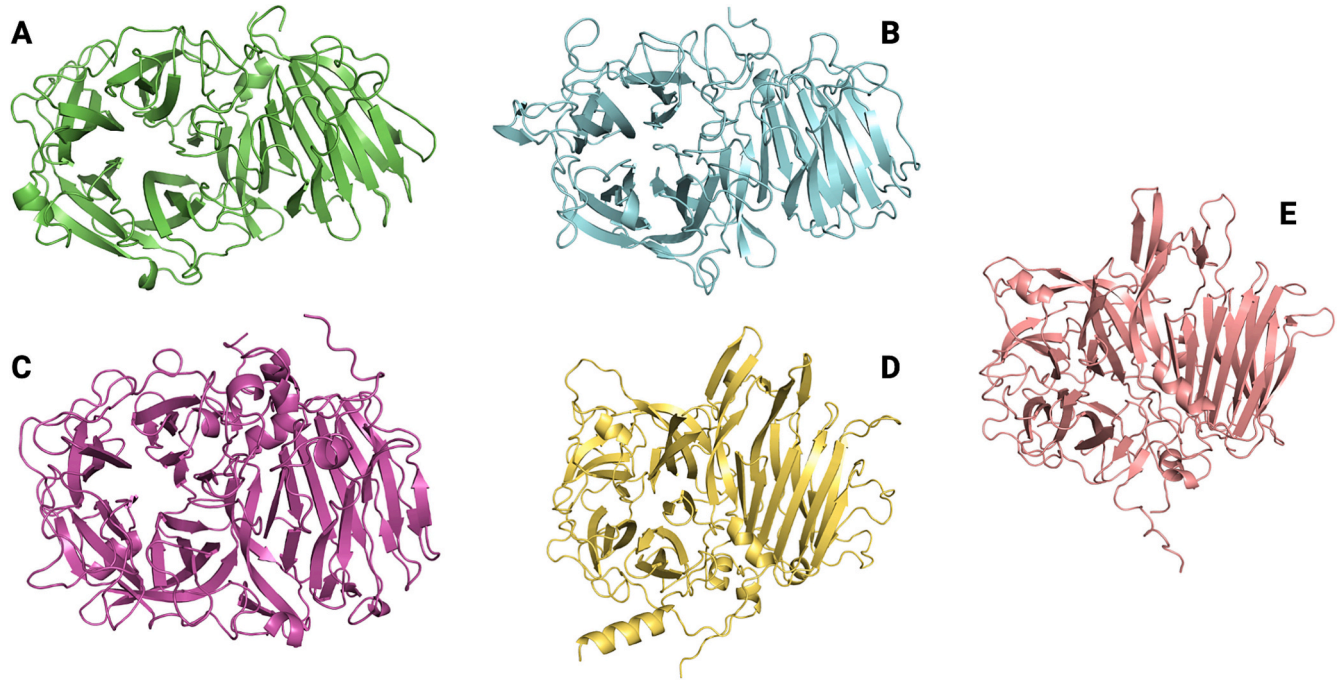

Fig. 1. 3D modeled structures from *A. welwitschiae* CCMB663 inulinases. **A:** CCMB663_NODE_2gene2, **B:** CCMB663_NODE_31gene3, **C:** CCMB663_NODE_53gene1, **D:** CCMB663_NODE_76-gene0, **E:** CCMB663_NODE_106-gene0.

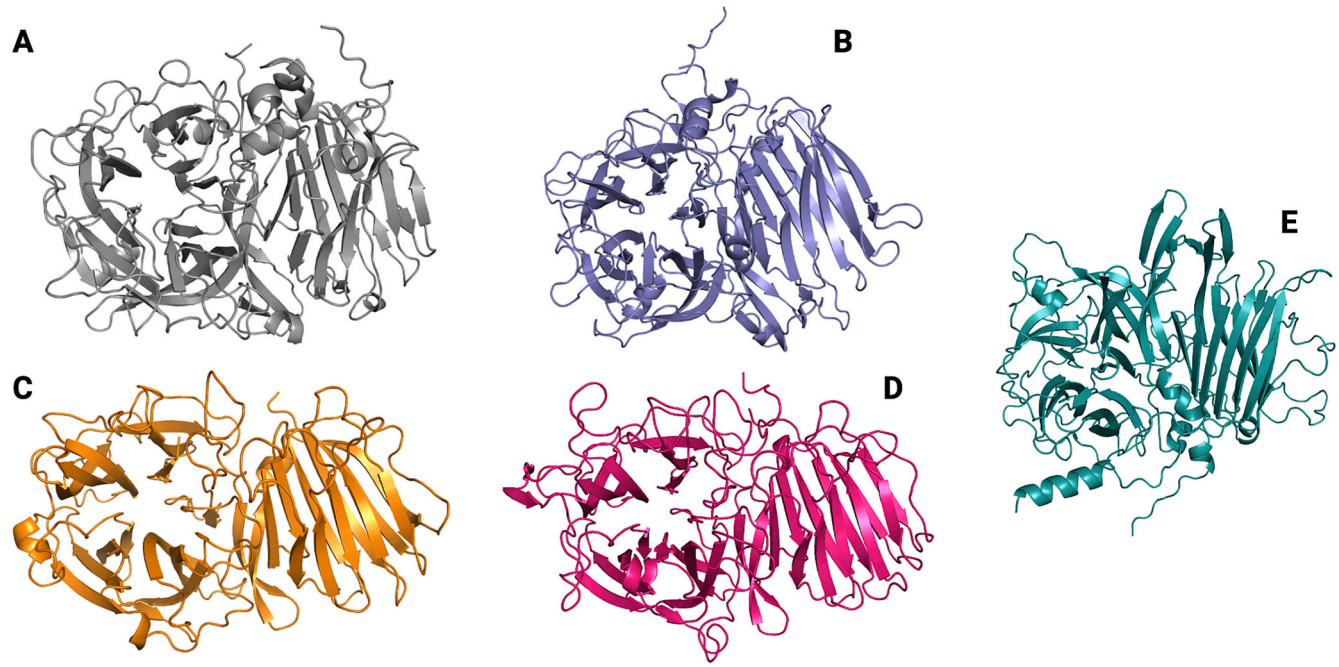

Fig. 2. 3D modeled structures from *A. welwitschiae* CCMB674 inulinases. **A:** CCMB674_NODE_7-gene7, **B:** CCMB674_NODE_7-gene8, **C:** CCMB674_NODE_12-gene6, **D:** CCMB674_NODE_19-gene3, **E:** CCMB674_NODE_38-gene2.

tropical regions and can be easily incorporated into solid-state fermentation processes, which have already been shown to be effective in increasing enzyme yields (Melo Neto et al., 2021).

The *Aspergillus* genus is widely explored for biotechnological studies and includs a wide variety of species. In the case of *A. welwitschiae*, there are few studies related to biotechnological applications and enzyme production for this species of fungus, but production of inulinases by *A. welwitschiae* MN056175 has been recently reported (Saleh et al., 2020; Stojanović et al., 2022). However, *A. welwitschiae* CCI and CSDI were studied for the first time in this work for the production of inulinases with potential for biotechnological applications.

