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Prospecting Potential Filamentous Fungi that Produce Cellulase for Production of 2G Ethanol

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HIGHLIGHTS

- Nineteen filamentous fungi were isolated from lignocellulosic samples.
- Macromorphological characterization of the isolates was performed on solid culture.
- Five potentially cellulolytic fungi were selected.

Abstract: The production of second-generation ethanol has been recognized as a promising alternative for reducing greenhouse gas emissions. To achieve this, the hydrolysis of lignocellulosic residues by cellulases and hemicellulases is required, facilitating the release of monosaccharides that are subsequently fermented for ethanol production. In this study, the prospecting and macroscopic characterization of filamentous fungi were conducted, along with the evaluation of potential cellulase producers. Four distinct samples were collected and filamentous fungi were isolated using three different methods. Macromorphological characteristics were assessed in two solid culture media, CMC and oat flour, under incubation at 30 °C. Qualitative cellulase production was analyzed in CMC medium, with enzymatic halos visualized through Congo red staining. To determine the effect of temperature on fungal growth, the growth rates of the isolates were measured at 30 °C, 35 °C, and 40 °C in CMC containing medium. A total of nineteen filamentous fungi were successfully isolated. Limited macromorphological variation was observed in CMC containing medium; however, greater morphological diversity was noted when isolates were cultivated in oat flour medium. The highest growth rates were recorded for B.D.1.3.A at 30 °C, B.D.1.4.B and B.I.3.8.2 at 35 °C, and B.I.3.8.2 at 40 °C. Additionally, the isolates B.D.1.1, B.D.1.4.B, B.I.3.8.2, V.D.7.1, and V.I.2.7.1 exhibited the highest cellulolytic activity. The findings of this study contribute to the identification of potential cellulase producing

filamentous fungi, improving the understanding of their biotechnological potential and supporting the development of sustainable technologies.

Keywords: biotechnology; biofuels; microbiology; renewable resources; saccharification

INTRODUCTION

In light of climate change and as a consequence of the Paris Agreement, Brazil has committed to reducing greenhouse gas emissions. This commitment has led to initiatives such as RenovaBio, which plays a fundamental role as a government policy recognizing the strategic value of biofuels, such as first-generation (1G) and second-generation (2G) ethanol, in Brazil's energy matrix [1].

In this context, 2G ethanol is a promising solution due to its eco-friendly nature, as it results in lower CO_2 emissions [2]. It is produced from lignocellulosic biomass (LCB), a promising biofuel source that does not directly compete with food production. LCB is a heterogeneous material composed mainly of three plant polysaccharides: cellulose, hemicellulose, and lignin [3]. Its composition varies depending on the plant species, typically consisting of 40–60% cellulose, 20–40% hemicellulose, and 10–24% lignin [4, 5, 6]. LCB is found in agricultural and forestry residues, grasses, and energy crops [7].

Cellulose, in turn, is a homopolymer composed of D-glucose monomers linked by linear *β*-(1→4) *O*-glycosidic bonds. Hemicellulose is also a carbohydrate polymer, made up of different monosaccharide molecules, primarily linked by *β*-(1→4) glycosidic bonds, including D-xylose, D-mannose, D-arabinose, among others. Lignin, on the other hand, is formed through coupling reactions involving the three main phenolic polymer units: p-coumaryl, coniferyl, and sinapyl alcohols [8].

Cellulose, a high-molecular-weight, unbranched polymeric material, is considered the most abundant renewable material on the planet. Its annual production through photosynthesis from CO_2 and water exceeds 10^{10} tons. However, despite this availability, only about $6x10^9$ tons are exploited in industries such as materials, chemicals, textiles, and paper [9,10].

To produce 2G ethanol from lignocellulosic biomass, the hydrolysis of polysaccharides by enzymatic cocktails, mainly cellulases and hemicellulases is required, along with other processes to deconstruct LCB into its monomers, generating fermentable sugars [7,8,9,10,11]. These sugars serve as a substrate for yeast fermentation, leading to ethanol production, energy generation, biomass formation, CO_2 release, and other byproducts [12,13].

The enzymes used in hydrolysis are powerful catalysts that accelerate reaction rates by lowering activation energy more efficiently and specifically than synthetic or inorganic catalysts, without altering reaction equilibrium [14]. Studies have been conducted to enhance the efficiency and robustness of enzymatic cocktails for biomass hydrolysis to produce biofuels and other bioproducts, aiming to improve industrial economics [15,16].

Cellulose hydrolysis is performed by cellulases, which belong to the glycoside hydrolase family. These proteins have three different structural arrangements: *α*-helix, *β*-sheet, and *β/α*-barrel, and their function is to cleave the *β*-(1→4) glycosidic bond in cellulose. The catalytic modules of cellulases are classified into different hydrolase families based on their structure and amino acid sequence [17,18].

Cellulases are classified into three main categories: endoglucanases, exoglucanases, and *β*-glucosidases, which act on different parts of cellulose and work synergistically to convert cellulose into glucose through the hydrolysis process [19].

Endoglucanase, also known as carboxymethylcellulase (CMCase), primarily hydrolyzes carboxymethylcellulose (CMC). The endoglucanase complex consists of endo-*β*-1,4-D-glucanase and endo-*β*-1,4-D-glucan-4-glucan-hydrolase. Its main function is to reduce the length of the cellulose polymer by hydrolyzing glycosidic bonds in the amorphous region, without activity in the crystalline region, acting as an intermediary in the cellulose hydrolysis process.

Exoglucanase, also known as cellobiohydrolase or 1,4-*β*-D-glucan cellobiohydrolase, acts on the reducing (mainly CBHI) and non-reducing (CBHII) ends of cellulose, releasing cellobiose as the primary product. This enzyme is active on microcrystalline substrates but does not act on CMC [20]. Meanwhile, *β*-glucosidases or cellobiases convert the cellobiose produced by exoglucanase into glucose, completing the hydrolysis process [21].

Notably, filamentous fungi are among the most widely used microorganisms in industrial enzyme production. These fungi, belonging to a broad group of eukaryotes, are heterotrophic and acquire nutrients by hydrolyzing complex substances into simple molecules for absorption and use in biosynthesis and energy production [22]. Due to their intrinsic ability to process a wide range of complex substrates, filamentous fungi

can synthesize a vast array of enzymes in significant quantities, including cellulases, amylases, xylanases, pectinases, proteases, lipases, among others [23].

