



A novel *Thermothelomyces heterothallicus* PA2S4T fungus isolated from the soil induces chitinase production using orange peel flour

Um novo fungo *Thermothelomyces heterothallicus* PA2S4T isolado do solo induz produção de quitinase utilizando farinha da casca de laranja

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Chitinases are enzymes capable of hydrolysing the β -1,4 bonds of chitin releasing chitooligosaccharides and N-acetylglucosamine and are widely used in food, pharmaceutical, and agricultural industries. Microorganisms are potential producers of this enzyme; however, there are no reports in the literature on the production of chitinase by fungi of the genus *Thermothelomyces*. Thus, this work aimed to investigate the production of extracellular chitinase using alternative carbon sources by the fungus isolated from soil, *Thermothelomyces heterothallicus* PA2S4T. The fungus was cultivated in a liquid medium supplemented with carbon sources and incubated at 40°C under stationary conditions for seven days. Orange peel flour was the best inducer for extracellular chitinase, with 82.3 U/mL of enzymatic activity. The highest production of chitinase was obtained on the tenth day, and the optimum pH and temperature for enzyme activity were 4.5 and 50°C, respectively. Therefore, the fungus *T. heterothallicus* PA2S4T proved to be promising in the production of extracellular chitinase, which presents pH and temperature characteristics favourable to biotechnological application.

Keywords: thermophilic fungi, agro-industrial waste, enzyme.

Quitinases são enzimas capazes de hidrolisar as ligações β -1,4 de quitina liberando quitooligossacarídeos e N-acetilglicosamina, e muito utilizadas na indústria alimentícia, farmacêutica e agrícola. Os microrganismos são potenciais produtores dessa enzima, entretanto não há relatos na literatura sobre a produção de quitinase por fungos do gênero *Thermothelomyces*. Assim, este trabalho teve como objetivo investigar a produção da quitinase extracelular utilizando fontes alternativas de carbono pelo fungo isolado do solo *Thermothelomyces heterothallicus* PA2S4T. O fungo foi cultivado em meio líquido suplementado com fontes de carbono e incubado a 40°C em condições estacionárias por 7 dias. A farinha da casca de laranja foi o melhor indutor para quitinase extracelular com 82,3 U/mL de atividade enzimática. A maior produção da quitinase foi obtida no décimo dia, sendo o pH e temperatura ótimos de atividade da enzima foram 4,5 e 50 °C, respectivamente. Além disso, sua atividade se manteve estável entre 40-50 °C e na faixa de pH de 4.0-5.0. Portanto, o fungo *T. heterothallicus* PA2S4T demonstrou ser promissor na produção de quitinase extracelular, a qual apresenta características de pH e temperatura favoráveis à aplicação biotecnológica.

Palavras-chave: fungo termófilo, resíduo agroindustrial, enzima.

1. INTRODUCTION

Industrial biotechnology uses a variety of microbial enzymes composed mainly of amylases, proteases, pectinases, xylanases, lipases, and cellulases. In recent years, chitinases (EC 3.2.1.14) have gained prominence in this scenario because these enzymes are responsible for hydrolysing chitin, a linear polysaccharide composed of β -1,4 N-Acetylglucosamine (GlcNAc) found abundantly in nature, especially in arthropod exoskeletons and fungal cell walls [1-3].

Chitinases are classified based on their mode of action into endochitinases (EC 3.2.1.14) and exochitinases. Endochitinases are responsible for randomly hydrolysing the β -1, 4 internal bonds

of the chitin polymer, releasing chitooligosaccharides of lower molecular weights, such as chitobioses, chitotrioses, chitotetraoses, while exochitinase or β -N-acetylglucosaminidases (EC 3.2.1.52) cleave the products generated by the action of endochitinases and chitobioses releasing the monomers N-Acetylglucosamine (GlcNAc) [1, 4]. Fungal chitinases are grouped into the GH18 family based on the amino acid sequence of the enzyme, where its basic structure is composed of five domains: catalytic domain, chitin-binding domain, serine/threonine rich region, N-terminal region, and C-terminal region [5]. In addition, the molecular mass of these enzymes range from 30 to 200 kDa, and the isoelectric point in the range from 3 to 8 [4, 6].