The high efficiency of the fermentation process for enzymatic production is directly linked to adequate cultivation conditions. Despite the advantages and wide applicability, the biggest problem with enzyme production lies in the high costs (Ilgın, Germec, & Turhan, 2019). The use of low-cost substrates, such as natural substrates and agro-industrial residues, offers some advantages such as cost reduction in the enzymatic production process, in addition to reducing potential impacts related to

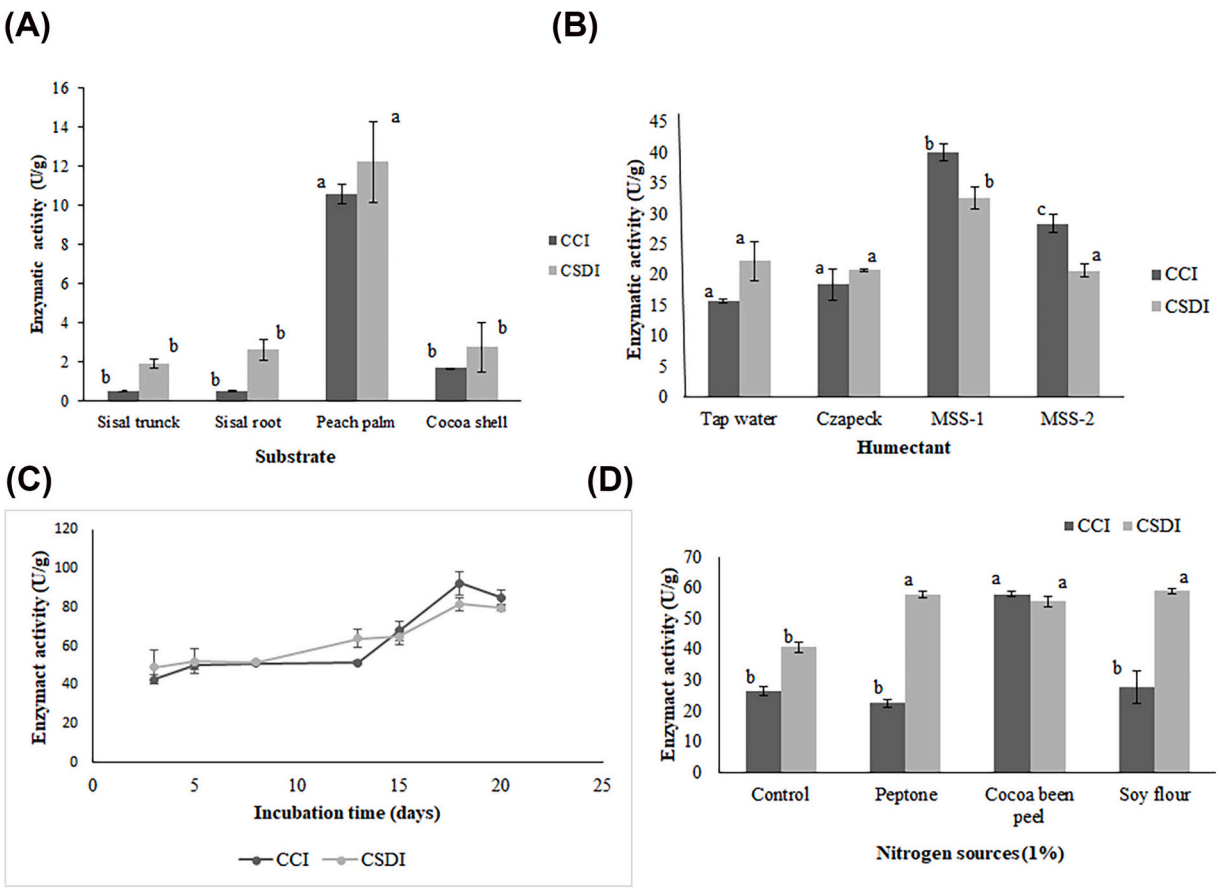

Fig. 3. Enzymatic activity of inulinase produced by *A. welwitschiae* (CCI and CSDI) after cultivation in FES for 5 days at 28 °C. **(A)** Fermentation using different natural substrates as a carbon source moistened with Czapec salts. **(B).** Effect of moisturing agent as tap water, Czapeck, MSS-1 and MSS-2. **(C)** Effect of fermentation time 2–20 days, and **(D)** and effect of different nitrogen sources on inulinase production by *A. welwitschiae* (CCI and CSDI) after cultivation under FES using peach-palm waste as a carbon source for 5 days at 28 °C. The data is presented as the average of three experiments, and the average values followed by different letters (a, b, c) differ statistically ($p < 0.05$), confirmed by the Tukey test.

disposal in the environment (de Cássia Spacki et al., 2022). The chemical composition of peach-palm favored fungal growth as it is rich in minerals and contains easily fermentable nutrients, such as carbohydrates (Carvalho et al., 2018). Previous work from our research group also demonstrated that peach-palm waste was efficient for the production of other enzymes, such as xylanases (Carvalho et al., 2018), cellulases (Bezerra et al., 2021) and polyphenol oxidases (De Carvalho et al., 2023).

The lower enzyme production in sisal substrates could be due to the presence of saponin in this substrate, which has antifungal action, limiting fungal growth (Carneiro, Silva, Santos, Lima, & Costa, 2021). Cocoa husk also showed lower results than peach-palm due to the residue's composition, which possibly meets the nutritional needs of the fungus to a lesser extent (Andoh-Mensah et al., 2023). As peach-palm waste showed the best results for enzyme production compared to other tested substrates, it was used as substrate for further tests. To determine the best moisturing agent for enzyme production in SSF, a test was conducted with four different wetting agents. The moisturing agent that best favored enzyme production, for both isolates, was MSS-1 (Fig. 3B).

The choice of the ideal moisturing agent for the substrate and microorganism used in the fermentation process is important, as the presence of metal ions in these solutions helps in the full expression of the catalytic activity of the enzyme, the growth, and reproduction of the microorganisms, since these micronutrients act as enzyme cofactors (Jana et al., 2014). The metal ions present in MSS-1 favored the production of inulinase by the fungi more than MSS-2 and Czapeck. Tap water has a low composition of these salts, which may have made it less effective. The MSS-1 salt medium was also used along with yacon juice and proved to be a good combination for the growth of *A. kawachii*, as well as an inducer of enzymatic synthesis of inulinase (Chesini et al., 2013). In this study, the use of MSS-1 promoted a two-fold increase in enzymatic activity of inulinase from *A. welwitschiae*, suggesting that these components are important for the growth of the fungus and enzyme production when using peach-palm waste as substrate.

Finding the best production time for the enzyme under study is of great importance to achieve optimal conditions, along with other parameters, for the enzyme of interest. Regarding the production time of the enzymes, it was observed that enzyme production was best at 18 days, for both isolates of *A. welwitschiae* (CCI and CSDI), as shown in Fig. 3C.

Although there was no significant difference between 18 and 20 days of incubation for the production of inulinase by both isolates, the 18-day period promoted the highest production of the enzymes (92.3 U/g for CCI and 81.5 U/g for CSDI). This incubation period is longer than that found in previous studies, where the average enzyme production time is 72 h. However, that production was already under optimized conditions, which tends to reduce the incubation time in SSF, such as for *Penicillium funiculosum* where the best time was between 72 and 120 h (Danial, Ayaz, & Alnahdi, 2015), and for the production of inulinase from *Penicillium oxalicum* using corn, optimal time was between 4 and 6 days (Singh, Chauhan, & Jindal, 2018). As the fungus also showed good enzyme production in five days (50.1 U/g for CCI and 52.3 U/g for CSDI), subsequent experiments were conducted during this incubation

period, which allows for quicker results for laboratory-scale tests.