Despite the abundance of these resources worldwide, their contribution to the energy sector remains limited. This can be attributed to the lack of economically viable technologies that can be applied on an industrial scale [23,24].

Thus, this study aimed to prospect and macroscopically characterize filamentous fungi from lignocellulosic samples to gain a better understanding of these microorganisms and their potential biotechnological applications. Additionally, the study analyzed the isolated fungi with potential cellulase production capabilities to release fermentable sugars for the generation of 2G ethanol.

MATERIAL AND METHODS

The experiments were conducted at the Laboratory of Mycology, Enzymology, and Product Development (LMEDP) of the Federal University of the Jequitinhonha and Mucuri Valleys (UFVJM), at the Institute of Science and Technology (ICT) in Diamantina, Minas Gerais, Brazil. The microorganisms used were registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under the registration number A64AD93.

Sample Collection

Four distinct samples of lignocellulosic residues were collected: sugarcane bagasse, ensiled sugarcane bagasse, filter cake, and vinasse, all sourced from the company Agropéu Agro Industrial de Pompéu S/A, located in Pompéu, Minas Gerais, Brazil. The samples were collected aseptically using sterile surgical gloves, 70% alcohol, and pre-autoclaved glass flasks at 120 °C, 1.0 atm, for 30 minutes. After collection, the samples were transported in an isothermal box sanitized with 70% alcohol and stored in the laboratory at 4 °C until use.

Isolation of Filamentous Fungi from the Collected Samples

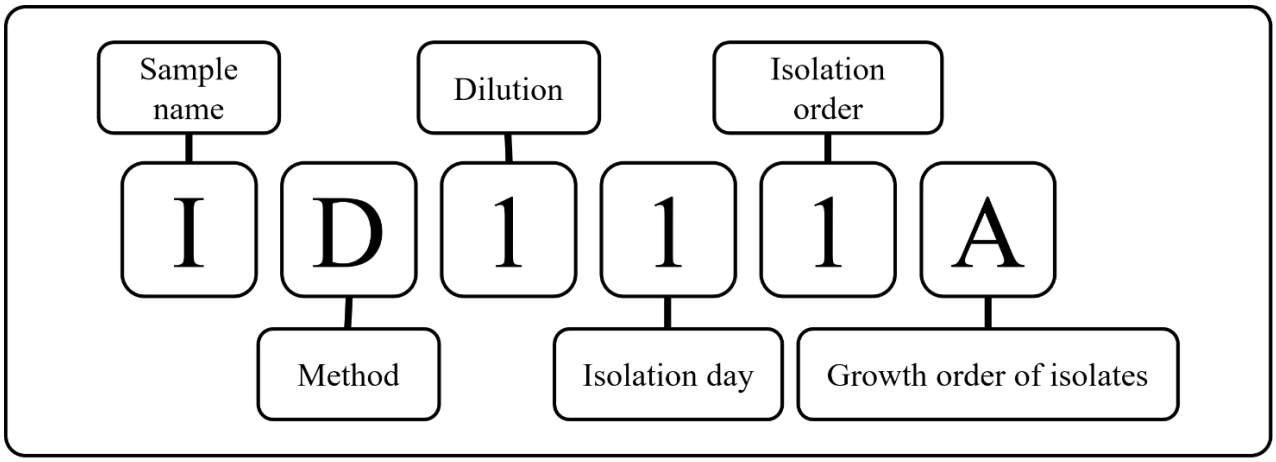
Filamentous fungi were isolated on a solid culture medium composed of 1% (w/v) carboxymethylcellulose (CMC) (Êxodo Científica®) and 2% (w/v) bacteriological agar (Êxodo Científica®). Initially, the culture medium was prepared, and, along with the Petri dishes, was autoclaved at 120 °C, 1.0 atm, for 20 minutes. Then, 20 mL of sterile medium was poured into the plates near a Bunsen burner under a laminar flow hood. After solidification, the procedure for isolating filamentous fungi began using three distinct methods: direct contact, pour plate, and serial dilution.

For the direct method, a small portion of the collected samples was taken using a sterile tweezer and placed onto the culture medium. The sample was gently spread over the medium and then positioned at the center of the plate. For the pour plate technique, the solid culture medium was prepared as previously described. After autoclaving, it was left to cool near a Bunsen burner until reaching approximately 40 °C. Then, using sterile tweezers, part of the collected sample was added to the culture medium still in the Erlenmeyer flask, mixed, and poured into pre-sterilized Petri dishes. The third cultivation method was based on serial dilution. Initially, a small portion of the collected sample was added to 10 mL of autoclaved distilled water in a 15 mL Falcon tube. Subsequently, a 1 mL aliquot of this solution was transferred to a new Falcon tube containing 9 mL of autoclaved distilled water. The dilution was repeated two more times, and 250 µL of the third dilution sample was used to inoculate a solid culture medium containing CMC.

The plates were incubated in a bacteriological oven at 30 °C for eight days, with filamentous fungi growth monitored every 24 hours. As the fungal species grew, point isolation was performed by transferring a portion to the center of another Petri dish containing the same culture medium. This procedure was repeated until complete fungal strain isolation was achieved.

Code of the Isolated Filamentous Fungi

The isolated fungi were assigned codes consisting of letters and numbers to indicate the sample from which they were isolated, the isolation method used, the dilution level (when applicable), the isolation day, and the order in which the fungi were isolated each day (Figure 1).



Sample	
Acronym	Meaning
B	Sugarcane bagasse
V	Vinasse
E	Sugarcane bagasse silage
T	Filter cake

Method	
Acronym	Meaning
D	Direct contact
P	Pour plate
I	Serial dilution

Dilution	
Acronym	Meaning
1	10x
2	100x
3	1000x

Figure 1. Coding method for isolated fungi.

Maintenance of Isolated Filamentous Fungi

The isolated microorganisms were maintained in the laboratory in test tubes containing inclined solid culture medium composed of 1% (w/v) CMC and 2% (w/v) bacteriological agar. Initially, they were stored in a bacteriological incubator at 30 °C until full fungal growth was achieved. Subsequently, the tubes were stored at 4 °C, with periodic subculturing.