Despite advances in studies on the structure and mechanism of action of chitinases, there is little availability of these enzymes on the market for use in various biotechnological processes [7]. In agriculture, chitinases are used in biological control against fungi and insects, as an indicator of fungal activity in the soil [1]. In the pharmaceutical industry, the enzyme is added to antifungal creams or combined with drugs for the treatment of fungal diseases [1, 3]. Chitin or chitosan hydrolysates, chitooligosaccharides, are also widely used in the medical sector, such as chitohexaosis and chitoheptaosis, which exhibit antitumor activities, and glucosamine, which is commonly used in the treatment of arthritis [1, 3]. Chitooligosaccharides are still classified as prebiotics and these are added to foods such as yogurt, milk, juice, and tofu [3].

Fungi usually produce chitinases to infect other microorganisms, defend against other pathogens, and also in the process of cell differentiation [3, 4, 6]. Among the microorganisms, filamentous fungi can secrete a wide variety of enzymes, and consequently facilitate their growth in the environment [8]. Among the chitinase-producing mesophilic fungi are *Trichoderma*, *Penicillium*, *Mucor*, *Aspergillus*, and *Metharhizium* [4]. Thermophilic fungi, which grow in temperatures ranging between 40°C and 80°C, comprise the fungi belonging to the order Sordariales, Eurotiales and Mucorales [9]. In the order Sordariales only thermophilic fungi of the family Chaetomiaceae are members, including *Myceliophthora heterothallica*, whose taxonomy was reclassified in a new genus called *Thermothelomyces heterothallicus*, reported as a producer of xylanase and cellulase enzymes [10-12]. However, there are no reports in the literature on chitinase production by this microorganism.

In addition, the use of agricultural by-products/agro-industrial residues as a supplement in bioprocesses for the production of fungal enzymes contributes to the use of low-cost carbon sources and avoids environmental problems due to inadequate disposal. Thus, this study aimed to investigate the production of extracellular chitinase using alternative carbon sources by the fungus isolated from soil, *Thermothelomyces heterothallicus* PA2S4T.

2. MATERIALS AND METHODS

2.1 Isolation and strain identification

The fungus was isolated from soil samples collected in the region of Nova Aurora, Parana state, Brazil, which is located at 24°30'9" S 53°15'18" W. Taxonomic identification was performed by analysing products amplified with primers for ribosomal genes using the methodology described by White et al. (1990) [13]. The isolation of the genomic DNA of this fungus occurred from the mycelial mass obtained from a two-day liquid culture supplemented with 1% glucose. The fungus mycelium was collected by centrifugation (5.000 x g, 10 min), and the pellet was used for DNA extraction using the hexadecyltrimethylammonium bromide (CTAB) method [14]. Primers ITS1 and ITS4 were used to amplify the regions of ITS1, ITS2 and 5.8S. The polymerase chain reaction (PCR) mixture included 0.6 μ M of each primer, 0.2 mM deoxynucleotides, 1.5 mM MgCl₂, 5x green GoTaq® Flexi buffer (Promega), and 2.5 U of GoTaq® Flexi DNA polymerase (Promega). The conditions of the PCR cycles consisted of an initial denaturation step at 95 °C for 2 minutes, 35 cycles at 94 °C for 45 seconds, 50 °C for 45 seconds and 72°C for 45 seconds, followed by a final phase extension at 72 °C for 5 minutes. The PCR products were analysed on 1.0% Tris-acetate-EDTA agarose gels (1.0% agarose, 0.04 M Tris-acetate, and 1 mM Na-EDTA), developed with ethidium bromide, and viewed under UV lighting. The determined sequences (ITS1, ITS2, and 5.8S) were then compared with other

sequences deposited in the database of the National Biotechnology Information Center (NCBI) using the basic local alignment tool.

2.2 Maintenance of the fungus

The routine maintenance of the fungal strain in the laboratory was carried out in test tubes containing 5 ml of potato dextrose agar (PDA), incubated at 40°C for seven days, and then kept in the refrigerator for a maximum period of 30 days.

2.3 Cultivation conditions

The fungus *T. heterothallicus* PA2S4T was grown in a Czapek liquid medium supplemented with 1% alternative carbon sources (sugarcane bagasse, sorghum bagasse, lemon peel, pear peel, tilapia fish scale, cicada exuvia, orange peel flour, apple flour, passion fruit fibre, and corn straw). Then 1.0 mL of the spore suspension (2×10^5 spores/mL) was inoculated in sterile distilled water in Erlenmeyer flasks (125 mL), containing 25 mL of liquid medium. The cultures were incubated at 40°C under stationary conditions for seven days. After growth, the cultures were filtered and the cell-free filtrates after dialysis were used to determine enzyme activity.