Depending on the substrate composition, supplementation with a nitrogen source can be essential for fungal growth and the consequent increase in enzymatic production. In the current work, three nitrogen sources were tested for the production of inulinase using peach-palm waste as the substrate, and an increase of five times in enzymatic activity was observed with the supplementation of the substrate with cocoa husk for the CCI isolate (57.89 U/g) and for CSDI, the Tukey test did not indicate a significant difference between peptone, cocoa husk, and soy flour, showing a difference only from these treatments compared to the control, demonstrating that there is an increase in enzyme production using any of these nitrogen sources (Fig. 3D). Soy contains about 36 % protein (Souza, Zanon, Pedroso, & Andrade, 2009); no previous study was found using soy for inulinase production, but it has been used to increase the production of other enzymes, such as the production of xylanase by *Trichoderma stromaticum* AM7 at a ratio of 0.75 g/g of soy bran, promoting an increase of about two times in enzymatic activity (Carvalho et al., 2018). Peptone is a semi-digested protein that serves as a source of nitrogen and carbon and is also widely used as a nitrogen source, including for inulinase production, being, along with yeast extract and meat extract, considered one of the best nitrogen sources for enzyme production for *Kluyveromyces marxianus* ATCC 36907 (Yupanqui-Mendoza et al., 2022).

Cocoa husk has been successfully used for the production of polyphenol oxidases from *Pleurotus pulmonarius* (De Carvalho et al., 2023), yet there are no reports in the literature of its use for inulinase production. Economically, the use of cocoa husk would be more advantageous as it involves an agro-industrial residue, thus not burdening the enzyme production process.

3.3. Optimization of Inulinase production using Doehlert matrix

In this study, the Doehlert statistical tool was used to obtain optimized conditions for the production of inulinase by *A. welwitschiae* strains CCI and CSDI varying temperature and humidity of SSF. Since peach-palm waste was considered the most suitable substrate (Fig. 3A) and MSS-1 the best moisturing agent (Fig. 3B) for the production of inulinase by *A. welwitschiae* under SSF, these were used for the optimization studies of enzyme production.

The experimental design carried out allowed the identification of specific parameters that positively influenced the production of inulinase. The minimum and maximum values of inulinase activity obtained were 23.87 and 45.04 U/g (CCI); 21.84 and 54.86 U/g (CSDI) respectively (Fig. 4). The optimized conditions of temperature (27 °C for CCI and 25 °C for CSDI) and humidity (82 % for CCI and 74.5 % for CSDI) corroborate with results found in previous studies, such as the production of inulinase by *Kluyveromyces marxianus* ATCC 36907 which showed the best results at 75 % humidity and 25–35 °C, with the maximum enzymatic activity found of 66.9 ± 2.1 U/g (Yupanqui-Mendoza et al., 2022). In the optimization study of inulinase production by an *A. tubingensis*, the best conditions for enzyme production were found with 71.2 % humidity at 30 °C (Trivedi et al., 2012).

The results were analyzed by ANOVA, and the second-order regression equation provided the levels of inulinase activity as a function of temperature and humidity. The correlation between predicted and observed experimental values showed a good fit for the mathematics proposed by the model, which explained 97.35 % of the variation of the observed data (R2 = 0.97353) for CCI and 98.42 % (R2 = 0.98421) for CSDI.

By applying various regression analyses to the experimental data, the second-order polynomial equation was obtained in terms of process variables as can be observed in eqs. (1) for *A. welwitschiae* CCI and (2) for *A. welwitschiae* CSDI.

$$Z = -109.945 + 1.6096\,U - 0.0092\,U^{\wedge}2 + 6.644\,T - 0.11695\,T^{\wedge}2 - 0.0038\,U^{*}T \quad (1)$$

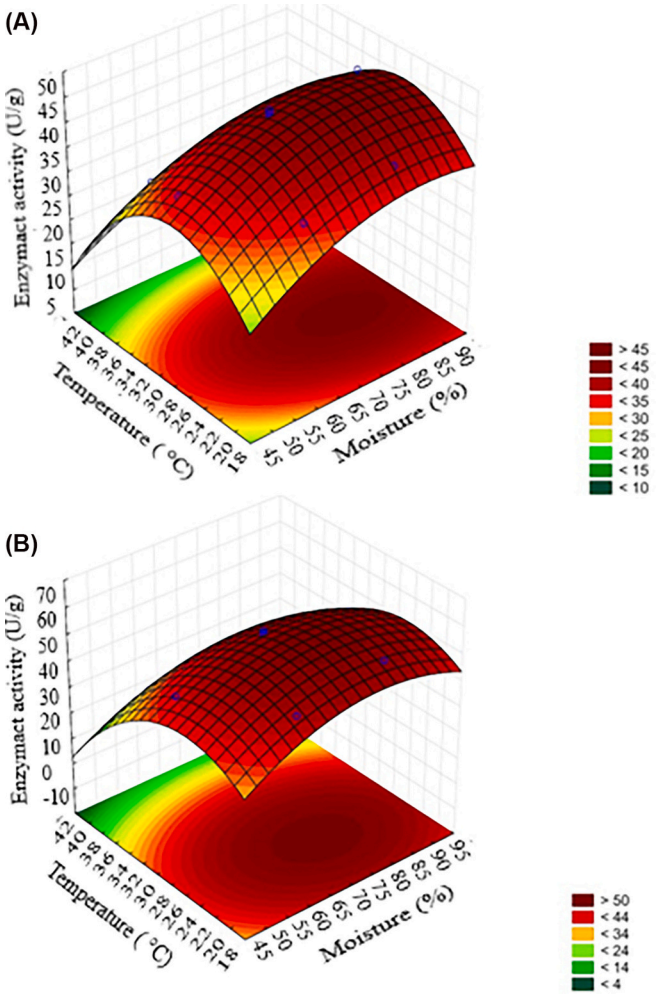

Fig. 4. Response surfaces generated by the Doehlert method showing the interaction between temperature and humidity for the production of inulinase by the *A. welwitschiae*. **(A)** *A. welwitschiae* CCI, and **(B)** *A. welwitschiae* CSDI under FES using peach-palm waste as a carbon source and moistened with MSS-1.

$$Z = -0.0184125\,U^{\wedge}2 + 2.84058333\,U - 0.1445625\,T^{\wedge}2 + 7.56575\,T - 0.003825\,U^{*}T - 143.443333 \quad (2)$$

where Z is the yield of inulinase (U/g), U is the moisture content (%) and T is the temperature (°C).

The ANOVA of the second-order regression model demonstrated that the model was significant with $p < 0.05$. The quadratic model did not show a lack of fit, which is confirmed by the F-test (Faj presents F < Ftab), left smaller residues and, therefore, has a good predictive capability. The adjusted response for the regression model was plotted in Figs. 4A (*A. welwitschiae* CCI) and 4B (*A. welwitschiae* CSDI). The ideal conditions found for maximum production of inulinase were 82 % humidity and 27 °C for CCI and 75 % humidity and 25 °C for CSDI. By substituting the factor levels in the regression equation, the maximum predicted inulinase production was determined to be 45.04 U/g (CCI) and 54.86 U/g (CSDI).

The validation of fermentation parameters for the two isolates, is of great importance to ensure the reliability of the results obtained from the experimental design and to assess the adequacy of the predictive models. In the case of the CCI isolate, validation under the conditions of 30 °C and 80 % moisture resulted in an observed enzymatic activity of 38.1 U/g, corresponding to 84.9 % of the predicted value, demonstrating a good approximation to the expected result. By optimizing the

parameters to 27 °C and 82 % moisture, the observed activity increased to 46.3 U/g, surpassing the prediction and reaching 100.9 %. This validation indicates that fine adjustments in fermentation conditions can maximize enzyme production.