The strains were also preserved in silica gel [25]. A spore suspension was prepared with 2 mL of powdered milk (200 $g.L^{-1}$ of autoclaved distilled water). From this suspension, approximately 1 mL was added to screw-cap test tubes containing 7 g of silica gel and then shaken. These tubes were sealed and stored at 4 °C.

Analysis of Macromorphological Characteristics of Filamentous Fungi in Solid Culture Medium Containing Carboxymethylcellulose and Oat Flour

Initially, solid culture media were prepared, consisting of 1% (w/v) CMC, as previously described, and solid culture media containing 4% (w/v) Quaker® oat flour and 2% (w/v) bacteriological agar (14). The media were autoclaved along with Petri dishes. After sterilization, 20 mL of medium was poured into the plates under laminar flow near a Bunsen burner. Once solidified, the isolated filamentous fungi were spot inoculated at the center of the Petri dish. The plates were incubated at 30 °C in a bacteriological incubator for eight days for the CMC medium and four days for the oat flour medium.

After incubation, macromorphological characteristics of the fungi were analyzed, including texture, surface, border, topography, colony color, and overall appearance.

Qualitative Analysis of Cellulase Production by Isolated Fungi

To qualitatively analyze cellulase production, the isolated filamentous fungi were spot inoculated at the center of Petri dishes containing CMC culture medium, as previously described, and incubated at 30 °C for eight days. After incubation, the fungal mycelium diameter was measured using a digital caliper, with measurements recorded in centimeters.

Subsequently, cellulolytic halo determination was performed by opening the Petri dishes and adding a revealing solution composed of congo red Verter® 1% (w/v), sodium chloride (NaCl) 1 M, and acetic acid (CH_3COOH) 0.5% (v/v).

Initially, 10 mL of congo red solution was added to the plates for 15 minutes. Afterward, the solution was discarded, and 10 mL of NaCl solution was added for another 15 minutes. Finally, the plates were washed with CH_3COOH, which was immediately discarded. The presence of clear zones around the mycelium indicated cellulase activity. The halo dimensions were measured using a digital caliper, and the enzymatic halo was recorded in centimeters. From these measurements, the enzymatic index (e.i.) was calculated as the ratio between the sum of the enzymatic halo size and the fungal mycelium diameter to the mycelium diameter (Equation 1).

$$\text{(enzymatic halo + mycelium diameter) / mycelium diameter = enzymatic index} \quad (1)$$

Effect of temperature on the growth of filamentous fungi

In order to infer the growth rate of the isolated fungi at different temperatures, they were cultured at 30 °C, 35 °C, and 40 °C for four days in a solid culture medium containing CMC, as described previously. After the incubation period, the mycelial radius was measured in centimeters using a digital caliper, and the growth rate was calculated in centimeters per hour (cm.hora-1).

Analysis of the quantitative production of cellulases by isolated filamentous fungi

Initially, the fungi selected in the previous experiment as the highest potential cellulase producers were spot inoculated onto Petri dishes containing a solid culture medium composed of 4% (m/v) oat flour and 2% (m/v) bacteriological agar [26], with growth in a bacteriological incubator at 30 °C for use in submerged culture inoculation.

Next, 25 mL of submerged culture medium (Carvalho-Peixoto, CP) [27] containing wheat bran as a carbon source were prepared in 125 mL Erlenmeyer flasks, autoclaved at 120 °C, 1.0 atm, for 20 minutes. Under laminar flow behind the Bunsen burner, the microorganisms were inoculated into the submerged culture medium using a 10 mm diameter disc from the selected strains, with the media maintained in a bacteriological incubator under stationary conditions at 30 °C for five days. After the incubation period, the crude extract containing the cellulases was obtained by vacuum filtration with the help of a Büchner funnel. From the extract, the carboxymethylcellulase (CMCase) activity was quantified.

Determination of carboxymethylcellulase activity by the saccharification method

The activity of cellulases was determined using 1% (m/v) CMC as the substrate, dissolved in 100 mM sodium citrate buffer, pH 5.0. For the reaction, 250 µL of substrate and 250 µL of enzymatic extract were pipetted into test tubes. The reaction was performed in a water bath at 55 °C for 10 minutes. Then, 50 µL of the reaction medium were removed and added to 50 µL of 3',5'-dinitrosalicylic acid (DNS), followed by boiling at 100 °C for 5 minutes. After cooling, 500 µL of distilled water were added [28]. The absorbance was measured in an Elisa plate reader Loccus LMR-96 at 540 nm, against a blank that consisted of the zero time of the reaction.

The method was previously standardized using a glucose standard curve (0.1 to 1.0 mg/mL). The unit of activity (U) was defined as the amount of enzyme that hydrolyzes one µmol of CMC per minute under the assay conditions, represented as U.mL-1.

RESULTS

Isolation of filamentous fungi

Using the pour plate, serial dilution, and direct contact methods, a total of nineteen filamentous fungi with different macromorphological characteristics were isolated over eight days at 30 °C. Based on the daily monitoring and isolation of the fungi, it was recorded that the day with the highest microbial growth was the seventh day, while the day with the fewest isolations was the third (Table 1).

Table 1. Monitoring of microorganism isolation over eight days at 30 °C in solid culture medium containing carboxymethylcellulose.

Days of filamentous fungi growth	Number of isolated fungi
1st	4
2nd	2
3rd	1
4st	2
7st	6
8st	4
Total	19

It is worth noting that ten filamentous fungi were isolated using the direct contact method, six by serial dilution, and three by the pour plate method. Using the pour plate method, the isolated fungi were coded as T.P.2.1, T.P.2.2, obtained from filter cake on the second day, and E.P.8.1, obtained from sugarcane bagasse silage on the eighth day. Regarding the serial dilution method, the isolated fungi were identified as V.I.2.7.1, obtained from vinasse on the second day; T.I.3.7.2, obtained from filter cake on the third day; E.I.1.7.2, obtained from sugarcane bagasse silage on the seventh day; B.I.1.8.1, B.I.3.8.3, and B.I.3.8.2, obtained from sugarcane bagasse on the eighth day.

Meanwhile, from the direct contact method, the fungi were isolated with the following codes: B.D.1.1, B.D.1.2, B.D.1.3.A, B.D.1.4.B, and B.D.4.1 from sugarcane bagasse; V.D.7.1 from vinasse; T.D.3.2 and T.D.7.1 from filter cake; and E.D.4.1, E.D.7.1 from sugarcane bagasse silage, on different growth days.