2.4 Enzymatic determination

Chitinase activity was determined through a mixture of 90 μ l of chitin substrate (Sigma-Aldrich), 0.2 mg/ml in a 50 mM sodium acetate buffer, a pH of 6.0, and 10 μ l of diluted enzyme extract when necessary and incubated at 40°C for 30 minutes. The reaction was stopped with the addition of 200 μ l of 0.4 M Na_2CO_3 solution and, the products were measured in a spectrophotometer at 410 nm. The chitinase unit was defined as the amount of enzyme required to produce 1 μ mol of p-nitrophenol per minute of reaction.

2.5 Determination of proteins

Extracellular proteins were measured using the Bradford method (1976) [15] and using bovine serum albumin (BSA) as a standard. The unit was defined as mg of protein per ml.

2.6 Influence of time on the production of extracellular chitinase

Cultures containing the Czapek liquid medium were supplemented with 1% orange peel flour carbon source, inoculated with fungal spores, and incubated at 40°C for 12 days. Each day the cultures were filtered to obtain the crude extract to carry out enzymatic assays and protein determination.

2.7 Optimum pH and temperature and chitinase stability

The optimal chitinase temperature was determined by incubating the enzyme with the substrate 0.2 mg/mL (w/v) dissolved in a McIlvaine buffer (0.1M, pH 4.5) at temperatures ranging from 35 to 70°C for 30 minutes. Thermal stability was carried out by incubating the enzyme at temperatures of 40, 50, 60, and 70°C and then aliquots were withdrawn at 0, 10, 30, 60, 90, and 120 minutes. The optimal pH of chitinase was determined using the McIlvaine 0.1M buffer in the pH range of 3.0 to 8.0. The pH stability was determined by incubating the enzyme in the McIlvaine buffer (pH 3.0 to 6.0) in an ice bath for 24 hours and the residual chitinase activity was measured.

2.8 Statistical analysis

All the experiments were performed in triplicate, and the results were presented as mean \pm standard error. Significant differences between the means of enzymatic activities were determined by an analysis of variance (ANOVA) followed by the Tukey test at the 5% level of significance ($p < 0.05$).

3. RESULTS AND DISCUSSION

3.1 Strain identification

The identification of fungal isolate PA2S4T was performed by sequencing the products amplified with the primers ITS1 and ITS4. The 523bp sequence of the ITS regions of the ribosomal DNA showed 99% similarity with the species *Thermothelomyces heterothallicus* (Klopotek) (van den Brink & Samson, 2012). The sequence of the region from ITS1 to ITS4 of this fungus is deposited and available in the NCBI database by accession number MW204601.1 (Figure 1). The fungi belonging to the Chaetomiaceae family are of great interest due to their thermostable enzymes, and are commonly addressed in the literature in studies of biomass degradation and biofuel production [16].