For the CSDI isolate, the observed activity of 46.6 U/g under conditions of 30 °C and 80 % moisture represented 86.9 % of the predicted value, suggesting a robust match between the observed and predicted results. When conditions were optimized to 26 °C and 70 % moisture, the activity increased to 58.7 U/g, slightly exceeding the predicted value (102.6 %). These results reinforce the importance of validating predictive models with experiments under real conditions, not only to verify the accuracy of the models but also to identify adjustments that can lead to more efficient production.

While theoretical models and simulations can predict certain outcomes, experimental validation ensures that these predictions can be replicated in real-world conditions. This is essential for ensuring that the models are accurate and reliable. Thus, experimental validation allows to test the impact of these adjustments in a controlled environment, helping them identify the best conditions to enhance enzyme production. In this specific case, experimental validation helps confirm whether optimized parameters significantly improve the efficiency of inulinase production from peach-palm waste. This has direct implications for sustainability by making the biotechnological process more resource-efficient and eco-friendly.

3.4. Semi-purification and characterization of Inulinase

The extracts subjected to semi-purification with peach-palm charcoal were obtained through SSF for 5 days, 80 % humidity and 27 °C for *A. welwitschiae* CCI and 75 % humidity and 25 °C for *A. welwitschiae* CSDI, showing enzymatic activities of 23.36 U/g for CCI and 50.17 U/g for CSDI. Partial purification with peach-palm charcoal promoted an increase in specific activity, with a purification index of 1.3 and yield of 108 % for CCI and purification index of 1.57 and yield of 125 % for CSDI (Table SM1).

The increase in specific activity can be justified by the fact that activated charcoal is an adsorbent, promoting direct and indirect adsorption of dye components resulting from the fermentation process, which may contain contaminants that interfere with the activity of inulinases (Koohestani, Salem, & Salem, 2023). These results corroborate with other semi-purification techniques of inulinase found in the literature. With partial purification of *A. niger* inulinase, a purification

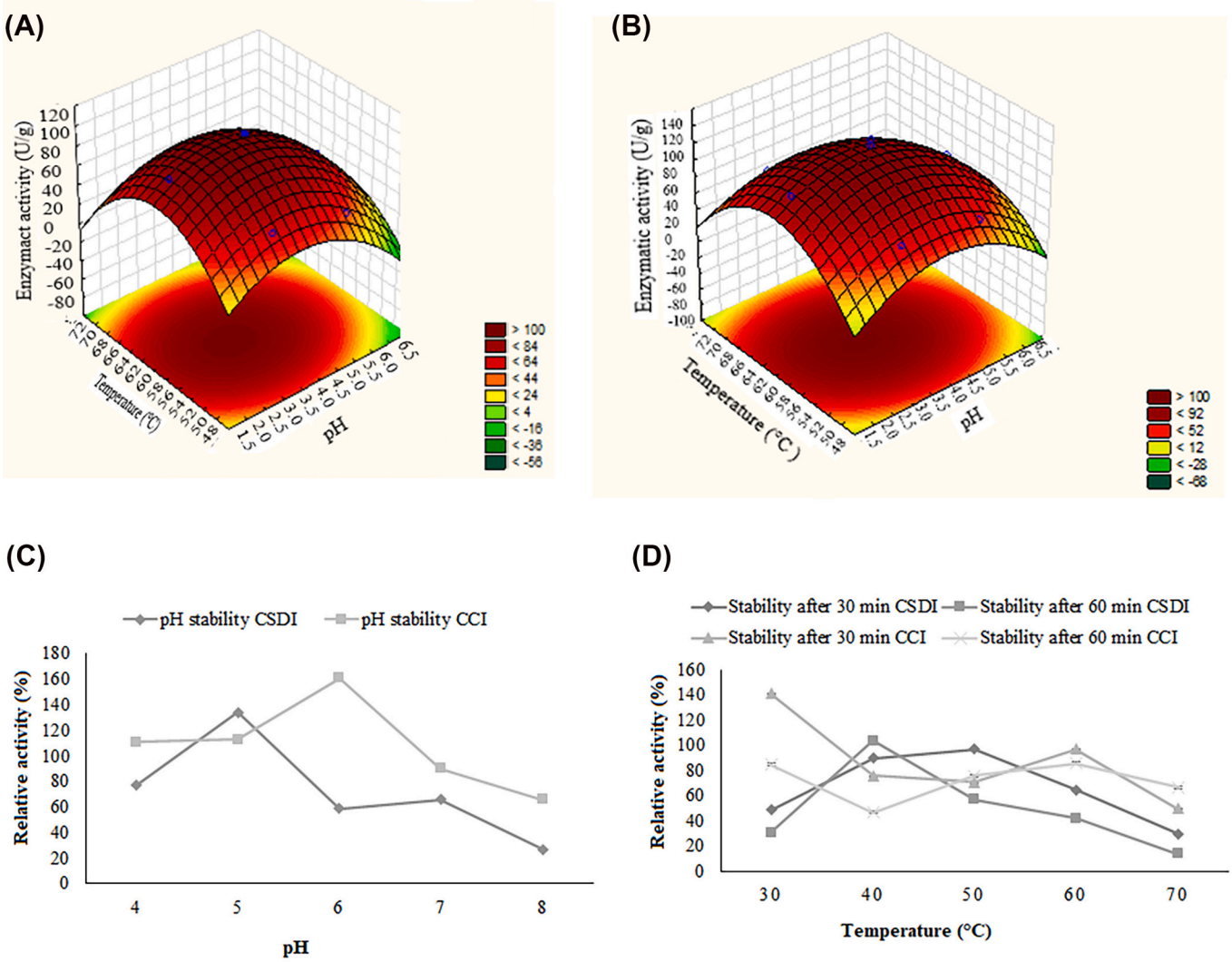

Fig. 5. Response surface showing the interaction between temperature and pH for the activity of inulinase by the fungus *A. welwitschiae*. **(A)** *A. welwitschiae* CCI and **(B)** *A. welwitschiae* CSDI. **(C)** pH stability after 2 h of incubation and **(D)** thermal stability after 30 min and 0 min of incubation at 30–70 °C for *A. welwitschiae* CCI and *A. welwitschiae* CSDI.

coefficient between 1.3 and 5.33 was obtained, and an increase in enzymatic activity of about 3.7 to 10 times through centrifugation and ultrafiltration techniques (Germec & Turhan, 2020). In the semi-purification of *A. niger* inulinase using the ultrafiltration technique, purification indices of 0.59 to 1.05 were obtained, with a reduction of about 2.4 times in the total enzyme activity in some steps and an increase of 1.1 times in other steps (Ilgın et al., 2019).

3.5. Characterization of Inulinase

The interaction between pH and reaction temperature demonstrated that the optimal pH and temperature points for the action of CCI inulinase were 3.4 and 58.8 °C, respectively. For the CSDI isolate, the pH and temperature at which the enzyme showed the best action were 3.5 and 59.1 °C, respectively. The application of the Doehlert tool to find the best pH and temperature points for enzyme action led to an increase in enzymatic activity of about 4.7 times for CCI and 2.4 times for CSDI, demonstrating that the experimental design allowed for the identification of specific parameters that positively influenced the activity of inulinases, which can be confirmed through the obtained response surfaces, where the reach of the optimal points can be observed (Fig. 5).