Segmenting the isolated fungi by sample, it was observed that eight of the nineteen fungi were isolated from sugarcane bagasse samples, totaling 42% of the isolates (Table 2). This distribution can be explained by several factors related to the intrinsic characteristics of each sample.

Table 2. Fungi isolated by collected sample.

Collected sample	Quantity of filamentous fungi isolated
Sugarcane bagasse	8
Sugarcane bagasse silage	4
Filter cake	5
Vinasse	2
Total	19

Macromorphological characteristics of filamentous fungi isolated in a culture medium containing carboxymethylcellulose and oat flour

Regarding the macromorphological characteristics of the filamentous fungi grown in solid medium containing CMC, it was observed that only one isolate (B.D.4.1) exhibited a cottony texture, six presented a velvety texture (B.D.1.3.A, B.D.4.1, E.P.8.1, T.D.7.1, V.D.7.1, and V.I.2.7.1), three creamy textures (E.D.7.1, T.I.3.7.2, and T.P.2.2), two powdery (E.I.1.7.2 and T.D.3.2), three serous (B.D.1.4.B, B.I.1.8.1, and B.I.3.8.2), and the texture of four fungi could not be defined due to inadequate mycelial growth (Table 3 and Figure 2).

Regarding pigment production in the culture medium, it was observed that the isolate V.D.7.1 produced a green pigment and the fungus T.D.7.1 produced a yellow pigment (Table 3 and Figure 2). Regarding the surface of the isolated fungi, four different types were recorded, with five fungi (B.D.1.4.B, B.I.1.8.1, B.I.3.8.2, E.I.1.7.2, and V.D.7.1) showing concentric streaks, three isolates with radial streaks (E.D.7.1, E.P.8.1, and T.D.3.2), only one fungus with a fissured surface (B.D.1.3.A), and five fungi with a smooth surface (E.D.4.1,

T.D.7.1, T.I.3.7.2, T.P.2.2, and V.I.2.7.1). The surface of five fungi could not be defined due to inadequate mycelial growth (Table 3 and Figure 2).

Regarding the edges, regular and smooth edges were observed in fungi B.D.1.4.B, B.I.1.8.1, E.D.4.1, and T.D.7.1. Three fungi had regular and undulated edges (B.I.3.8.2, E.I.1.7.2, and V.D.7.1), three had irregular and lobed edges (E.D.7.1, E.P.8.1, and T.I.3.7.2), one had a filamentous edge (V.I.2.7.1), one irregular and smooth (T.P.2.2), one irregular and undulated (B.D.1.3.A), and one regular and filamentous (T.D.3.2). The edge of five fungi could not be defined due to inadequate mycelial growth (Table 3 and Figure 2).

The flat topography was prominent with fourteen occurrences, only two fungi had a smooth and convex topography (B.D.1.4.B and E.P.8.1), one aerial (B.D.4.1), and one smooth and convex with a central peak (V.D.7.1). The topography of five fungi could not be defined due to inadequate mycelial growth (Table 3 and Figure 2).

Regarding the color of the isolated fungi, there was not much variation, most of them had hyaline mycelium, with white, brown, and green coloration (Table 3 and Figure 2).

It is worth mentioning that the fungi were also cultivated in solid medium composed of oat flour, at 30°C, for four days, where a greater pigmentation of the microorganisms was observed. Regarding the texture, two fungi exhibited a cottony texture (B.D.1.3.A and B.D.4.1), while the others had a velvety texture (Table 4 and Figure 2). It was also observed that only the isolate V.D.7.1 pigmented the culture medium.

Regarding the surface, all nineteen fungi exhibited a granular surface. As for the edges, only one fungus presented undulated edges (T.D.3.2), while the others had filamentous edges. Upon examining the topography, flattened, pleated, and crateriform isolates with central folds were observed, mostly with a dry appearance, and the fungus B.I.3.8.3 had an opaque appearance (Table 4 and Figure 2).

The results obtained in this study highlight the diversity of colors, textures, topographies, and edges exhibited by the isolated filamentous fungi. Additionally, some of the isolates with the highest cellulolytic activity showed macromorphological features similar to those described for species of the Aspergillus genus, such as A. flavus and A. fumigatus, as reported by Picková and coauthors (2021), Abdulzahra et al. (2021), and Ameen (2023) [29, 30, 31]. This morphological resemblance suggests a potential taxonomic relationship with this group, although not definitive. However, molecular identification of the fungal isolates was not performed, which limits the taxonomic precision, reproducibility, and biotechnological applicability of the findings. This limitation is acknowledged in the present study, and molecular analyses are planned for future work to confirm fungal identities and provide a more comprehensive phylogenetic characterization.

Table 3. Macromorphological characteristics of fungi in culture medium containing carboxymethylcellulose.

Fungi	Texture	Surface	Edges	Topography	Color
B.D.1.1	Undefined	Undefined	Undefined	Flat	Hyaline with black spores
B.D.1.2	Undefined	Undefined	Undefined	Flat	Hyaline with white spores
B.D.1.3.A	Velvety	Cracked	Irregular, wavy	Flat	Brown center, white border
B.D.1.4.B	Serous	Concentric striation	Regular, smooth	Smooth and convex	Hyaline with black spores
B.D.4.1	Cottony	Undefined	Undefined	Aerial	White with black spores
B.I.1.8.1	Serous	Concentric striation	Regular, smooth	Flat	Hyaline with black spores
B.I.3.8.2	Serous	Concentric striation	Regular, wavy	Flat	Hyaline with black spores
B.I.3.8.3	Undefined	Undefined	Undefined	Undefined	Hyaline with black spores
E.D.4.1	Velvety	Smooth	Regular, smooth	Flat	White with black spores
E.D.7.1	Creamy	Radial striations	Irregular, lobulated	Flat	White
E.I.1.7.2	Powdery	Concentric striation	Regular, wavy	Flat	White
E.P.8.1	Velvety	Radial striation	Irregular, lobulated	Smooth and convex	White

Cont. Table 3

T.D.3.2	Powdery	Radial striation	Regular, filamentous	Flat	Beige
T.D.7.1	Velvety	Smooth	Regular, smooth	Flat	Hyaline with black spores
T.I.3.7.2	Creamy	Smooth	Irregular, lobulated	Flat	White
T.P.2.1	Undefined	Undefined	Undefined	Flat	Hyaline with black spores
T.P.2.2	Creamy	Smooth	Irregular, smooth	Flat	White
V.D.7.1	Velvety	Concentric striation	Regular, wavy	Smooth and convex with central peak	Green center, white borders
V.I.2.7.1	Velvety	Smooth	Filamentous	Flat	Hyaline with black spores

Table 4. Macromorphological characteristics of fungi in culture medium containing oat flour.