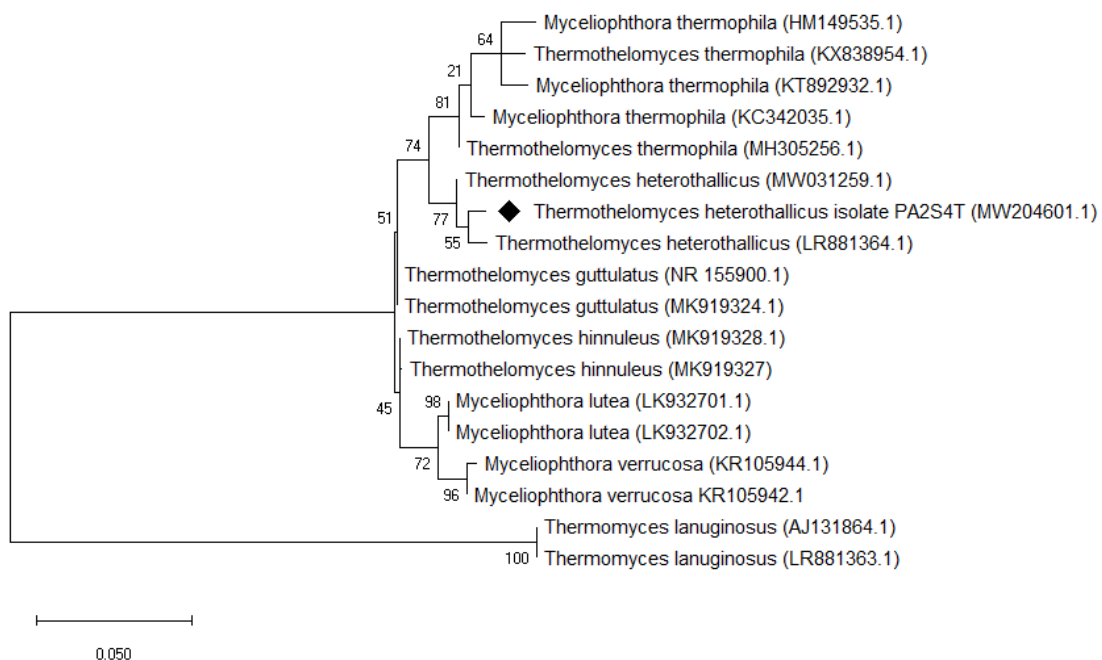


Figure 1 - A phylogenetic tree constructed from sequences of the ITS regions of the ribosomal DNA from *Thermothelomyces heterothallicus* PA2S4T and compared to sequences of other strains obtained from the NCBI GenBank database. The *Thermomyces lanuginosus* strain was used as an out-group. Fungus species are in bold symbols, followed by the Genbank accession number.

3.2 Influence of carbon sources on chitinase production

The production of extracellular chitinase by the fungus *T. heterothallicus* PA2S4T obtained from liquid cultivation showed that orange peel flour was the source of carbon that statistically showed the highest production of extracellular chitinase (82.3 U/mL), followed by lemon peel (27.9 U/mL). The chitinolytic activities considered statistically similar were obtained with corn straw, pear peel, and sugarcane bagasse, while lower chitinase induction values were observed with the carbon sources: apple flour, cicada exuvia, tilapia fish scale, sorghum bagasse, and

passion fruit fibre (Figure 2). In general, the carbon sources used to induce chitinase production are compounds that contain chitin, such as waste from the fishing industry [16-18]. However, studies have shown the use of residues from orange to induce enzyme production, whether the bagasse or the peel of the fruit, such as in the production of pectinase and polygalacturonase by the saprophytic fungus *Aspergillus fumigatus* [19], the production of laccases by the fungus *Peyronellaea pinodella* [18], and pectinase production by the fungus *Aspergillus niger* [19]. This increase in the production of these enzymes may be related to the great presence of pectin (42.5%) and soluble sugars, such as glucose, fructose, and sucrose (16.9%), which together with cellulose (9.21%) and hemicellulose (10.5%) make up the orange peel, one of the main by-products generated by the citrus industry [20, 21].

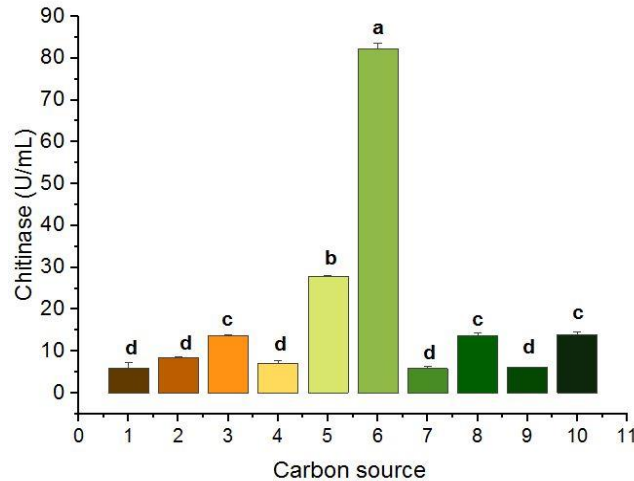


Figure 2 - Influences of carbon sources on chitinase production by the fungus *Thermothelomyces heterothallicus* PA2S4T. The liquid culture was supplemented with 1% (w/v) of different carbon sources and incubated under static conditions at 40°C for 7 days. The carbon sources are: apple flour (1), cicada exuvia (2), corn straw (3), tilapia fish scale (4), lemon peel (5), orange peel flour (6), passion fruit fibre (7), pear peel (8), sorghum bagasse (9) and sugarcane bagasse (10).