The ANOVA of the second-order regression model showed that the model was significant with $p < 0.05$. The model did not show a lack of fit, which is confirmed by the *t*-test (Faj presents F < Ftab). The adjusted response for the regression model above was plotted in Figs. 5A (CCI) and 5B (CSDI). The ideal conditions found for maximum production of inulinase were very similar for both tested isolates, with a pH of 3.4 and temperature of 59 °C for CCI, and pH of 3.5 and temperature of 59 °C for CSDI. By substituting the factor levels in the regression equation, the maximum predictable response for inulinase production was 101.6 U/g (CCI) and 115.1 U/g (CSDI).

The experimental validation of the model demonstrated that the observed enzymatic activity was 86.6 % (92.8 U/g) of the predicted value for the CSDI isolate and 69 % (70.2 U/g) for the CCI isolate, indicating that the model was relatively effective but requires further refinement. The smaller deviation for the CSDI isolate suggests that the model more accurately captured the interaction between pH and temperature in this case, while additional factors may be affecting the enzymatic activity of the CCI isolate. This validation is critical, as it highlights the necessity of model adjustments to improve predictive accuracy and optimize reaction conditions. Moreover, it facilitates the identification of potential model limitations and ensures the reproducibility of predictions under real-world conditions, thereby supporting the model's iterative enhancement for biotechnological applications demanding high enzymatic efficiency.

The strain *A. welwitschiae* CCI exhibited stability in the pH range of 4.0 to 7.0, maintaining up to 90 % of enzymatic activity for up to 2 h. The *A. welwitschiae* CSDI isolate showed optimal inulinase stability at pH 5.0, with a significant difference from this pH value compared to other tested ranges ($p < 0.05$), maintaining 80 % of its activity for up to 2 h (Fig. 5C).

The optimal pH values and stability found are similar to those reported in other studies, such as inulinase from *Penicillium oxalicum* BGPUP-4 in its free form, which showed optimal pH and stability for up to 4 h at pH 5.0 (Singh et al., 2018). This result is close to that observed for inulinase from *Kluyveromyces marxianus*, which showed an optimal pH of 5.5, maintaining up to 50 % of its activity at pH 7.5 (Trapala, Bustos-Jaimes, Manzanares, Bárzana, & Montiel, 2020).

However, the temperature at 40 °C, maintaining activity above 75 % after 30 and 60 min in the temperature range of 30–60 °C. The inulinase from *A. welwitschiae* CSDI maintained activity above 80 % after 30 min of incubation in the 40–50 °C range, and after 1 h of incubation, the enzyme remained stable only at the temperature of 40 °C (Fig. 5D). These results indicate that the inulinase produced by the *A. welwitschiae* can be used at higher temperatures.

Inulinase from *A. welwitschiae* MN056175 also showed higher enzyme activity at 60 °C and greater thermal stability at 40 °C (Saleh et al., 2020). Most described inulinases demonstrated optimal activity when subjected to temperatures between 40 and 60 °C (Ramapriya, Thirumurugan, Sathishkumar, & Manimaran, 2018; Trapala et al., 2020; Trivedi et al., 2012).

After analyzing the influence of different ionic salts and chemical compounds on inulinase activity, it was observed that the inulinase from the CSDI isolate was activated by almost all the tested compounds, particularly by $MnCl_2$ (95 %), $FeCl_3$ (51 %), $CoCl_2$ (61 %), and EDTA (60 %). However, it was inhibited by 40 % in the presence of 0.1 % SDS.

Regarding the action of ionic salts and other additives on the inulinase from the CCI isolate, it was noted that the enzyme was strongly activated by KCl_2 (155 %) at 1 mM and SDS 0.1 % (146 %), and to a lesser extent by $MnCl_2$ (28 %) and CoCl2 (23 %) at 1 mM, and experienced greater inhibition in the presence of NaCl (41 %), $CuSO_4$ (47 %), and EDTA (34 %) (Table 2).

The inhibition of inulinase from the CSDI isolate by SDS may suggest an interaction with the enzyme's hydrophobic amino acids, altering the conformation of the catalytic site. The decrease in inulinase activity of the CCI isolate following the addition of EDTA may have occurred because this substance is a chelating agent, sequestering ions, suggesting that this inulinase could be a metalloenzyme (Gonçalves, Riul, Terenzi, Jorge, & Guimarães, 2011). The increase in enzymatic activity of inulinase by the addition of the ionic salt $MnCl_2$ was also observed by Germec and Turhan (2020) in their study of *A. niger* inulinase. Exposure of the enzyme to $MnSO_4$ in a study with *A. tritici* BGPUP6O inulinase also demonstrated increased activity, while $CuSO_4$, $ZnSO_4$, and EDTA inhibited enzyme activity (Singh et al., 2020).

3.6. Hydrolysis of commercial inulin and sisal

In the saccharification of sisal, *A. welwitschiae* inulinase produced a maximum of 302.8 mg/g of reducing sugar within a 7 h of incubation using 4 % substrate and 36 U/g enzyme (Table SM2). The TLC analysis (Fig. 6A) showed a decrease in the amount of polysaccharide in the controls compared to the tests. It is also possible to observe a greater formation of disaccharides and monosaccharides in the tests (1, 2, and 3 T) compared to the controls (1, 2, and 3C).

Incubation of commercial inulin with the enzyme yielded a maximum production of 260 mg/g of reducing sugar, using 3 % inulin within a 6 h at 50 °C (Table SM2). The TLC (Fig. 6B) showed a decrease in the amount of polysaccharide in the controls compared to the tests. There is also a greater formation of oligosaccharides, disaccharides, and monosaccharides in the tests (1, 2, and 3 T) compared to the controls (1, 2, and 3C), which confirms the action of enzyme in breaking down inulin at a substrate concentration similar to that used in saccharification.

Agave sisalana (sisal) is a good source for the production of inulin, producing a type of linear β (2 → 1) inulin, which is why this substrate

Table 2
Effect of different additives observed in triplicate on the inulinase activity of *A. welwitschiae*.

Compounds (1 mM)	(CSDI)	Relative activity (%)	(CCI)	
Control	100.0	± 0.1	100.0	± 0.0
NaCl	123.8	± 0.3	59.2	± 0.2
KCl_2	126.9	± 0.0	254.7	± 0.2
$CuSO_4$	128.2	± 2.1	53.4	± 0.8
$ZnSO_4$	120.2	± 0.5	74.6	± 0.3
$CaCl_2$	117.9	±1.2	64.9	± 0.1
$MgCl_2$	135.0	± 1.1	91.1	± 0.2
$MnCl_2$	195.0	± 2.9	128.0	± 0.3
$FeCl_3$	150.6	± 3.8	86.2	± 0.4
$CoCl_2$	160.7	± 3.5	122.7	± 0.2
β-mercaptoethanol	133.7	± 0.0	75.3	± 0.1
EDTA	160.3	± 0.0	65.8	± 0.1
SDS (0.1 %)	60.2	± 0.0	245.6	± 0.4

* Control: no ions or reagents added. Mean ($n = 3$) ± mean standard error.