Fungi	Texture	Surface	Edges	Topography	Mycelium Color
B.D.1.1	Velvety	Granular	Filamentous	Crateriform with central folds	Green
B.D.1.2	Velvety	Granular	Filamentous	Crateriform with central folds	Green
B.D.1.3.A	Cottony	Granular	Filamentous	Crateriform with central folds	Black
B.D.1.4.B	Velvety	Granular	Filamentous	Crateriform with central folds	Green
B.D.4.1	Cottony	Granular	Filamentous	Crateriform with central folds	White
B.I.1.8.1	Velvety	Granular	Filamentous	Crateriform with central folds	Green
B.I.3.8.2	Velvety	Granular	Filamentous	Crateriform with central folds	Green
B.I.3.8.3	Velvety	Granular	Filamentous	Crateriform with central folds	Green
E.D.4.1	Velvety	Granular	Filamentous	Crateriform with central folds	Green
E.D.7.1	Velvety	Granular	Filamentous	Crateriform with central folds	Green
E.I.1.7.2	Velvety	Granular	Filamentous	Crateriform with central folds	Green
E.P.8.1	Velvety	Granular	Filamentous	Crateriform with central folds	Green
T.D.3.2	Velvety	Granular	Undulated	Flattened and folded	Yellowish-green
T.D.7.1	Velvety	Granular	Filamentous	Crateriform with central folds	Green
T.I.3.7.2	Velvety	Granular	Filamentous	Crateriform with central folds	Green
T.P.2.1	Velvety	Granular	Filamentous	Crateriform with central folds	Green
T.P.2.2	Velvety	Granular	Filamentous	Crateriform with central folds	Green
V.D.7.1	Velvety	Granular	Filamentous	Crateriform with central folds	Light green
V.I.2.7.1	Velvety	Granular	Filamentous	Crateriform with central folds	Green

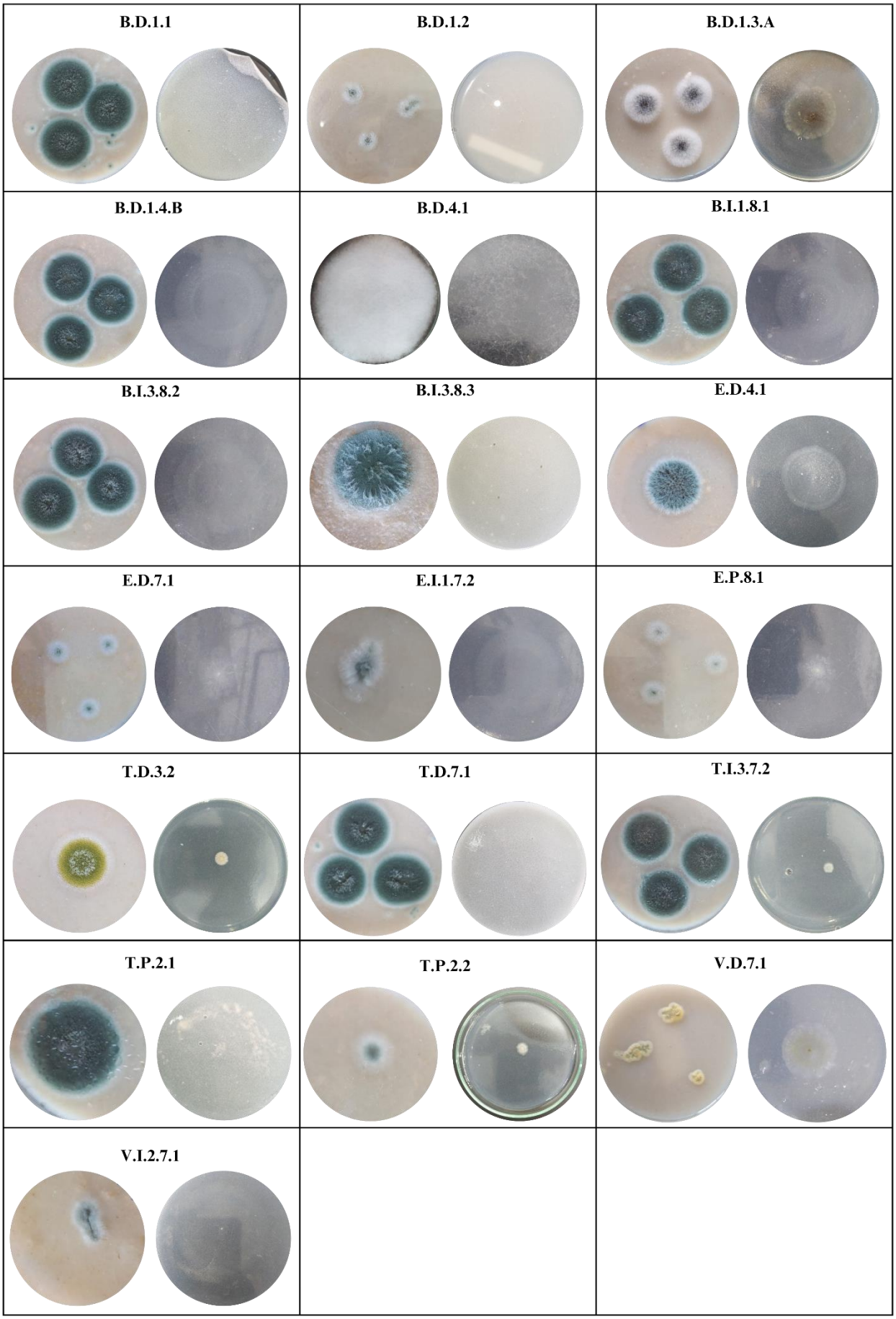


Figure 2. Representative image of the isolated filamentous fungi. The photograph on the right shows the filamentous fungi cultivated in medium containing carboxymethylcellulose, and on the left, the medium containing oat flour.