Table 1 shows the chitinases produced by different species of fungi and their sources of inducing carbons. Chitinases from thermophilic microorganisms are still lacking; there are only reports of *Humicola grisea* [22], *Thermoascus aurantiacus* var. *levisporus* [23], *Chaetomium thermophilum* [23], *Myceliophthora thermophila* C1 [24], and *Thermomyces lanuginosus* SSBP [6]. This study reports for the first time on the production of thermostable chitinases from the fungus *Thermothelomyces heterothallicus*.

Table 1 - Chitinase production by fungi using different carbon sources.

Fungus	Carbon source	Chitinase	Reference
<i>Alternaria alternata</i>	Shrimp shells	15.6 ^(c)	[25]
<i>Aspergillus niger</i> LOCK 62	Yeast Extract + Colloidal Chitin	82 ^{(c)*}	[26]
<i>Aspergillus terreus</i>	Shrimp shell powder	4.82 ^(a)	[27]
<i>Aspergillus terreus</i> CBNRKR KF529976	Fish scale	3.7 ^(d)	[28]
<i>Beauveria bassiana</i>	Colloidal chitin	0.585 ^(d)	[29]
<i>Chaetomium thermophilum</i>	Colloidal chitin	2.32 ^(d)	[23]
<i>Humicola grisea</i>	Chitin + colloidal chitin	213.57 ^(d)	[22]
<i>Isaria fumosorosea</i>	N-acetylglycosamine + colloidal chitin	284.09 ^(d)	[30]
<i>Metarhizium anisopliae</i> IBCB 348	Sugarcane bagasse	12.44 ^(b)	[31]
<i>Metarhizium anisopliae</i> IBCB 360	Silkworm	7.14 ^(b)	[32]
<i>Myceliophthora thermophila</i> C1	Glucose	3.5 ^(a)	[24]
<i>Paecilomyces</i> sp.	Colloidal chitin	90 ^(c)	[33]
<i>Thermoascus aurantiacus</i> var. <i>levisporus</i>	Colloidal chitin	1.37 ^(c)	[23]
<i>Thermothelomyces heterothallicus</i> PA2S4T	Orange peel flour	82.3 ^(c)	[This study]
<i>Trichoderma asperellum</i> UTP-16	Starch	2.05 ^(b)	[34]
<i>Trichoderma harzianum</i> TUBF 966	Colloidal chitin	14.7 ^(c)	[35]
<i>Trichoderma viride</i>	Colloidal chitin	23.8 ^(c)	[36]
<i>Trichoderma viride</i> AUMC 13021	Colloidal chitin	38.3 ^(a)	[37]

U/mg: (a); Ug⁻¹: (b); U/mL: (c); *nmol/h.

3.3 Influence of time on chitinase production

The maximum chitinase production was obtained on the 10th day and supplemented with orange peel flour as a carbon source in cultivation under stationary conditions (Figure 3). Rustiguel et al. (2012) (32) also reported that the maximum chitinase production of *Metarhizium anisopliae* using silkworm chrysalises occurred between the 8th and 12th days of culture.

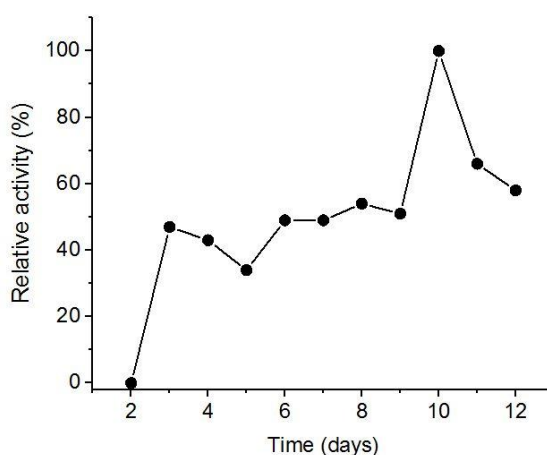


Figure 3 - Influence of time on chitinase production by the fungus *Thermothelemyces heterothallicus* PA2S4T in cultivation supplemented with orange peel flour, incubated at 40°C for 12 days under stationary conditions.