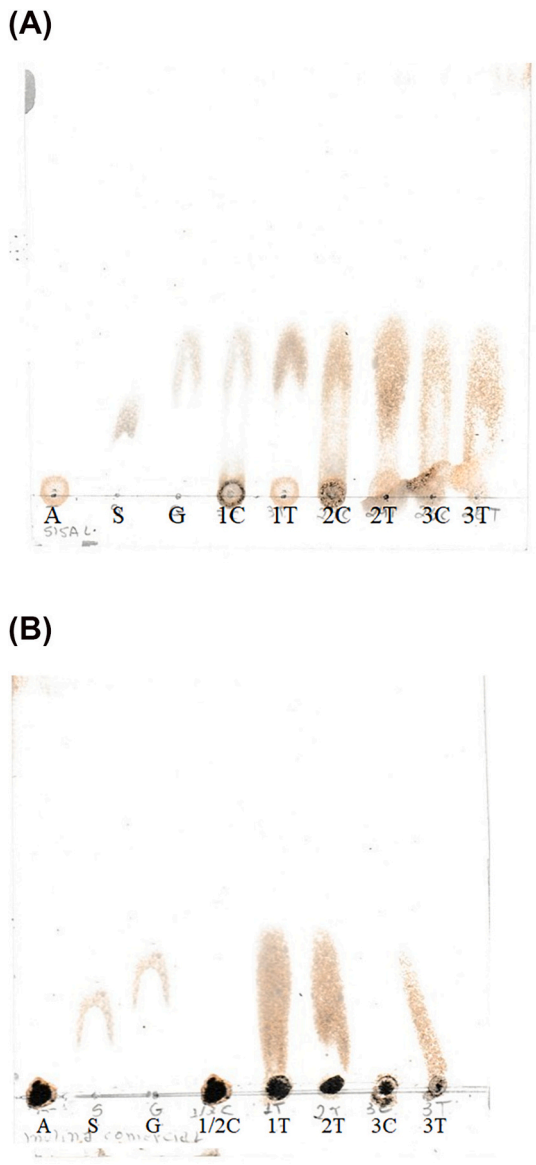

Fig. 6. TLC analysis of hydrolysis products by inulinase from *A. welwitschiae* CCI. **(A)** - in sisal root: starch (A), sucrose (S), glucose (G), 3 % sisal incubated for 5 h at 40 °C without enzyme (1C) and with enzyme (1 T); 3 % sisal incubated for 6 h at 50 °C without enzyme (2C) and with enzyme (2 T) and 4 % sisal incubated for 7 h at 55 °C without enzyme (3C) and with enzyme (3 T). **(B)** - in commercial inulin: standard starch (A), standard sucrose (S), standard glucose (G), 3 % inulin incubated for 5 h at 40 °C without enzyme (1/2C) and with enzyme (1 T); 3 % inulin incubated for 6 h at 50 °C with enzyme (2 T); 4 % inulin incubated for 7 h at 55 °C without enzyme (3C) and with enzyme (3 T).

was chosen for saccharification with inulinase (López & Salomé-Abarca, 2024). These results are corroborated by findings in the literature, where Saleh et al. (2020) and colleagues in their study with inulinase from *A. welwitschiae* MN056175 achieved between 35.55 and 555.13 mg of reducing sugar depending on the conditions of enzymatic hydrolysis, using artichoke leaves.

4. Conclusion

The molecular modeling experiments conducted with the proteins utilized in this study facilitate the elucidation of the structural dissimilarities between the fungal *endo*- and *exo*-inulinases, in addition to those observed between them and the inulinases of other organisms. Moreover, this study represents a significant innovation in the field, as it marks the first structural characterization of this class of enzyme in *A. welwitschiae*. Furthermore, the combination of kinetic and physicochemical data with structural information will facilitate additional computational studies for the engineering of these enzymes using artificial intelligence (AI) approaches to enhance their performance in inulin hydrolysis and the degradation of other sisal residues. This could facilitate the utilization of these molecules in the modern bioeconomy. The isolates of *A. welwitschiae* (CCI and CSDI) were observed to produce inulinase enzymes when cultivated with solely agro-industrial residues as substrates, including peach-palm waste in solid-state fermentation (SSF). Moreover, the semi-purified inulinases exhibited high activity at elevated temperatures and thermostability, as well as action at more acidic pH. These physicochemical characteristics in conjunction with their capacity to hydrolyze commercial inulin and sisal residues to produce oligosaccharides and reducing sugars, illustrate their potential for biotechnological applications, particularly in the production of fructooligosaccharides, which can replace sugar in dessert formulations in food industries.

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CRediT authorship contribution statement

Emanuelle Santos de Carvalho Cardoso: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Nívea Queiroz Martins:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Raquel Araújo Azevedo:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Lucas Sousa Palmeira:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis. **Gabriel Quintanilha-Peixoto:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis. **Bruno Andrade:** Writing – review & editing, Validation, Supervision, Investigation, Funding acquisition. **Mateus Pereira Flores Santos:** Writing – review & editing, Validation, Investigation. **Ana Paula Trovatti Uetanabaro:** Writing – review & editing, Investigation, Formal analysis. **Erik Galvão Paranhos da Silva:** Writing – review & editing, Investigation, Formal analysis. **Aristóteles Góes-Neto:** Writing – review & editing, Investigation, Funding acquisition, Conceptualization. **Andréa Miura da Costa:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.141978.

References

Andoh-Mensah, E., Anthonio, C. K., Sossah, F. L., Fianko, D. A., & Yankey, E. N. (2023). Integrated soil fertility management using cocoa bean shells improves soil chemical properties, coconut yield and mitigates environmental pollution. *Journal of Cleaner Production, 428*, 139418. https://doi.org/10.1016/j.jclepro.2023.139418

Apolinário, A. C., de Carvalho, E. M., de Lima Damasceno, B. P. G., da Silva, P. C. D., Converti, A., Pessoa, A., Jr., & da Silva, J. A. (2017). Extraction, isolation and characterization of inulin from *Agave sisalana* boles. *Industrial Crops and Products, 108*, 355–362. https://doi.org/10.1016/j.indcrop.2017.06.045

Basmak, S., & Turhan, I. (2024). Production of β-mannanase, inulinase, and oligosaccharides from coffee wastes and extracts. *International Journal of Biological Macromolecules, 261*(Mar. 2024), Article 129798. https://doi.org/10.1016/j.ijbiomac.2024.129798

Bezerra, C. O., Carneiro, L. L., Carvalho, E. A., das Chagas, T. P., de Carvalho, L. R., Uetanabaro, A. P. T., & da Costa, A. M. (2021). Artificial intelligence as a combinatorial optimization strategy for Cellulase production by *Trichoderma stromaticum* AM7 using peach-palm waste under solid-state fermentation. *Bioenergy Research, 14*(4), 1161–1170. https://doi.org/10.1007/s12155-020-10234-4

Blum, M., Chang, H.-Y., Chuguransky, S., Grego, T., Kandasaamy, S., Mitchell, A., & Finn, R. D. (2020). The InterPro protein families and domains database: 20 years on. *Nucleic Acids Research, 49*(D1), D344–D354. https://doi.org/10.1093/nar/gkaa977

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

Carneiro, J.d. S., Silva, M. C.d. J., Santos, E. N.d., Lima, F. L. O., & Costa, M. S. F. (2021). Biological activities of *Agave sisalana* with an emphasis on antimicrobial action: A literature review. *Research, Society and Development, 10*(3), Article e2510312734. https://doi.org/10.33448/rsd-v10i3.12734

Carvalho, E. A., Nunes, L. V., Goes, L. M.d. S., Silva, E. G. P.d., Franco, M., Gross, E., & Costa, A. M.d. (2018). Peach-palm (*Bactris gasipaes* Kunth.) waste as substrate for xylanase production by *Trichoderma stromaticum* AM7. *Chemical Engineering Communications, 205*(7), 975–985. https://doi.org/10.1080/00986445.2018.1425208

de Cássia Spacki, K., Corrêa, R. C. G., Uber, T. M., Barros, L., Ferreira, I. C. F. R., Peralta, R. A., … Peralta, R. M. (2022). Full exploitation of peach palm (*Bactris gasipaes* Kunth): State of the art and perspectives. *Plants, 11*, 3175. https://doi.org/10.3390/plants11223175