Qualitative analysis of cellulase secretion in solid culture medium containing CMC

Considering the enzymatic index criteria, the analysis results indicated that the fungi with the highest enzymatic index were T.D.3.2, V.D.7.1, T.I.3.7.2, B.D.1.4.B, and B.I.1.8.1. In terms of hydrolysis enzyme halo size, the five largest were observed in the fungi V.D.7.1, T.D.3.2, B.D.1.4.B, B.I.1.8.1, and B.D.1.1. The fungi V.D.7.1, T.D.3.2, B.D.1.4.B, and B.I.1.8.1 performed remarkably in both analyses. Regarding mycelial diameter, the fungi B.D.4.1, B.D.1.3.A, T.D.7.1, V.I.2.7.1, and B.D.1.4.B showed the greatest growth, although they did not form hydrolysis halos, demonstrating good development in the carboxymethylcellulose medium (Table 5 and Figure 3).

Table 5. Data obtained from the qualitative analysis of enzymatic hydrolysis.

Fungi	Enzymatic Halo (cm)	Mycelial Diameter (cm)	i.e.
B.D.1.1	0,40	3,20	1,125
B.D.1.2	0,00	2,90	0,000
B.D.1.3.A	0,30	3,50	1,086
B.D.1.4.B	0,50	3,30	1,152
B.D.4.1	0,00	6,40	0,000
B.I.1.8.1	0,40	3,00	1,133
B.I.3.8.2	0,30	2,80	1,107
B.I.3.8.3	0,00	0,00	0,000
E.D.4.1	0,20	3,00	1,067
E.D.7.1	0,20	2,00	1,100
E.I.1.7.2	0,20	2,75	1,073
E.P.8.1	0,20	1,80	1,111
T.D.3.2	1,10	0,70	2,571
T.D.7.1	0,20	3,50	1,057
T.I.3.7.2	0,20	0,80	1,250
T.P.2.1	0,00	0,00	0,000
T.P.2.2	0,10	0,80	1,125
V.D.7.1	1,20	1,80	1,667
V.I.2.7.1	0,20	3,35	1,060

Effect of temperature on the growth of filamentous fungi isolated in solid culture medium containing carboxymethylcellulose

Regarding the effect of temperature on the growth of the isolates, it was observed that five fungi exhibited the highest growth rate at 30 °C: B.D.1.3.A (0.0292 $cm.h^{-1}$), T.P.2.1 (0.0260 $cm.h^{-1}$), B.I.1.8.1 (0.0240 $cm.h^{-1}$), B.D.1.2 (0.0219 $cm.h^{-1}$), and E.D.4.1 (0.0208 $cm.h^{-1}$). The fungi that showed the highest growth at 35 °C were B.I.3.8.2 (0.0313 $cm.h^{-1}$), B.D.1.4.B (0.0313 $cm.h^{-1}$), T.P.2.1 (0.0271 $cm.h^{-1}$), B.D.1.2 (0.0271 $cm.h^{-1}$), and B.D.1.3.A (0.0260 $cm.h^{-1}$). Finally, the fungi with the highest growth at 40 °C were B.I.3.8.2 (0.0365 $cm.h^{-1}$), B.D.1.1 (0.0323 $cm.h^{-1}$), V.D.7.1 (0.0323 $cm.h^{-1}$), B.D.1.4.B (0.0313 $cm.h^{-1}$), and V.I.2.7.1 (0.0292 $cm.h^{-1}$) (Figure 4).

It is worth mentioning that the isolates B.I.3.8.2, B.D.1.1, V.D.7.1, and V.I.2.7.1 increased their growth rate as the temperature increased. On the other hand, the fungi T.P.2.1, B.D.1.3.A, E.D.4.1, T.D.3.2, T.I.3.7.2, and T.P.2.2 showed decreased growth as the temperature increased, indicating microorganisms with low tolerance to temperature increases (Figure 4).