3.4 Influence of temperature and pH on enzymatic activity and stability

The temperature and optimal pH of chitinase activity of *T. heterothallicus* PA2S4T were 50°C (Figure 4A) and pH 4.5 (Figure 4B), respectively. Similarly, the optimum temperature (50°C) and the optimum pH in the range from 4.0 to 5.5 were observed for chitinase from *M. anisopliae* [32]. However, the pH values of *B. bassiana* chitinases were 5.5, 6.0, and 8.0 for the different isoforms

of the enzyme produced by the fungus [38]. Chitinase from *T. heterothallicus* PA2S4T exhibited thermal stability at temperatures of 40 and 50°C during 120 min; however, at temperatures of 60 and 70 °C, there was a decrease in enzyme activity after 10 min of incubation (Figure 4C). The enzyme showed pH stability for 24 h at pH 4.0 to 5.0, with 100% stable activity at pH 4.5, as shown in Figure 4D. Rustiguel et al. (2012) [32] reported that chitinase from entomopathogenic fungus *Metarhizium anisopliae* exhibited thermostability at 50°C and 100% activity at pH 7.

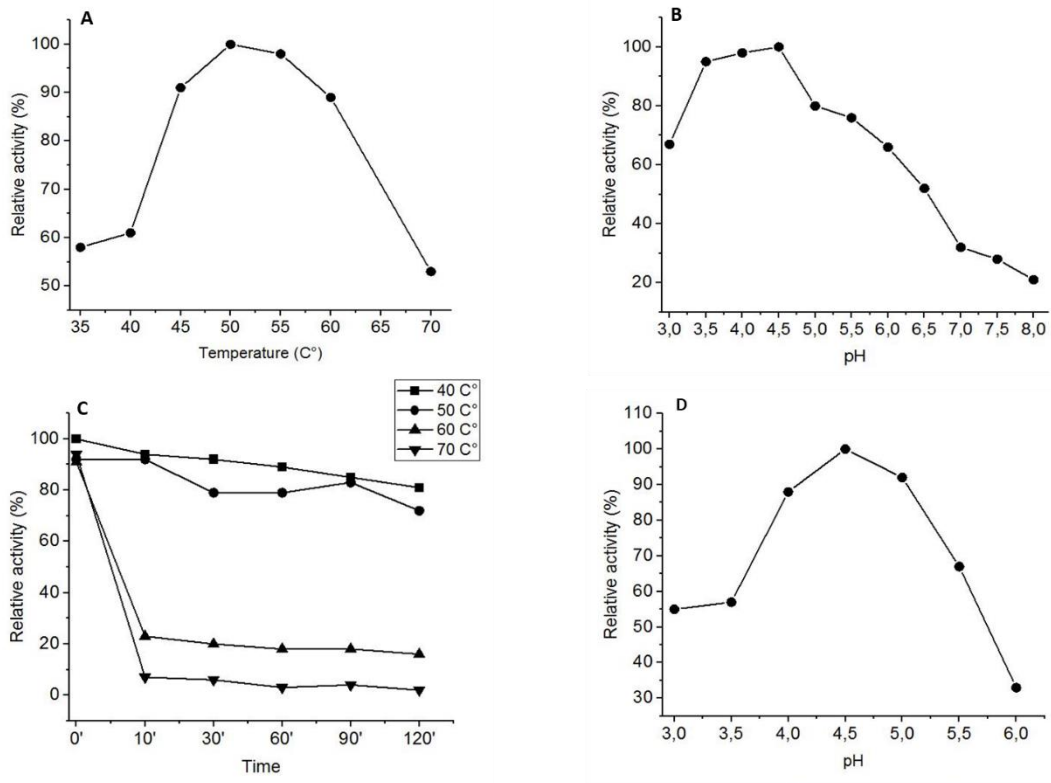


Figure 4 - Influence of temperature (A); Influence of pH (B); Thermal stability of chitinase (C); on the chitinase activity incubated at different temperatures 40°C (■), 50°C (●), 60°C (▲), 70°C (▼) for up to 120 min; Stability at pH of chitinase of the fungus *Thermothelomyces heterothallicus* PA2S4T (D).

4. CONCLUSION

The thermophilic fungus *Thermothelomyces heterothallicus* PA2S4T isolated from the soil of western Parana state has great potential as a producer of extracellular chitinase using alternative carbon sources, mainly orange peel flour, which is a waste product of the orange juice industry. In addition, this is the first report on chitinase production by this fungal species using carbon sources with a chitin-free composition or its derivatives. The enzyme also exhibited optimal pH and temperature characteristics that provide biotechnological application and could be promising for the degradation of residues that contain chitins.

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