Casteliano, G. A., Santos, T. T., Bezerra, C. O., Costa, A. M.d., Pólvoras, C. E. M., Figueredo, A. F. R., & Uetanabaro, A. P. T. (2022). Alkalinization and moist heat treatments of substrates for cultivation of edible mushrooms in pupunha and cocoa residues. *African Journal of Agricultural Research, 18*(5), 368–375. https://doi.org/10.5897/ajar2021.15911

Chen, H.-Q., Chen, X.-M., Chen, T.-X., Xu, X.-M., & Jin, Z.-Y. (2011). Optimization of solid-state medium for the production of inulinase by *aspergillus ficuum* JNSP5-06 using response surface methodology. *Carbohydrate Polymers, 86*(1), 249–254. https://doi.org/10.1016/j.carbpol.2011.04.044

Chesini, M., Neila, L. P., Parra, D. F.d.l., Rojas, N. L., Esquivel, J. C. C., Cavalitto, S. F., & Hours, R. A. (2013). *Aspergillus kawachii* produces an inulinase in cultures with yacon (*Smallanthus sonchifolius*) as substrate. *Electronic Journal of Biotechnology, 16*(3), 8. https://doi.org/10.2225/vol16-issue3-fulltext-13

Danial, E. N., Ayaz, N. O. A., & Alnahdi, H. S. O. (2015). Production of Inulinase by free and immobilized cells of *Penicillium funiculosum* p.36. *Brazilian Archives of Biology and Technology, 58*(4), 636–642. https://doi.org/10.1590/s1516-8913201500167

Das, D., Bhat, M. R., & Selvaraj, R. (2020). Optimization of inulinase production by a newly isolated Penicillium amphipolaria strain using solid-state fermentation of hardy sugarcane stems. *Biocatalysis and Agricultural Biotechnology, 30*(Nov. 2020), Article 101875. https://doi.org/10.1016/j.bcab.2020.101875

De Carvalho, L. R., Santos, D. R., dos Santos Lima, C. S., Peralta, R. M., de Souza, C. G. M., Uetanabaro, A. P. T., & da Costa, A. M. (2023). Stable polyphenol oxidase produced by *Pleurotus pulmonarius* from fermented peach-palm and cocoa wastes. *Bioenergy Research, 17*(1), 198–207. https://doi.org/10.1007/s12155-023-10628-0

Dotsenko, A., Denisenko, J., Zorov, I., Wasserman, L., Semenova, M., Korolev, A., & Sinitsyn, A. (2023). Single substitution in α-helix of active center enhanced thermostability of *Aspergillus awamori* exo-inulinase. *Journal of Molecular Graphics and Modelling, 119*, Article 108381. https://doi.org/10.1016/j.jmgm.2022.108381

Dotsenko, A., Denisenko, Y., Zorov, I., Rozhkova, A., & Shashkov, I. (2025). N-linked glycosylation affects catalytic parameters and fluctuation of the active center of Aspergillus awamori exo-inulinase. *Protein Expression and Purification, 226*(Feb. 2025), Article 106613. https://doi.org/10.1016/j.pep.2024.106613

Germec, M., & Turhan, I. (2020). Partial purification and characterization of *aspergillus Niger* inulinase produced from sugar-beet molasses in the shaking incubator and stirred-tank bioreactors. *International Journal of Biological Macromolecules, 164*, 3789–3799. https://doi.org/10.1016/j.ijbiomac.2020.09.032

Gonçalves, H. B., Riul, A. J., Terenzi, H. F., Jorge, J. A., & Guimarães, L. H. S. (2011). Extracellular tannase from *Emericella nidulans* showing hypertolerance to temperature and organic solvents. *Journal of Molecular Catalysis B: Enzymatic, 71*(1–2), 29–35. https://doi.org/10.1016/j.molcatb.2011.03.005

Guerrero-Urrutia, C., Volke-Sepulveda, T., Figueroa-Martinez, F., & Favela-Torres, E. (2021). Solid-state fermentation enhances inulinase and invertase production by *Aspergillus brasiliensis*. *Process Biochemistry, 108*, 169–175. https://doi.org/10.1016/j.procbio.2021.06.014

Holt, C., & Yandell, M. (2011). MAKER2: An annotation pipeline and genome-database management tool for second-generation genome projects. *BMC Bioinformatics, 12*(1). https://doi.org/10.1186/1471-2105-12-491

Holyavka, M. G., Kayumov, A. R., Baydamshina, D. R., Koroleva, V. A., Trizna, E. Y., Trushin, M. V., & Artyukhov, V. G. (2018). Efficient fructose production from plant extracts by immobilized inulinases from *Kluyveromyces marxianus* and *Helianthus tuberosus*. *International Journal of Biological Macromolecules, 115*, 829–834. https://doi.org/10.1016/j.ijbiomac.2018.04.107

Hoshida, H., Kidera, K., Takishita, R., Fujioka, N., Fukagawa, T., & Akada, R. (2018). Enhanced production of extracellular inulinase by the yeast *Kluyveromyces marxianus* in xylose catabolic state. *Journal of Bioscience and Bioengineering, 125*(6), 676–681. https://doi.org/10.1016/j.jbiosc.2017.12.024

Ilgın, M., Germec, M., & Turhan, I. (2019). Inulinase production and mathematical modeling from carob extract by using *Aspergillus niger*. *Biotechnology Progress, 36*(1). https://doi.org/10.1002/btpr.2919

Jana, A., Halder, S. K., Banerjee, A., Paul, T., Pati, B. R., Mondal, K. C., & Das Mohapatra, P. K. (2014). Biosynthesis, structural architecture and biotechnological potential of bacterial tannase: A molecular advancement. *Bioresource Technology, 157* (Apr. 2014), 327–340. https://doi.org/10.1016/j.biortech.2014.02.017

Jayapal, N., Samanta, A. K., Kolte, A. P., Senani, S., Sridhar, M., Suresh, K. P., & Sampath, K. T. (2013). Value addition to sugarcane bagasse: Xylan extraction and its process optimization for xylooligosaccharides production. *Industrial Crops and Products, 42*, 14–24. https://doi.org/10.1016/j.indcrop.2012.05.019

Kamble, P. P., Suryawanshi, S. S., Jadhav, J. P., & Attar, Y. C. (2019). Enhanced inulinase production by fusarium solani JALPK from invasive weed using response surface methodology. *Journal of Microbiological Methods, 159*, 99–111. https://doi.org/10.1016/j.mimet.2019.02.021

Koohestani, M., Salem, A., & Salem, S. (2023). Decolorization of corn syrup in a dynamic system by activated carbon bed: Microwave-assisted impregnation of H3PO4 over cherry and date stones for efficient purification. *Sustainable Chemistry and Pharmacy, 36*, Article 101298. https://doi.org/10.1016/j.scp.2023.101298

Korf, I. (2004). Gene finding in novel genomes. *BMC Bioinformatics, 5*(1). https://doi.org/10.1186/1471-2105-5-59

López, M. G., & Salomé-Abarca, L. F. (2024). The agavins (Agave carbohydrates) story. *Carbohydrate Polymers, 327*, Article 121671. https://doi.org/10.1016/j.carbpol.2023.121671