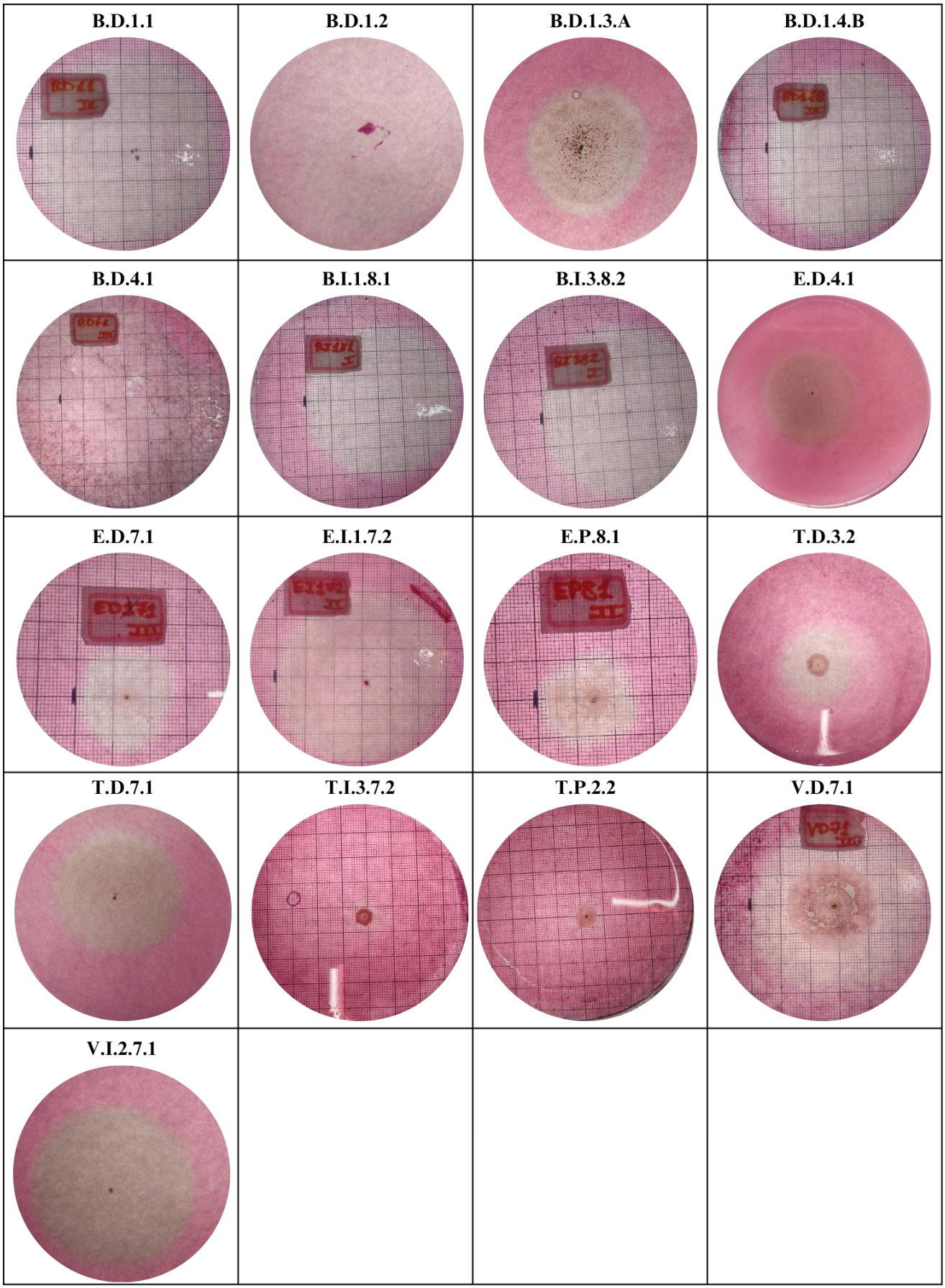


Figure 3. Enzymatic halo by the action of cellulase enzymes on the solid medium composed of carboxymethylcellulose and the consequent formation of clear regions around the fungi.

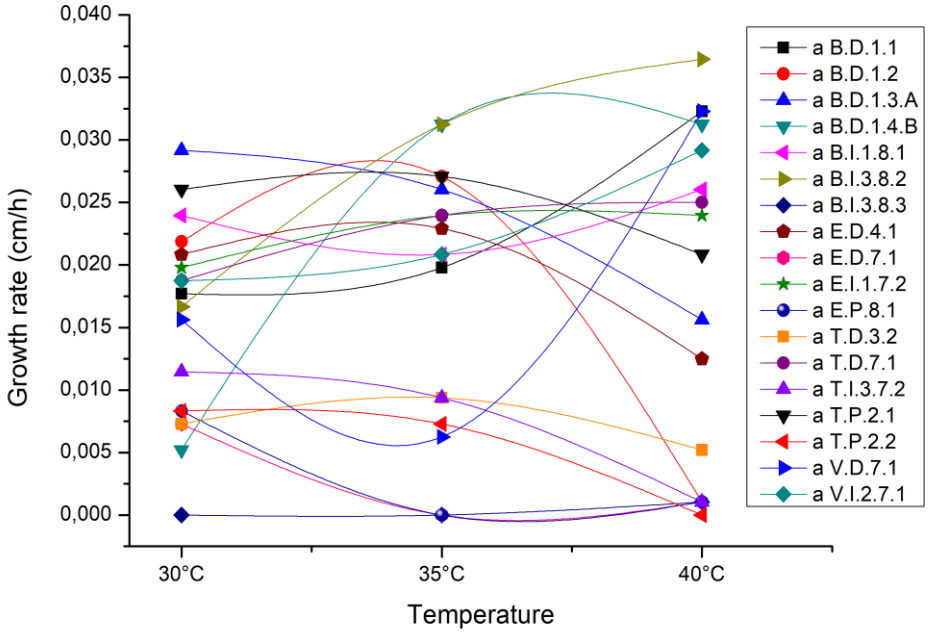


Figure 4. Growth rates of the filamentous fungi isolates in solid culture medium at different temperatures.

Quantitative analysis of cellulase production by selected isolated fungi

The quantitative analysis of enzymatic hydrolysis using Congo red, followed by the evaluation of fungal growth at different temperatures, allowed for the selection of five microorganisms for the determination of endoglucanase (CMCase) activity by cultivating these specimens in submerged culture medium containing wheat bran as a carbon source.

It was observed that the fungus B.I.3.8.2 exhibited the highest enzymatic activity, 0.208 $U.mL^{-1}$, and also stood out in the qualitative production at 35 °C and 40 °C (Figure 4), followed by the fungus B.D.1.1, with activity of 0.205 $U.mL^{-1}$, B.D.1.4.B, with 0.152 $U.mL^{-1}$, and V.D.7.1 and V.I.2.7.1, which presented lower enzymatic activities, with 0.117 $U.mL^{-1}$ and 0.176 $U.mL^{-1}$, respectively. Based on the obtained results, it can be observed that the isolated microorganisms have potential for CMCases production.

DISCUSSION

It is well established that bacteria and yeasts, as unicellular microorganisms, exhibit faster growth rates compared to filamentous fungi, which are multicellular organisms composed of filamentous hyphae. The development of microorganisms is influenced by environmental factors such as humidity, pH, and temperature. Additionally, the diversity of microorganisms inhabiting these materials may be affected by the intrinsic characteristics of the substrates' composition. Furthermore, fungi interact with one another and respond to environmental stimuli through a complex network of extracellular signaling and cellular responses [29,30].

During the isolation of filamentous fungi, only a limited number of microorganisms were observed to grow under the analyzed conditions, with a total of 19 filamentous fungi isolated from four distinct samples. This limited growth is likely attributed to factors such as the low initial concentration of microorganisms in the samples, which are dry and rich in lignocellulosic material (sugarcane bagasse, ensiled sugarcane bagasse), the low pH of certain samples (filter cake and vinasse), and the composition of the culture medium used, carboxymethylcellulose (CMC), among other factors.

Among the isolation methods evaluated, the pour plate method yielded the fewest isolates, possibly due to its oxygen demand. This method is more suitable for the growth of anaerobic and facultative aerobic microorganisms since the sample remains submerged in the culture medium. While the increased surface area enhances nutrient accessibility, oxygen exposure is simultaneously restricted.