Lukashin, A. (1998). GeneMark.Hmm: New solutions for gene finding. *Nucleic Acids Research, 26*(4), 1107–1115. https://doi.org/10.1093/nar/26.4.1107

Mao, W., Han, Y., Wang, X., Zhao, X., Chi, Z., Chi, Z., & Liu, G. (2019). A new engineered endo-inulinase with improved activity and thermostability: Application in the production of prebiotic fructo-oligosaccharides from inulin. *Food Chemistry, 294*, 293–301. https://doi.org/10.1016/j.foodchem.2019.05.062

Melo Neto, B. A.d., Carvalho, E. A., Costa, A. M.d., Uetanabaro, A. P. T., & Melo, G. J. A. d. (2021). Using peach-palm waste to produce enzymes: A review. In *Agriculture and agro-industry in the context of sustainable rural development* (pp. 52–69). Editora Científica Digital. https://doi.org/10.37885/210705544. Retrieved from.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry, 31*(3), 426–428. https://doi.org/10.1021/ac60147a030

Mohamed, S. A., Salah, H. A., Moharam, M. E., Foda, M. S., & Fahmy, A. S. (2015). Characterization of two thermostable inulinases from *Rhizopus oligosporus* NRRL 2710. *Journal of Genetic Engineering and Biotechnology, 13*(1), 65–69. https://doi.org/10.1016/j.jgeb.2014.12.001

Prado-Acebo, I., Cubero-Cardoso, J., Lu-Chau, T. A., & Eibes, G. (2024). Integral multi-valorization of agro-industrial wastes: A review. *Waste Management, 183*, 42–52. https://doi.org/10.1016/j.wasman.2024.05.001

Quintanilha-Peixoto, G., Torres, R. O., Reis, I. M. A., Oliveira, T. A. S., Bortolini, D. E., Duarte, E. A. E., … Branco, A. (2019). Calm before the storm: A glimpse into the secondary metabolism of *Aspergillus welwitschiae*, the etiologic agent of the sisal bole rot. *Toxins, 11*(11), 631. https://doi.org/10.3390/toxins11110631

Ramapriya, R., Thirumurugan, A., Sathishkumar, T., & Manimaran, D. R. (2018). Partial purification and characterization of exoinulinase produced from *Bacillus* sp. *Journal of Genetic Engineering and Biotechnology, 16*(2), 363–367. https://doi.org/10.1016/j.jgeb.2018.03.001

Saleh, S. A. A., Abd El-Galil, A. A., Sakr, E. A. E., Taie, H. A. A., & Mostafa, F. A. (2020). Physiochemical, kinetic and thermodynamic studies on *Aspergillus wewitschiae* MN056175 inulinase with extraction of prebiotic and antioxidant Cynara scolymus leaves fructo-oligosaccharides. *International Journal of Biological Macromolecules, 163*, 1026–1036. https://doi.org/10.1016/j.ijbiomac.2020.07.036

Santos, M. P. F., da Silva, J. F., da Costa Ilhéu Fontan, R., Bonomo, R. C. F., Santos, L. S., & Veloso, C. M. (2020). New insight about the relationship between the main characteristics of precursor materials and activated carbon properties using multivariate analysis. *The Canadian Journal of Chemical Engineering, 98*(7), 1501–1511. https://doi.org/10.1002/cjce.23721

Schrödinger. (2023, October 24). Copyright © 2024 Inc.Release download. Retrieved June 16, 2024, from Schrödinger website: https://www.schrodinger.com/release-download/.

Silva, M. F., Golunski, S. M., Rigo, D., Mossi, V., Di Luccio, M., Mazutti, M. A., … Treichel, H. (2013). Fructooligosacharides production in aqueous medium with inulinase from *Aspergillus niger* and *Kluyveromyces marxianus* NRRL Y-7571 immobilized and treated in pressurized CO2. *Food and Bioproducts Processing, 91*(4), 647–655. https://doi.org/10.1016/j.fbp.2013.04.009

Singh, R. S., Singh, T., Hassan, M., & Kennedy, J. F. (2020). Updates on inulinases: Structural aspects and biotechnological applications. *International Journal of Biological Macromolecules, 164*, 193–210. https://doi.org/10.1016/j.ijbiomac.2020.07.078

Singh, R. S., Chauhan, K., & Jindal, A. (2018). Response surface optimization of solid state fermentation for inulinase production from *Penicillium oxalicum* using corn bran. *Journal of Food Science and Technology, 55*(7), 2533–2540. https://doi.org/10.1007/s13197-018-3173-3

Singh, R. S., Chauhan, K., & Kennedy, J. F. (2017). A panorama of bacterial inulinases: Production, purification, characterization and industrial applications. *International Journal of Biological Macromolecules, 96*, 312–322. https://doi.org/10.1016/j.ijbiomac.2016.12.004

Souza, L. C. F.d., Zanon, G. D., Pedroso, F. F., & Andrade, L. H. L.d. (2009). Teor de proteína e de óleo nos grãos de soja em função do tratamento de sementes e aplicação de micronutrientes. *Ciência e Agrotecnologia, 33*(6), 1586–15993. https://doi.org/10.1590/s1413-70542009000600018

Stanke, M., Steinkamp, R., Waack, S., & Morgenstern, B. (2004). AUGUSTUS: A web server for gene finding in eukaryotes. *Nucleic Acids Research, 32*(Web Server), W309–W312. https://doi.org/10.1093/nar/gkh379

Stojanović, S., Ristović, M., Stepanović, J., Margetić, A., Duduk, B., Vujčić, Z., & Dojnov, B. (2022). *Aspergillus welwitschiae* inulinase enzyme cocktails obtained on agro-material inducers for the purpose of fructooligosaccharides production. *Food Research International, 160*, Article 111755. https://doi.org/10.1016/j.foodres.2022.111755

Trapala, J., Bustos-Jaimes, I., Manzanares, P., Bárzana, E., & Montiel, C. (2020). Purification and characterization of an inulinase produced by a *Kluyveromyces marxianus* strain isolated from blue agave bagasse. *Protein Expression and Purification, 176*, Article 105718. https://doi.org/10.1016/j.pep.2020.105718

Trivedi, S., Divecha, J., & Shah, A. (2012). Optimization of inulinase production by a newly isolated *Aspergillus tubingensis* CR16 using low cost substrates. *Carbohydrate Polymers, 90*(1), 483–490. https://doi.org/10.1016/j.carbpol.2012.05.068

Versluys, M., Porras-Domínguez, J. R., Voet, A., Struyf, T., & Van den Ende, W. (2024). Insights in inulin binding and inulin oligosaccharide formation by novel multi domain endo-inulinases from Botrytis cinerea. *Carbohydrate Polymers, 328*(Mar. 2024), Article 121690. https://doi.org/10.1016/j.carbpol.2023.121690

Yafetto, L. (2022). Application of solid-state fermentation by microbial biotechnology for bioprocessing of agro-industrial wastes from 1970 to 2020: A review and bibliometric analysis. *Heliyon, 8*(3), Article e09173. https://doi.org/10.1016/j.heliyon.2022.e09173

Yupanqui-Mendoza, S. L., Vaz de Arruda, P., & Castelo da Silva, G. M. (2022). Statistical sequential optimization of process parameters for inulinase production by *Kluyveromyces marxianus* ATCC 36907 in solid-state fermentation using beer residue. *Biocatalysis and Agricultural Biotechnology, 39*, Article 102252. https://doi.org/10.1016/j.bcab.2021.102252