Regarding the serial dilution method, filamentous fungi were observed to grow at later stages of the experiment, which is likely a consequence of sample dilution before inoculation. A primary goal of this technique is to enable the growth of microorganisms present in lower concentrations.

The direct contact isolation method demonstrated greater efficiency in comparison to the other methods analyzed. This efficiency is attributed to the aerobic nature of fungi and the absence of dilution effects on fungal concentrations within the samples. Notably, 50% of the isolates were obtained exclusively from sugarcane bagasse samples, which may be explained by the specific chemical composition of this substrate. In terms of isolation timing, most isolates were obtained at the beginning of the study, likely due to the exposure of microorganisms to oxygen and their physicochemical characteristics.

The chemical characteristics of each sample varied according to their origin, processing, and storage conditions, which in turn influenced microbial diversity and growth potential. The pH of the substrates was also a critical factor; for example, sugarcane bagasse silage typically exhibits pH values below 3.88, creating favorable conditions for material preservation by inhibiting the growth of undesirable microorganisms [31].

Regarding filter cake and vinasse, both substrates possess lower organic matter content and acidic pH values, which directly impact fungal growth while maintaining high moisture levels. Filter cake contains 24 to 28% organic matter and 6 to 8% mineral constituents, while vinasse has a pH range of 4.0 to 4.8 [32].

The slow growth of filamentous fungi observed during isolation in this study may be attributed to the composition of the solid culture medium, which consisted solely of CMC. CMC is an anionic, water-soluble derivative of cellulose produced through the reaction of cellulose with chloroacetic acid under alkaline conditions, introducing carboxymethyl groups at positions C2, C3, or C6 of glucose units. The abundance of hydroxyl and carboxyl groups in CMC results in high water absorption capacity; however, the polysaccharidic nature of its glycosidic linkages provides a limited nutrient source for microbial growth [33].

CMC hydrolysis requires the presence of cellulases to break down cellulose into simple sugars (D-glucose). However, the production of these enzymes is a time-consuming process, and not all fungi are capable of secreting them in sufficient quantities over short incubation periods. Additionally, the exclusive use of CMC as a carbon source may not provide all essential nutrients for fungal growth. In more complex culture media containing a variety of nutrients, fungi may experience more favorable conditions for development.

The macromorphological characteristics and growth patterns of fungi are strongly influenced by the composition of the culture medium. Due to the nutrient deficiency of CMC, significant morphological diversity was not observed among the isolates, with most exhibiting hyaline mycelium. In contrast, oat flour, a more nutrient-rich substrate, provided essential growth factors such as carbohydrates, dietary fibers, proteins, and fats, leading to increased fungal growth and predominantly greenish pigmentation.

The enzymatic index serves as an important parameter for screening enzyme-producing fungi, as it establishes a relationship between the fungal mycelium size and the hydrolyzed region influenced by enzyme secretion. A larger enzymatic halo and smaller fungal colony diameter indicate more pronounced enzymatic activity.

Based on the criteria analyzed, the isolate B.D.1.4.B exhibited the greatest potential for cellulase production, excelling in all evaluation parameters. Other promising cellulase-producing fungi included T.D.3.2, V.D.7.1, and B.I.1.8.1.

The findings of this study align with those reported by Ang et al. (2015) [37], who isolated 32 filamentous fungi from palm oil waste, including Trichoderma, Aspergillus, Rhizopus, Talaromyces, and Penicillium. The enzymatic screening performed by these authors revealed activity indices ranging from 1.04 to 1.62, values similar to those observed in the present study [34]. Similarly, the study by Cyrus and coauthors (2015) [38] reported the isolation of 67 fungi from agricultural residues and mountain soil, with the highest enzymatic index recorded at 1.90 (SWTS0X) and the lowest at 1.04 (SMS41-R) [35]. The results obtained in this research are therefore consistent with existing literature, demonstrating enzymatic indices between 1.057 and 2.571 cm.

When evaluating the effect of temperature on fungal growth in solid medium, variation in thermal tolerance among the isolates was observed. Additional tests are required to classify the isolates as psychrophiles (optimal growth at 0 °C to 20 °C), mesophiles (25 °C to 40 °C), or thermophiles (40 °C to 60 °C) [36]. These findings are critical for selecting microorganisms suitable for biotechnological applications under specific thermal conditions.

In this study, the isolate B.I.3.8.2 exhibited enzymatic activity of 0.208 U/mL in submerged culture using wheat bran as the carbon source. This value closely aligns with that reported by Santos and coauthors (2022) [39], who cultivated Aspergillus niger on a mixture of dry urban tree leaves as a substrate for cellulase

production using solid, submerged, and sequential fermentation, obtaining CMCase activities of 0.137 U/mL, 0.237 U/mL, and 0.395 U/mL, respectively.

However, while Santos and coauthors (2022) [39] employed a mixed substrate and different fermentation methods, the fungi in this study were evaluated under standardized conditions, highlighting the need to explore operational variables that may enhance enzymatic production efficiency. Further studies are required to optimize culture conditions and maximize the industrial viability of these cellulolytic microorganisms in biotechnological processes.

CONCLUSION

An in-depth understanding of the macromorphological characteristics of filamentous fungi collected from lignocellulosic samples was obtained through the experiments. A total of nineteen fungi were isolated, with eight from sugarcane bagasse, four from ensiled sugarcane bagasse, five from filter cake, and two from vinasse.

Further insights into fungal development and growth in culture media with varying compositions and nutrient availability were gained, as these factors are directly related to the macromorphological characteristics expressed by the microorganisms. Additionally, the qualitative analysis of cellulase secretion, utilizing congo red staining, was identified as a promising tool for the rapid screening of a large number of fungal isolates, facilitating the identification of those with higher cellulolytic potential.

The influence of growth temperature on the microorganisms was also a critical factor, revealing their tolerance to different temperature ranges, with optimal development observed at 30 ºC. Furthermore, the quantification of endoglucanase activity highlighted the fungal isolates B.D.1.1, B.D.1.4.B, B.I.3.8.2, V.D.7.1, and V.I.2.7.1 as notable producers of this enzyme.

This study underscores the importance of prospecting filamentous fungi from diverse materials for the isolation of microorganisms with enzymatic potential for biotechnological applications. Moreover, the findings contribute to a broader understanding of the Brazilian microbiota, advancing knowledge of fungal diversity and their functional characteristics.

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